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Insights into Genetic and Physiological Characteristics of Clover Rhizobia in Afghanistan Soils

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Abstract: Livestock production in Afghanistan highly relies on grazing and clover feed, which is a key component of pastures and forage crops. This study elucidated the genetic diversity of clover-nodulating rhizobia in different ecological regions and their effects on clover growth. A total of 57 rhizobia were isolated and their genetic diversities were studied through 16S rRNA and *nifD* genes. The isolates were inoculated to clover (Afghan local variety), to investigate the potential of nitrogen fixation and influences of clover growth. The 16S rRNA gene analysis showed two distinct groups of *Rhizobium* (94.7%) and *Ensifer* (5.3%) species. The *nifD* phylogenetic relationship revealed a high similarity to *Rhizobium* and a novel lineage group close to *Rhizobium leguminosarum* species. In the plant test, different genotypes significantly ($p < 0.01$) exhibited an increase in plant biomass production, compared to the un-inoculated plants. Among genotypes, the highest plant biomass was recorded in PC8 (1769.0 mg/plant) and PC9 (1409.2 mg/plant) isolates as compared to un-inoculated plants (144.0 mg/plant). Moreover, these isolates showed maximum nitrogen fixation rates of 8.2 and 6.5 µM/plant, respectively. These isolates were identified as the most promising rhizobial strains for developing biofertilizers in the context of Afghanistan.

Keywords: Afghanistan; *nifD*; rhizobia; clover; 16S rRNA

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1. Introduction

Trifolium species are among the most vital and valuable forage legumes globally [1]; they belong to the world's third-largest plant family, called Fabaceae (Leguminosae) [2]. *Trifolium* contains more than 250 species and plays a significant role as nitrogen fixers, enhancing pasture quality in natural and cultivated grasslands [3]. Among the species within the genus, white clover (*Trifolium repens*) and red clover (*T. pratense*) are the most extensively utilized. As legumes and valuable feed plants, they have long played a crucial role in contributing significantly to agricultural and animal production in both Europe and America [4]. Livestock production serves as a primary source of livelihood and family sustenance in Afghanistan [5]. Clover plays a significant role as one of the major feed sources, utilized both in its fresh form and as hay to nourish animals [6]. However, the cultivation area and production amount still cannot cater to the demand and soil fertility requirement. The diverse plant species within the Fabaceae family possess the capability to establish nitrogen-fixing symbiotic associations with soil bacteria, commonly known as rhizobia [7].

This process, known as biological nitrogen fixation (BNF), offers an ecological and cost-effective alternative for supplying nitrogen to legume crops. BNF reduces the reliance on synthetic nitrogen fertilizers in agriculture, thereby mitigating their negative impacts on natural ecosystems [8–10]. Legume crops, on average, annually fix atmospheric nitrogen from 20 to 200 kg/ha through a symbiotic relationship with rhizobia in their root nodules [11], while the symbiotic relationships are host-specific [12]. Rhizobia are distinguished by their large and complex genomes, typically ranging from 6 to 9 Mbp, comprising a chromosome alone or alongside several substantial plasmids [13,14]. These bacteria can exist as free-living organisms in the soil, plant endophytes, and endosymbionts inside legume root nodules [15].

Nitrogen (N) is a vital component of soil fertility and a primary element in chlorophyll, crucial for the photosynthesis process in plants [16]. However, the excessive use of chemical nitrogen sources has led to severe environmental contamination, adversely affecting the abundance of soil microbial communities through acidification [17] and an increased level of N in the water and air as well [17]. Also, rhizobia can promote root development, provide protection against soil-borne pathogens, increase stress tolerance, and induce systemic resistance in plants [18]. In addition to the above influences, rhizobia plays a role in promoting plant growth and productivity. This includes the synthesis of plant growth-promoting phytohormones such as indole-3-acetic acids (IAA), cytokinins, gibberellins, riboflavin, lumichrome, and Nod factors [19].

Sufficient quantities of chemical fertilizers are essential to enhance fodder production in arable field conditions and achieve a desirable biomass yield [20,21]. Given the considerable costs and environmental implications associated with nitrogenous fertilizer application, along with the aim of establishing sustainable agriculture in Afghanistan, biofertilizers, particularly promising rhizobial inoculants, are regarded as an effective alternative for optimizing fodder production [22,23]. The study of rhizobial diversity as a biological resource and identifying promising bacterial strains with exciting features is a valuable strategy to maximize agricultural productivity [24]. However, rhizobia's genetic makeup and population composition may be linked to geographical and environmental conditions [25]. Given the significance of clover as the predominant forage crop in Afghanistan, no documentation is currently available regarding the root nodule bacteria associated with clover crops in Afghan soils and prospective biofertilizer development. Moreover, studying Afghan clover-nodulating rhizobia assists researchers and biofertilizer production companies in articulating rhizobial diversity and efficient utilization.

Hence, this is the first attempt to elucidate the physiological properties and genetic characteristics of root nodule bacteria associated with clover crops in Afghan soils. To achieve this goal, we collected six soil samples from diverse agricultural-ecological-climatic regions in Afghanistan [26], isolated their root nodulating rhizobia, and examined their genetic diversity (16S rRNA and *nifD*) and nitrogen fixation potential. Additionally, we assessed the isolates for abiotic tolerance, considering factors such as pH, salt, and temperature. The most promising isolates, determined by their symbiotic performances as well as tolerance to the abiotic stresses, would be selected for the development of biofertilizers tailored for clover crops in Afghanistan.

2. Materials and Methods

2.1. Soil Sampling

Various environmental factors such as climate, soil properties, and plant growth and type influence soil microorganisms' diversity and community structure. Therefore, six soil samples were gathered at a depth of 0 to 20 cm from diverse agricultural climatic regions (hot desert climate to cold and semi-arid climate) and agricultural and forage crop fields to isolate native clover-nodulating rhizobia from Afghan soils, as reported by Habibi et al. [26]. Each soil sample was a composite sample from their sampling site, and the specifics of the soil samples can be found in Table 1. It is noteworthy that the sites where soil samples were taken have no previous history of microbial inoculation.

Table 1. Soil sampling sites and numbers of nodules obtained from clover plants after inoculation with soil samples.

Soil Samples No	Soil Sampling Sites	Climate	Latitude and Longitude	Elevation (m)	Previous Crop	pH	EC (ms/cm)	The Number of Nodules * Obtained from Clover Plants
								Trifolium resupinatum
1	Nangarhar	Hot desert climate	34°25′ N–70°27′ E	826	*Vigna radiata* L. (Mung bean)	7.66 ± 0.02	0.57 ± 0.01	13
2	Kabul	Semi-arid climate	34°31′ N–69°11′ E	1791	*Glycine max* L. (Soybean)	8.10 ± 0.70	2.29 ± 0.16	0
3	Parwan	Cold semi-arid climate	35°07′ N–69°14′ E	1916	*Medicago sativa* L. (Alfalfa)	7.70 ± 0.05	0.61 ± 0.15	17
4	Baghlan	Semi-arid climate	36°08′ N–68°42′ E	528	Mung bean	7.85 ± 0.05	1.28 ± 0.03	17
5	Kunduz	Semi-arid climate	36°43′ N–68°52′ E	351	Mung bean and *Zea mays* L. (Maize)	7.65 ± 0.06	1.68 ± 0.01	10
6	Bamyan	Cold arid and semi-arid	34°49′ N–67°49′ E	2550	Alfalfa	8.25 ± 0.06	4.10 ± 0.43	0

* The number of nodules represented by the number of isolates.

2.2. Isolation of Clover-Nodulating Rhizobia

To isolate microsymbionts associated with clover, seeds of a local Afghan clover variety (Persian clover: *Trifolium resupinatum* L.) underwent surface sterilization. This process involved pre-treating the seeds with 70% ethanol for 30 s, followed by immersion in a 3% sodium hypochlorite solution for 3 min. Subsequently, the seeds were thoroughly rinsed with sterilized reverse osmosis (RO) water and allowed to germinate for 2 days at 25 °C. Soil suspensions (1 g/5 mL) were prepared for each sample and utilized for the isolation of rhizobia. Each soil suspension inoculant was then applied to pots containing germinated clover seeds and vermiculite.

The pots were arranged in a growth chamber, and the plants were cultivated under controlled conditions, with a 16 h light/8 h dark photoperiod and day/night temperatures set at 25 °C/18 °C. Maintaining a soil moisture level of 60%, a sterilized nitrogen-free nutrient solution was introduced to the pots. After seven weeks, the plants were carefully uprooted from the pots, washed, and vermiculite was removed. The nodules were harvested from the plant roots and subjected to surface sterilization using 70% ethanol and 3% sodium hypochlorite. Each nodule was then macerated in a glycerol solution (15%, *v*/*v*), with 10 µL of the solution streaked onto yeast extract mannitol (YEM) agar plates. Subsequently, the plates underwent incubation for 4–7 days at 28 °C. Pure single colonies were stored for the long term in 15% glycerol at −80 °C and for the short term on slants stored at 4 °C.

2.3. Screening of Rhizobia Strains against Abiotic Stress

The growth potential of the isolate was elucidated under varied salinity, pH, and temperature conditions to assess stress tolerance, following the methodology outlined by Djedidi et al. [27]. Initially, the isolates were cultured in YEM broth medium for two days at 28 °C. Subsequently, 10 µL (10^6 cells mL^{-1}) of each culture was inoculated onto YEM agar plates and incubated for three days. Temperature tolerance experiments included incubation at 25 °C, 28 °C (as a positive control), 40 °C, and 45 °C. For salinity tolerance, isolates were exposed to 0% (0.1 g L^{-1} NaCl) as a positive control, followed by increasing NaCl concentrations to 2%, 3%, and 4%. In terms of pH tolerance, the medium's pH was adjusted to 4.5, 6.8, 9, and 10, with a pH of 6.8 serving as the positive control. Each of these stress-tolerance experiments was conducted in triplicate for every isolate. Based on the abiotic stress elucidation, 19 isolates were selected for further study.

2.4. Genomic DNA Extraction

Rhizobial isolates were cultured in YEM broth medium at 28 °C for a duration of 4–7 days. Following cultivation, bacterial cells were collected through centrifugation at 10,000 rpm for 10 min and subjected to two washes with TNE buffer (10 mM Tris, 0.1 M

NaCl, and 1 mM EDTA, pH 8). Genomic DNA extraction was carried out using the protocol outlined by Yokoyama et al. [28]. The concentration and purity of the extracted DNA were assessed using a UV-Vis Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Subsequently, the extracted DNA, at a specific concentration of 200–250 ng/µL, was employed for the amplification of 16S rRNA and *nifD* genes.

2.5. Genes Amplification and Sequencing

The PCR amplification and sequencing of 16S rRNA and *nifD* were performed as explained by Habibi et al. [26]. A set of 1F (5′-AGT TTG ATC CTG GCT C-3′) and 3R (5′-AAG GAG GTG ATC CAG CC-3′) was used for sequencing the 16S rRNA, and *nifD* 161F (TGCGRSGTRAAGTCSAAYAT) and *nifD* 1435R (TCCATGTCKCGSGCGAARAT) primers were used for *nifD*. For PCR amplification, 50 µL reaction mixtures containing a primer set of F and R (10 µM each), 0.5 µL Taq DNA polymerase (ExTaq polymerase 5 UmL^{-1}, Takara Bio, Otsu, Japan), and 200 ng DNA/µL, 5 µL 10× reaction buffer, 4 µL dNTP mixture were used. PCR conditions were as follows: denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 7 min. Then, bands related to 16S rRNA and *nifD* were purified using a QIAEX II agarose gel extraction kit (Qiagen, Valencia, CA, USA). Eventually, according to the manufacturer's protocols, PCR products were sequenced using an ABI PRISM 3500 genetic analyzer (Applied Biosystems, Waltham, MA, USA). The sequences of 16S rRNA and *nifD* have been deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers LC787678–LC787696 for 16S rRNA and LC787659–LC787677 for *nifD*. Phylogenetic trees of sequenced genes were constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 11.

2.6. Symbiotic Performance

Seed sterilization, cultivation, and growing conditions are detailed in Section 2.2. In this context, rhizobial cells were collected through centrifugation at 10,000 rpm for 10 min at 4 °C and subjected to two washes with TNE solution. Subsequently, a cell density of 10^8 CFU was administered to two germinated clover seeds in pots. Roots were separated from shoots, placed in jars, and assessed for nodules' nitrogen fixation ability. Ten percent of the jar's air was replaced with acetylene and incubated for one hour at 25 °C. The amount of ethylene in the jar was measured using a gas chromatograph (Shimadzu 2014AF, Kyoto, Japan).

2.7. Statistical Analysis

The data underwent analysis through one-way analysis of variance (ANOVA), and differences among the means of the treatments were determined using Tukey's honestly significant difference (HSD) test at a significance level of 5%. All statistical analyses were performed using JMP Pro 16 (JMP, Cary, NC, USA).

3. Results

3.1. Isolation of Rhizobia from Clover Root Nodule

A total of fifty-seven isolates from clover nodules were obtained, as shown in Table 1. The induced root nodules by rhizobia were found in four soil samples (Nangarhar, Parwan, Baghlan, and Kunduz), while the soils of Kabul and Bamyan could not show nodule formation. High numbers of clover root nodules were found in the Baghlan and Parwan soils.

3.2. Rhizobial Tolerance for Abiotic Stresses

All clover isolates (100%) could grow at different pHs (4.5 to 10) and exhibited a high adaptation to the different soil pH (Table 2). Regarding high-temperature resistance, 40 and 45 °C influenced the growth of isolates. Only 12.5% of Baghlan isolates could survive at 45 °C, and they were the most sensitive isolates to temperature among the four soils,

whereas, in Nangarhar soil, approximately 36% of isolates were able to grow (Table 2). In the salinity test, the effect of salinity was wider than the other two abiotic stresses (pH and temperature). The frequency of clover isolates obtained from three soils (Kunduz, Baghlan, and Parwan) observed the same survival ratio from 50 to 58% at 4% of NaCl condition, while the Nangarhar isolates, only 21.4% were able to survive (Table 2). At a low percent of NaCl (2%) condition, all isolates (100%) obtained from Kunduz soil could survive.

Table 2. The growth potential of clover isolates at different pH, temperatures, and NaCl concentrations.

Soil Samples	pH				Temperature (°C)				NaCl Concentrations (%)			
	4.5	6.8	9	10	25 °C	28 °C	40 °C	45 °C	0	2	3	4
Nangarhar	100.0 [a]	100.0	100.0	100.0	100.0	100.0	74.0	35.7	100.0	64.0	57.1	21.4
Parwan	100.0	100.0	100.0	100.0	100.0	100.0	74.4	21.4	100.0	92.8	85.7	57.1
Baghlan	100.0	100.0	100.0	100.0	100.0	100.0	81.2	12.5	100.0	93.7	93.7	56.2
Kunduz	100.0	100.0	100.0	100.0	100.0	100.0	50.0	28.5	100.0	100.0	100.0	50.0

[a] frequency of isolates (%).

3.3. The 16S rRNA and nifD Genes Analysis

Based on the 16S rRNA, a total of nineteen clover isolates were divided into two groups, as shown in Figure 1. GI contained eighteen isolates (94.7% of the total), and GII consisted of only one isolate (5.3%). The GI group is divided into three subgroups [GIa (47.4%), GIb (5.3%), and GIc (42.1%)]. The GIa subgroup included nine isolates and showed a close relationship (100%) to the *Rhizobium hidalgonense* and *Rhizobium leguminosarum* species. The GIb subgroup consisted of one isolate (KC10) and was highly similar (100%) to the *Rhizobium leguminosarum* species. The GIc subgroup comprised eight isolates and revealed high similarity to the *Rhizobium etli* CFN 42. The GII group contained one isolate (KC3) and showed maximum similarity to the *Ensifer meliloti* 2011.

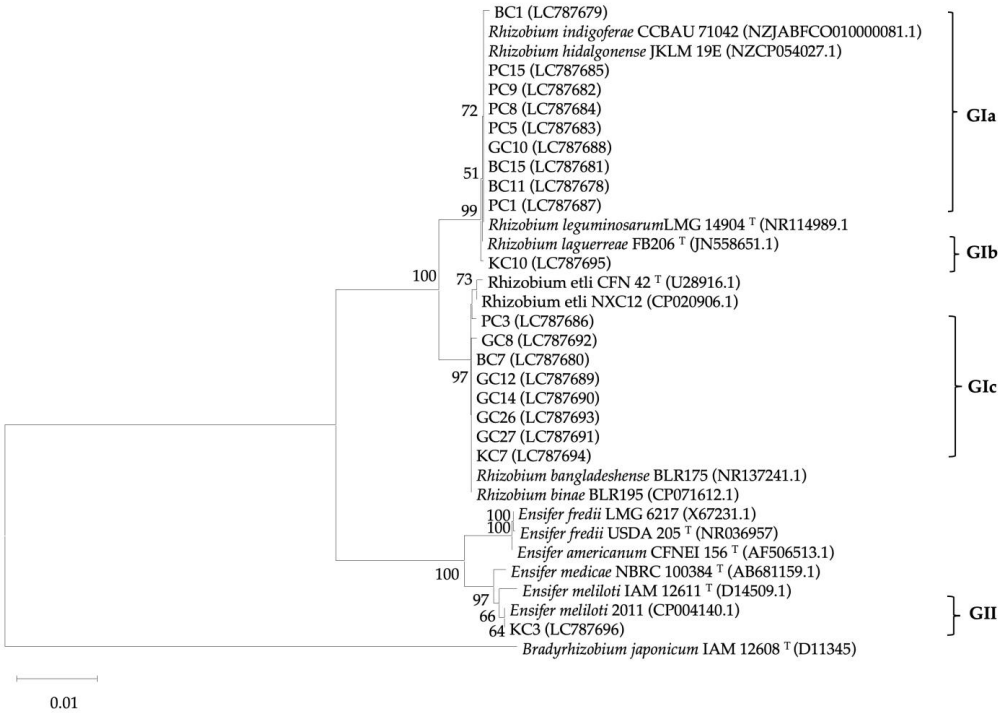

Figure 1. Neighbor-joining phylogenetic tree based on the partial sequence of the 16S rRNA gene (1383 bp) constructed using MEGA 11.0. The clover isolates, and relevant type strains with their accession numbers exhibited in the tree represent relationships among the rhizobial strains. *Bradyrhizobium japonicum* IAM 12608 was used as an outgroup. Bootstrap values are displayed for each node with 1000 replicates, and values less than 50 were ignored.

All 19 clover isolates based on the *nifD* gene sequence, were categorized into one group (GI), as shown in Figure 2. They showed close similarity to the *Rhizobium* species. The GI group was divided into two subgroups: the GIa subgroup contained eleven isolates (57.9% of the total), and the GIb subgroup consisted of eight isolates (42.1%). The GIa subgroup showed close similarity to the *Rhizobium leguminosarum* bv. *trifolii* species when compared to the other references, whereas the eight isolates were separated from *Rhizobium etli* and *Rhizobium leguminosarum* bv. *trifolii* species. The KC3 isolate, according to the 16S rRNA gene sequence, was categorized into *Ensifer meliloti* 2011. In the *nifD* sequence, the KC3 isolate showed maximum similarity to the *Rhizobium* species.

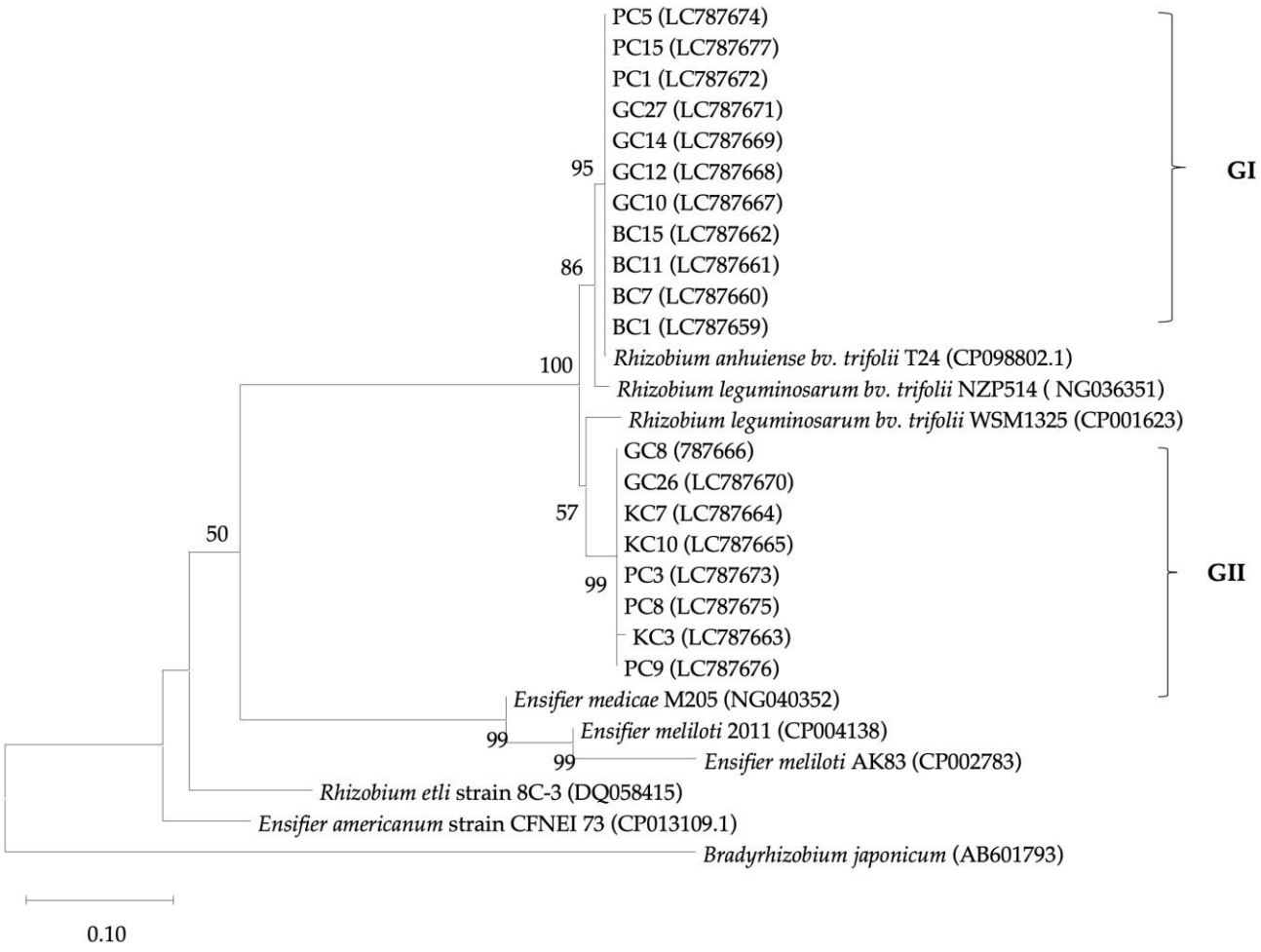

Figure 2. The phylogenetic relationship between isolated clover strains and relevant similar species based on the partial sequence of the *nifD* gene (1158 bp) and the neighbor-joining method. Bootstrap values are displayed for each node with 1000 replicates. Values less than 50 were ignored.

Table 3 presents the plant growth test results for the nineteen clover isolates. All plants inoculated with rhizobia exhibited effective nodules and increased biomass. Notably, all isolates demonstrated a statistically significant increase in plant biomass compared to the control (un-inoculated plants). Specifically, among various genotypes, PC8 and PC9, associated with GIa and GIb genotypes, displayed elevated acetylene reduction activity (ARA) (8.2 µM/plant, 6.5 µM/plant) and substantial biomass production (1769.0 mg/plant and 1409.2 mg/plant) (Table 3). The most promising isolates, characterized by different genotypes and showcasing the highest biomass production, include BC15, BC7, PC8, PC9, PC15, GC8, GC14, and KC7 (Table 3). In contrast, low biomass production was observed in PC1, KC10, GC12, GC27, and KC3 isolates.

Table 3. Growth performances of inoculated clover plants corresponding to the inoculation of 19 rhizobial isolates.

Isolates Name	Bacterial Species	Phylogenetic Groups of 16S rRNA	Accession Number (16S rRNA)	Phylogenetic Groups of *nifD*	Accession Numbers (*nifD*)	ARA (μM/Plant)	Biomass Production (mg/Plant)
BC1	*Rhizobium hidalgonense*	GIa	LC787679	GIa	LC787659	2.2 ± 1.1 a–d	1029.5 ± 90.5 c–i
BC11	*Rhizobium hidalgonense*	GIa	LC787678	GIa	LC787661	4.4 ± 2.3 a–d	1064.8 ± 137.7 b–i
BC15	*Rhizobium hidalgonense*	GIa	LC787681	GIa	LC787662	6.7 ± 0.7 a–d	1601.5 ± 74.3 a–c
GC10	*Rhizobium hidalgonense*	GIa	LC787688	GIa	LC787667	5.5 ± 0.5 a–d	1263.2 ± 130.3 b–h
PC15	*Rhizobium hidalgonense*	GIa	LC787685	GIa	LC787677	7.1 ± 3.1 a–c	1363.2 ± 60.2 a–g
PC1	*Rhizobium hidalgonense*	GIa	LC787687	GIa	LC787672	2.1 ± 1.1 a–d	867.8 ± 44.7 e–i
PC5	*Rhizobium hidalgonense*	GIa	LC787683	GIa	LC787674	1.1 ± 0.7 b–d	925.2 ± 58.6 e–i
PC8	*Rhizobium hidalgonense*	GIa	LC787684	GIb	LC787675	8.2 ± 2.3 a	1769.0 ± 109.0 a
PC9	*Rhizobium hidalgonense*	GIa	LC787682	GIb	LC787676	6.5 ± 3.7 a	1409.2 ± 114 a–g
KC10	*Rhizobium leguminosarum* bv. *viciae*	GIb	LC787695	GIb	LC787665	4.9 ± 1.1 a–d	854.7 ± 77.1 f–i
BC7	*Rhizobium bangladeshense*	GIc	LC787680	GIa	LC787660	5.3 ± 2.5 a–d	1531.5 ± 148.4 a–d
GC12	*Rhizobium bangladeshense*	GIc	LC787689	GIa	LC787668	0.9 ± 0.4 cd	761.0 ± 172.3 hi
GC14	*Rhizobium bangladeshense*	GIc	LC787690	GIa	LC787669	4.2 ± 0.9 a–d	1616.4 ± 48.9 ab
GC27	*Rhizobium bangladeshense*	GIc	LC787691	GIa	LC787671	1.9 ± 0.5 a–d	720.2 ± 79.4 hi
GC26	*Rhizobium bangladeshense*	GIc	LC787693	GIb	LC787670	3.1 ± 1.1 a–d	1104.5 ± 170.1 b–i
GC8	*Rhizobium bangladeshense*	GIc	LC787692	GIb	LC787666	7.1 ± 1.2 ab	1422.6 ± 169.1 a–f
KC7	*Rhizobium bangladeshense*	GIc	LC787694	GIb	LC787664	6.2 ± 2.7 a–d	1452.4 ± 59.5 a–e
PC3	*Rhizobium bangladeshense*	GIc	LC787686	GIb	LC787673	3.5 ± 0.9 a–d	964.5 ± 147.3 d–i
KC3	*Ensiefer meliloti*	GII	LC787696	GIb	LC787663	2.2 ± 0.8 a–d	827.1 ± 23.4 g–i
Control	-	-	-	-	-	0.04 ± 0.0 d	144.0 ± 15.2 j
p-value						**	***

The value represents means, followed by their standard errors. The letters show differences among treatments according to Tukey's HSD at a 5% level. The symbols ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively.

4. Discussion

Four of the six soil samples from different fields induced root nodules on clover plants. Notably, Parwan and Baghlan soils exhibited the dominance of nodules. However, the remaining two samples of Kabul and Bamyan soils failed to induce root nodulation in clover crops, possibly due to elevated soil salinity conditions (2.3–4.1 ms/cm) observed in these soils (Table 1). The challenge of nodule formation in legume crops under conditions of soil salinity has been documented in prior studies, underscoring salinity as a significant impediment to the normal growth of legume crops, especially clover [29–31].

The growth performance of clover isolates exhibited notable resilience across a range of pH levels (4.5–10). However, the isolates demonstrated heightened sensitivity to temperature and salinity. Elevated temperatures significantly impacted isolate growth, with approximately 50% unable to grow at 40 °C and 88% at 45 °C on the plates. Similarly, the presence of 2–4% NaCl inhibited the normal growth of colonies, with this effect being more pronounced in the Nangarhar soil sample (Mung bean field), characterized by a low electrical conductivity (EC) (Table 1). Additionally, about 95% of clover isolates displayed a close affiliation with the *Rhizobium* species. This suggests that *Rhizobium* sp. exhibits better adaptability to various pH levels, particularly at pH 4.5, compared to *Ensifer* or *Sinorhizobium* species. Despite acidic pH (4.0) tolerance, *Rhizobium* involves a normal nodulation process. *Rhizobium* strains display diverse levels of acid tolerance, with certain strains capable of thriving at pH 4.6 and below. Nevertheless, under low pH conditions, rhizobium attachment to root hairs, root colonization, and subsequent nodule formation may be adversely affected [32–34]. It is crucial to emphasize that distinct strains of rhizobium may demonstrate varying responses to low pH conditions, with some strains exhibiting greater tolerance than others [35]. Furthermore, elevated pH levels can influence the composition of nodulation factors produced by rhizobium, potentially impacting its capacity to form nodules and facilitate nitrogen fixation [36], while some strains can have tolerance under elevated pH conditions [33]. Moreover, in accordance with our findings, certain studies have demonstrated that high temperatures can have a detrimental impact on rhizobial growth and living conditions [37,38]. A similar effect can be observed with an increase in salt concentrations [39].

Using the 16S rRNA sequence, nineteen isolates were classified into two distinct groups, as illustrated in Figure 1. The GI group comprised eighteen isolates, constituting 94.7% of the total, while the GII group consisted of only one isolate, making up 5.3% of the total isolates. Further subdivision into three subgroups (GIa, GIb, and GIc) was observed within the GI group. Specifically, the GIa subgroup, which included nine isolates, exhibited a close relationship (100%) to the *Rhizobium hidalgonense* and *Rhizobium leguminosarum* species. The *Rhizobium laguerreae* FB206 seems to not only have alfalfa-nodulating rhizobia but is also able to nodulate clover (*Trifolium resupinatum*) effectively. GIb subgroup consisted of one isolate (KC10) and had high relatedness (100%) to the *Rhizobium leguminosarum* bv. *viciae* species. *Rhizobium leguminosarum* bv. *viciae* is a nodulating rhizobia of *Vicia faba*. *Rhizobium leguminosarum* bv. *viciae* showed effective nodules on clover roots. The GIc subgroup comprised eight isolates (42.1%) and revealed high similarity (100%) to different *Rhizobium* species (*Rhizobium bangladeshense* BLR175 and *Rhizobium binae* BLR195), including the *Rhizobium etli* CFN 42. Recently, Shamseldin et al. (2014) documented the capability of *Rhizobium etli* to form nodules on *Trifolium alexandrinum* L. roots in Egypt, highlighting *Rhizobium etli* as the predominant species responsible for nodulating Egyptian winter berseem clover (*Trifolium alexandrinum* L.). Furthermore, Castaingts et al. [40] identified Pvu-miR5942 as a newly discovered miRNA that plays a role in selecting *Rhizobium etli* strains for nodule colonization. Furthermore, *Rhizobium etli* CFN42 has the capacity to form a symbiotic association with the roots of *Phaseolus vulgaris* plants, resulting in the development of nitrogen-fixing nodules [41–43], in clover [44,45], and in Faba bean (*Vicia faba* L.), *T. semipilosum*, and white clover (*T. repens* L.). It seems that *R. etli* has high compatibility to nodulate various species of clover. Notably, within this investigation,

the GII group included a sole isolate (KC3) demonstrating maximum similarity (100%) to *Ensifer Meliloti* 2011, which can nodulate some clover species [46].

The 19 clover isolates were initially grouped together (GI) based on the *nifD* gene sequences. Subsequently, GI was further divided into two subgroups (GIa and GIb). The GIa subgroup consisted of 11 isolates (57.9%), showing high similarity to *Rhizobium leguminosarum* bv. *trifolii*. On the other hand, the GIb subgroup comprised eight isolates (42.1%), representing a novel lineage group of *Rhizobium leguminosarum* bv. *trifolii*. Additional analysis is required to explore and understand this *nifD* group further. One isolate (KC3) in the GIc subgroup showed high similarity to the genus of *Ensifer* based on the 16S rRNA gene sequence, while in the *nifD* sequence, it revealed maximum similarity to the *Rhizobium* genus and categorized in the new lineage group of *Rhizobium* species. This means the *nifD* gene transferred from *Ensifer* sp. to *Rhizobium* sp. That horizontal gene transfer occurred in Kunduz soil in the field of Mung bean and Maize and produced effective nodules on clover roots. It could be due to the lateral or horizontal gene transfer, which is a common event among prokaryotes and can occur among the different genera and species of bacteria [27,47], including nonsymbiotic bacteria [48]. However, phylogenetic variation at *nifD* describes a major variation in partner quality, particularly clover [49], and may have an effective role in plant and rhizobial symbiotic and mutual relationships.

In the context of clover isolates, all plants inoculated with rhizobia exhibited the formation of effective nodules and a notable increase in biomass production. All clover isolates demonstrated statistically significant enhancements in plant biomass compared to un-inoculated plants. Increasing plant biomass varied among the different genotypes (16S rRNA and *nifD*). PC8 and PC9, having the same genotypes (GIa, GIb), exhibited the highest plant biomass production and ARA among the various genotypes. More stable biomass production (75%) was observed in the genotypes of GIc and GIb. However, the KC3 isolate (*Ensifer meliloti*) showing the transferred *nifD* gene from *Rhizobium* species, produced low plant biomass. This result is consistent with Gordon et al.'s [49] report that *nifD* plays an important role in partner quality issues. In our study, lateral gene transfer of *nifD* was not effective as compared to *Rhizobium* species. The inoculation effect of *Rhizobium* species, especially *Rhizobium trifolii*, was studied individually and as co-inoculation with other microbial inoculants on different clover varieties in various fields and climatic conditions [50–54]. Tufail et al. [50] reported that inoculation of *Rhizobium trifolii* seed inoculation on berseem clover (*Trifolium alexandrinum*) significantly increased forage biomass and quality. He found that the number of stems/m^2 (348.2), plant height (24.4 cm), green forage yield (39.9 t/ha), dry matter yield (5.54 t/ha), and other parameters notably enhanced through *Rhizobium* seed inoculation. Also, he stated that *Rhizobium* inoculum increased soil fertility and gained net income compared to the non-inoculated plots. The efficacy of clover rhizobial inoculants seems to increase with the co-inoculation of other microbial inoculants. For instance, Furtak et al. [52] documented that the co-inoculation of red clover (*Trifolium pratense* L.) with *Rhizobium leguminosarum* and *Azospirillum* resulted in more root and shoot biomass when compared with plants inoculated with R. *leguminosarum* alone. Furthermore, he added that co-inoculation improved clover nodulation and growth under the condition of polycyclic aromatic hydrocarbon contamination.

Considering the environmental pollution due to excessive chemical fertilizer application and the tendency to increase organic and eco-friendly fertilizers, the application of effective microbial inoculants might be a virtuous strategy for enhancing clover green and dry biomass and feeding animals. In our study, based on the plant test results, isolates BC7, BC15, PC15, and PC8, representing various phylogenetic groups within clover, are identified as promising candidates for the formulation of biofertilizers in the Afghanistan context.

5. Conclusions

This study represents the first information on Afghanistan's clover-nodulating rhizobial genetic diversity and its potential for increasing plant growth. Around 60% of rhizobial

isolates in abiotic stress could not grow under 2–4% NaCl stress. A high-temperature effect on rhizobial isolates growth was observed at 45 °C (75.5% of total isolates), followed by 40 °C (30.1%). Genetic analysis based on 16S rRNA showed that *Rhizobium* species were the dominant group among the isolated rhizobia. The *nifD* results displayed close similarity to rhizobium species and a novel linage group to *Rhizobium leguminosarum* species. A lateral gene transfer of the *nifD* gene was observed from *Rhizobium* to *Ensifer* species in Kunduz soil. In the plant test, all isolates significantly increased plant biomass production in comparison to the negative control (un-inoculated plant). Among genotypes, PC8 and PC9 isolates displayed the height results of plant biomass and nitrogen fixation activity. All isolates, particularly PC8 and PC9, have the capacity to nodulate clover roots and enhance plant growth effectively.

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