CHARACTERIZATION OF NON-RHIZOBIAL ENDOPHYTIC BACTERIA IN ALGERIAN COWPEA ROOT NODULES: UNVEILING POTENTIAL FOR NITROGEN FIXATION

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Abstract. The study aimed to characterize the culturable endophytic bacteria present in cowpea nodules (*Vigna unguiculata* L. (Walp)). Five endophytic non-rhizobial isolates (SVU1, SVU3, SVU9, SUV15, and SUV18) were isolated from the root nodules of cowpea. Phylogenetic analysis of 16S rRNA gene sequences revealed that the isolates belonged to the genus *Paenibacillu*s, *Serratia*, *Microbacterium, Bacillus* and *Lysinibacillus*. Unlike rhizobia, all five isolates absorbed Congo Red contained in the Yeast Mannitol Extract medium. The nitrate reductase test was positive for all strains except SVU18and all strains utilized, mannitol, inositol and sucrose as substrates. The oxidase reductase test was negative. The results indicated that the root nodules of the legume could host a diverse endophytic community associated with cowpea, representing the first report of endophytic bacteria from Algerian cowpea nodules. These findings shed light on a part of the diversity of non-rhizobial within the cowpea nodules. The findings about the *nif*H gene showed that these bacteria open avenues for further exploration of the potential applications of endophytic bacteria in promoting nitrogen fixation and enhancing plant growth. Future studies will explore the potential application of these strains in nitrogen fixation and growth promotion for sustainable agriculture.

Key words: 16S rRNA; cowpea; endophytic bacteria; *nif*H; non-rhizobial

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is an important native African legume plant distributed throughout Latin America, Southeast Asia, Southern Europe and the Middle East [30, 42]. They are widely used as an economic source of protein as well as animal feed and crops in most rural and semi-urban areas [2]. Africa is a major production area where cultivation is crucial for low-input agriculture, which is the basic characteristic of most of the continent [8]. Cowpea production has increased dramatically in recent decades. Africa produced almost 9.4 million tons of cowpeas in 2022 [51]. In addition, plants contain proteins, lipids, carbohydrates, fiber and vitamins. It also contains interesting amounts of minerals, particularly iron, zinc, and calcium, in the seeds, leaves, and green pods of cowpea [39]. Cowpea is a water-deficiency tolerant crop that could be used as food for humans and livestock with most of its macro- and micronutrients [39].

As a legume, cowpea plays an essential role in global food security and agricultural sustainability. Its ability to form a symbiosis with rhizobia is well known and helps to fix atmospheric nitrogen in special structures in the roots called nodules [27]. In this way, up to 80% of nitrogen is fixed in the soil, reducing the need for nitrogen fertilizers and the cost of their production [34]. However, recent studies have shown that legume root nodules also harbor another category of endophytes of non-rhizobial endophytic bacteria, which have emerged in recent years as a key component of the legume root microbiome. These endophytes can colonize the interior of the nodules without damaging the plant or altering the rhizobia-legume symbiosis [16].

Nitrogen deficiency is a major nutrient stressor that has significantly impacted crop productivity worldwide [6]. Many studies have shown that endophytes can help plants by binding N_2 and making this element conveniently accessible to plants [4, 25, 36].

Bacterial endophytes, while living in plant tissues, can provide a favorable environment for nitrogen fixation by reducing competition with other rhizospheric microorganisms by creating a microaerobic condition for nitrogenase activity [49]. In addition, bacterial root endophytes enhance plant growth, act as biocontrol agents, naturally protect the host from pests, and support tolerance to numerous biotic/abiotic stresses. Endophytes capable of producing various growth hormones such as auxin or cytokinin and 1-aminocyclopropane-1-carboxylate deaminase, increased uptake of K ions into plant tissues and reduced ethylene levels are an alternative mechanism to alleviate stressful conditions in several plants [10, 22]. They can also improve the absorption of nutrients such as magnesium, zinc, sulfur and phosphorus from the soil and enable the host plant to have better growth and survival [22]. The use of endophytes as biofertilizers could be a better approach to improve the microbial status of the soil, which stimulates the natural soil microbiota by influencing the accessibility of nutrients and the degradation of organic matter and inevitably affected soil qualities [13, 45]. Endophytes have enormous potential to improve crop performance and reduce environmental

impacts caused by chemical inputs, as increasing agricultural productivity is a top priority worldwide [43]. Therefore, this study aimed to evaluate the diversity and N fixation efficiency of non-rhizobial endophytic bacteria isolated from local Algerian cowpea nodules. The results of this study could be a great resource to identify new approaches to sustainable agricultural practices and microbial applications as affordable biofertilizer alternatives to reduce the use of chemical N fertilizers, which are often expensive and pollute the environment.

MATERIAL AND METHODS

Plant material and culture conditions

The Algerian cowpea landrace NKB7 was used, and its characteristics were described in Fasusi *et al.* (2021) [14]. The trial was conducted in pots containing 3 kg of pre-sieved soil from the Djelfa region of Algeria 34°24'32.7"N 2°26'16.4"E. Three seeds were sown per pot, and irrigation was carried out manually regularly.

Nodule sampling and sterilization

Plants were harvested at the flowering stage, and nodules were detached from the roots, rinsed with distilled water, and then kept at 4°C in tubes containing $CaCl_2$, from which they were separated by carded cotton for later use. Harvested root nodules were first immersed in 12% sodium hypochlorite for 10 minutes, then in 96% pure ethanol for 5 minutes, followed by ten successive baths in sterile distilled water.

Bacterial isolation and purification of isolates

Sterile nodules were individually crushed in a drop of sterile distilled water on Petri dishes containing Yeast Extract Mannitol (YEM) agar medium (mannitol, 10g; K_2HPO_4, 0.5g; $MgSO_4$, 0.2g; Yeast Extract, 0.4g; NaCl, 0.1g; agar agar, 15g/L) supplemented with Congo Red [41]. From the nodular juice, depletion streaks were made on the agar medium. All plates were incubated at 30°C, with the incubation time varying according to the growth type of the isolates. After incubation, only colonies that have absorbed Congo Red were successively subcultured on YEM agar medium supplemented with Congo Red. To purify isolates, successive subcultures were performed on YEM agar media supplemented with Congo Red. Isolated colonies were seeded and incubated at 28°C for 48 to 120 hours. The operation was repeated until a single, pure colony is obtained.

Morphological and Gram characterization of bacteria

To confirm the Gram status of isolates, the Gram KOH test [44] was performed, a technique in which an isolated colony was brought into contact with a drop of 3% KOH solution. One minute later, the mixture is lifted with a sterile Pasteur pipette. If a filament was formed and carried away by the pipette, then the isolated colony was a Gram-negative bacterium; otherwise, it was a Gram-positive bacterium.

Biochemical characterization of bacteria

The Api 20E was used to identify the biochemical properties of the bacterial isolates, which provides valuable information about the metabolic and biochemical properties of the bacterial isolates, aiding in their identification and characterization [46].

Tolerance of isolates to high temperature, NaCl, and pH

After purification, all the isolates were tested for growth at elevated NaCl, and pH on Yeast Mannitol Broth (YMB) (mannitol, 10g; K_2HPO_4, 0.5g; $MgSO_4$, 0.2g; yeast extract, 0.4g; NaCl, 0.1g/L) and on YEM agar plates for high temperatures.

Two series of test tubes containing 10 mL of YMB are prepared, each tube is inoculated with 1mL of a fresh pre-culture of 10^8 bacteria mL^{-1}, then incubated at 28°C with 100 rpm rotary agitation for 48 h for various pH (2; 4; 10 and 12) and 72 h for various NaCl concentrations (1%; 4%; 8%; 11% and 15%) with three replications for each tube in both series. After 120 h of incubation, strain growth was estimated by measuring optical density at 620 nm.

For high temperature, freshly grown isolates were spotted three times as replications isolates were incubated at 38 °C, 42 °C, and 45 °C for 72 h. Bacterial growth was measured based on visible changes in growth and colony size on YEM agar plates.

Identification and phylogenetic analysis of endophytic non-rhizobial bacteria

DNA extraction

Genomic DNA was extracted from three-day-old bacterial cultures of each isolate grown in YMB at 28°C, using the FastDNA SPIN™ Kit for soil (MP Biomedical, Santa Ana, CA), with a modified protocol as described by [19]. For each isolate, DNA was extracted from the bacterial pellet obtained by centrifugation. The DNA was then quantified using PicoGreen. For a PCR with a final volume of 20 µL, 50 ng of template DNA was used.

Amplification of 16S rDNA gene

The partial 16S rRNA sequence, including the V1-V8 regions, was verified for the DNA of each bacterium individually using the universal primers BAC 27f (5' AGA GTT TGA TCM TGG CTC AG 3') and BAC 1492r (5' CGG TTA CCT TGT TAG ATT 3'). Genomic DNA (50 ng/mL) was amplified by PCR in a 20µL reaction volume containing 4 µL PCR 5X green buffer (Promega), a 1µL at 10 µM concentration of each primer, a 0.4µL of deoxynucleoside triphosphate, 1.2µL $MgCl_2$ (25mM), and 0.1µL of GoTaq DNA polymerase (Promega).

The PCR program was performed as recommended by [28] which consisted of an initial denaturation at 94°C for 6 minutes, followed by denaturation at 94°C for 45 seconds, annealing for 30 seconds at 59°C, and extension at 72°C for 1 minute (total 35 cycles), with a final extension at 72°C for 2 minutes. PCR amplification was performed using a Thermocycler (Eppendorf Personal, Germany). The expected amplicon size is 1446 bp.

Amplification of nifH gene

A nitrogenase complex, which consists of two separate proteins, catalyzes biological nitrogen fixation: the Fe protein encoded by *nif*H and the MoFe protein encoded by *nif*D and *nif*K [11]. The *nif*H gene was amplified using the primers PolF (5' GCT CAT GCA CGT GCC ACT CTA C 3') and PolR (5' GCT TAT ATT AGT GAC CGG AGG 3') [5]. Genomic DNA (50 ng/mL) was amplified by PCR in a 20µL reaction volume containing 4 µL PCR 5X green buffer (Promega), a 1µL at10 µM concentration of each primer, a 0.4µL of deoxynucleoside triphosphate, 1.2µL $MgCl_2$ (25mM), and 0.1µL of GoTaq DNA polymerase (Promega).

The PCR program was optimized to obtain a single amplicon per well and bacterium. The thermal profile for the detection of *nif*H consisted of an initial denaturation at 94°C for 7 minutes, followed by denaturation at 94°C for 1 minute, annealing for 30 seconds at 60°C, and extension at 72°C for 45 seconds (total 35 cycles), with a final extension at 72°C for 2 minutes. PCR amplification was performed using a Thermocycler (Eppendorf Personal, Germany). The expected amplicon size is 359 bp.

Sequencing and data analysis

The 16S rRNA and *nif*H amplicons were purified directly from PCR mix and sequenced at the Beckman Genewiz laboratory (England). The resulting sequences were cleaned using the MEGA v11.0.13 software. The Basic Local Alignment Search Tool (BLAST) was employed to identify closely related species using the BLASTn algorithm provided by the U.S. National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Subsequently, the complete sequence analysis was deposited in the NCBI GeneBank database to acquire accession numbers, followed by the construction of a phylogenetic tree of the two genes using the CLUSTAL W algorithm in the MEGA v11.0.13 software. Phylogenetic relationships were constructed using the Neighbor-Joining and Maximum Likelihood methods [40]. Phylogenetic trees were constructed using the Kimura 2-parameter model [40] present in the MEGA v11.0.13 software.

RESULTS

Morphological characterization of isolates

After isolates' purification, individual colonies were morphologically characterized on YEM agar medium based on their color, form, elevation, opacity, and margin according to [32]. The growth morphology and rate of isolates are listed in Table 1. Based on their reactivity to the KOH Test, isolates (SVU1, SVU9, SVU15, and SVU18) were tentatively classified as Gram-positive, when the KOH test was negative. In contrast, SVU3 was classified as Gram-negative. The appearance of colonies for each isolate within 48 hours is indicative of rapid bacterial growth.

Biochemical characters of isolates

The results varied considerably between the isolates (Table 2). The nitrate reductase test (reducing nitrates to nitrites) was positive for all isolates except SVU18, and all strains used glucose, mannitol, inositol, and sucrose as substrates. Whereas, the Oxidase reductase test, Arginine, Lysine utilization, Ornithine utilization, H_2S production, Urease, and Melibiose were negative for all of the isolates. A negative oxidase test indicates that the tested strain lacks cytochrome c oxidase. This enzyme is essential for aerobic bacteria which use oxygen as the final electron acceptor in their respiratory chain.

Tolerance of isolates to high temperature, salinity, and pH

All the isolates were tested for growth at high temperatures, under salinity, and extreme pH (Table 3). For the high-temperature tolerance tests (38 °C, 40 °C, and 45 °C), all isolates except SVU9 grew normally at 38 °C (Table 3). With the temperature rise, only SVU1 and SVU3 were able to grow at 42 °C and 45 °C. For the salinity and pH tests, all the isolates were grown at 28 °C (Table 3) with various NaCl concentrations (1, 4, 8, and 12 %) and pH levels (2, 4, 8 and 10), respectively. At a low concentration of NaCl (1%), the isolates were grown normally. In contrast, none of the isolates tolerate higher concentrations of NaCl (12%) SVU18 showed a tolerance at 4 and 8% of NaCl. All isolates were grown normally at pH 4 and 8, SVU15 and SVU18 tolerate low acidic pH (2). In contrast, only SVU9 tolerates Basic pH (10).

DNA extraction and PCR amplification of 16S rRNA and *nif*H genes

The size of DNA fragments of bacterial isolates is around 10000 bp (Fig. 1a). Amplicon intensity is proportional to the amount of DNA extracted, i.e. between 500 and 700 ng for SVU1-SVU15 isolates and 120 ng for SVU18.

The 16S rRNA gene, or bacterial universal gene, is mainly used because of its structure, which is highly conserved in all bacteria. Amplification of this gene enables bacteria to be detected and identified [31]. Fig.1b shows that all five isolates show specific

Table 1. Characterization of isolates based on their growth morphology and their growth rate on Yeast Extract Mannitol Agar medium, including KOH test.

Isolates	Colony Morphology	KOH Test	Growth Rate
SVU1	Cream, punctiform colonies, entire margin, glossy and convex elevation	-	48h
SVU3	Red, opaque, circular, entire margin, slightly raised elevation	+	48h
SVU9	Yellow, opaque, small, round, slightly raised elevation, convex	-	48h
SVU15	Large, white, opaque, circular, raised, entire margin	-	48h
SVU18	Pale-yellow, circular, entire margin, round and convex elevation	-	48h

amplification below 1,500 bp (the expected size of the gene being 1,446 bp). Fig. 1c shows that all bacterial isolates, except SVU18, have an amplicon between 300 and 400 bp specific to the *nif*H gene, whose expected size is 359 bp.

16S rRNA gene sequencing

The 16S rRNA gene sequencing of SVU1, SVU3, SVU9, SVU15, and SVU18 was carried out comprising a 904-bp-long sequence (NCBI gene bank accession number in parentheses). A phylogenetic tree was constructed using 16S rRNA-gene sequencing of SVU1, SVU3, SVU9, SVU15, and SVU18 and representative sequences from NCBI databases, which showed respectively 100% sequence similarity to *Paenibacillus polymyxa strain* R4.5 (CP097770), 100% sequence similarity to *Serratia marcescens* strain JW-CZ2(CP055161), 99% sequence similarity to *Microbacterium sp*. BH-3-3-3 (CP017674), and 99% sequence similarity to *Bacillus mycoides* strain TH26 (CP037992), and 100% sequence similarity to *Lysinibacillus sphaericus* strain KCCM 35418 (CP026120) (Fig. 2).

Table 2. Biochemical characters of bacterial isolates

Tests	SVU1	SVU3	SVU9	SVU15	SVU18
o-nitrophenyl –Pgalactoside	+	-	-	+	+
Arginine utilization	-	-	-	-	-
Lysine utilization	-	-	-	-	-
Ornithine utilization	-	-	-	-	-
Citrate utilization	-	-	+	-	-
H_2S production	-	-	-	-	-
Urease	-	-	-	-	-
Tryptophan	+	+	+	+	+
Indole	+	+	-	-	-
Sodium pyruvate	+	-	+	+	+
Nitrate reductase	+	+	+	+	-
Glucose	+	+	+	+	-
Mannitol	+	+	+	+	+
Inositol	+	+	+	+	+
Sorbitol	+	+	+	+	+
Rhamnose	+	-	+	+	+
Saccharose	+	+	+	+	+
Melibiose	-	-	-	-	-
Xylose	-	+	-	+	+
Arabinose	-	+	-	+	+
Oxidase	-	-	-	-	-

(+) color change and substrate degradation by the bacterium; (-) no color change, hence no substrate degradation.

Table 3. Tolerance of isolates at high-temperature, salinity and extreme pH

Isolates	T°C			pH				NaCl (%)			
	38	42	45	2	4	8	10	1	4	8	12
SVU1	++	++	+	-	++	+++	-	+	+	-	-
SVU3	++	++	+	-	++	+++	-	+	+	-	-
SVU9	-	-	-	-	+	+++	++	+	-	-	-
SVU15	++	-	-	+	++	+++	-	+	-	-	-
SVU18	++	-	-	+	++	+++	-	++	++	+	-

(-) not tolerant; (+) moderately tolerant; (++) tolerant; (+++) very tolerant.

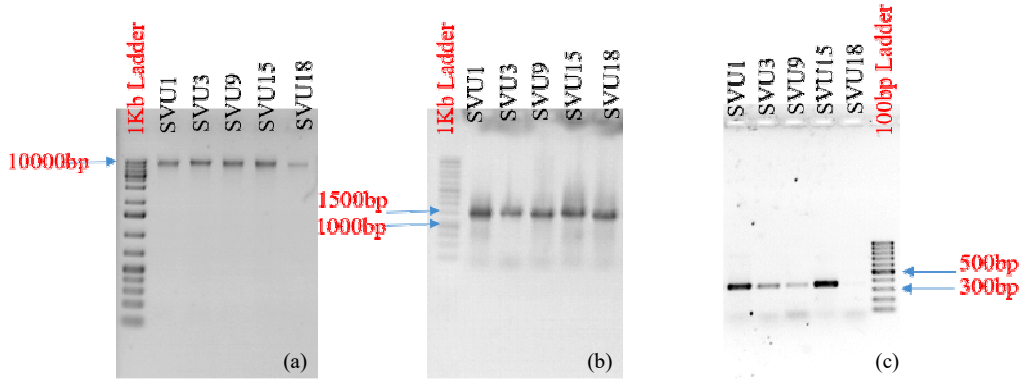

Figure 1. Agarose gel electrophoresis of genomic DNA extraction of bacterial isolates (a), PCR amplification of 16S rRNA gene (b) and PCR amplification of *nif*H gene (c). Lanes 1-5 –SVU1-SVU18 (Bacterial isolates).

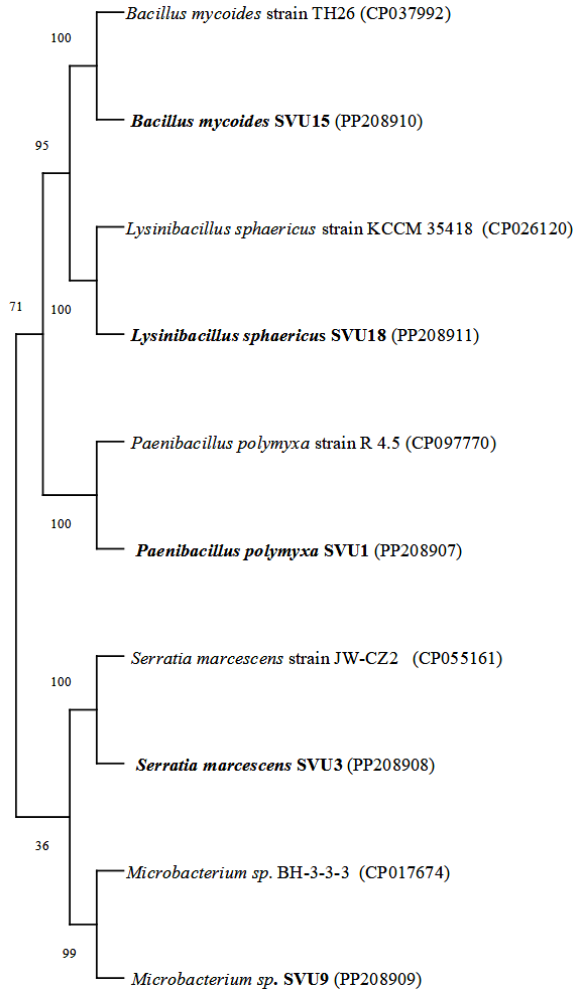

Figure 2. Phylogenetic analysis of *Paenibacillus polymyxa* SVU1 (PP208907), (*Serratia marcescens* SVU3 (PP208908), of *Microbacterium sp.* SUV9 (PP208909), of *Bacillus mycoides* SUV15 (PP208910) and *Lysinibacillus sphaericus* SVU18 isolated from nodules of *Vigna unguiculata* based on 16S rRNA gene sequences available online from the National Center for Biotechnology Information (NCBI) database. The tree was constructed after multiple alignments of sequence data by ClustalW. Distance and clustering with the Neighbor-Joining and Maximum Likelihood methods were performed using Mega 11. Bootstrap values based on 1000 replications, listed as percentages at the branching points. Isolated strains are in **bold**. (The numbers in parentheses are accession numbers).

*nif*H gene sequencing

Phylogenetic analysis of the aligned 268-bp *nif*H sequences resulted in the trees shown in Fig.3. The *nif*H sequences of the strains SVU1 (PP275890), SVU3 (PP275891) and SVU9 (PP275892) were aligned with *nif*H sequences of *Paenibacillus polymaxa* strains. Meanwhile, the *nif*H sequence of SUV15 (PP275893) was aligned with the *nif*H sequence of the strain *Klebsiella oxytoca.*

DISCUSSION

Only a few studies have focused on endophytes isolated from cowpea nodules in South Africa and northeastern Brazil, respectively [29, 47]. Recently,

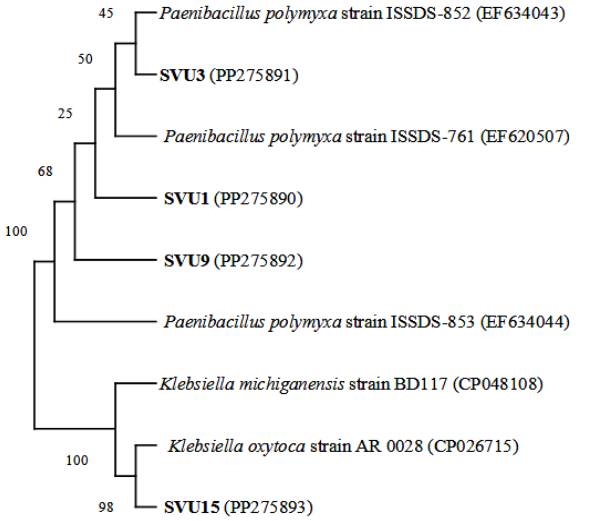

Figure 3. Tree phylogenetic analysis based on the *nif*H sequences of isolates SVU1 (PP275890), SVU3 (PP275891) SUV9 (PP275892), and SUV15 (PP275893) based on *nif*H gene sequences available online from the National Center for Biotechnology Information (NCBI) database. The tree was constructed after multiple alignments of sequence data by ClustalW. Distance and clustering with the Neighbor-Joining and Maximum Likelihood methods were performed using Mega 11. Bootstrap values based on 1000 replications are listed as percentages at the branching points. Isolates from this study are in **bold.** (The numbers in parentheses are accession numbers).

bacterial endophytes isolated from nodule cowpea genotypes belonging to the genus *Paraburkholderia*, *Enterobacter*, *Strenotrophomonas* and *Pseudomonas* cultivated in Kenya were characterized [37]. However, the isolation and characterization of endophytic bacteria from cowpea nodules is still largely unknown. In this study, we isolated and identified five indigenous non-rhizobia bacterial endophytes from Algerian landrace cowpea nodules. Bacterial isolates that absorbed the Congo red contained in YEM medium were selected to ensure the selection of endophytes (non-rhizobial). Molecular identification based on the analysis of 16S rRNA genes and their taxonomic classification revealed that the strains belonged to the genera *Paenibacillus*, *Serratia*, *Microbacterium*, *Bacillus* and *Lysinibacillus*. Phylogenetic analysis of the *nif*H sequences revealed that the strains *Paenibacillus polymyxa*, *Serratia marcesens* and *Microbacterium sp*. were aligned with the sequences of the *P. polymyxa* strains. While the *Bacillus mycoides* strain matched with *the Klebsiella oxytoca* sequence. In this study, the phylogenies of the 16S rRNA and *nif*H genes were incongruent, except for the SVU1 strain, which aligned with *P. polymyxa* strains. Our results suggest that *Serratia marcescens* and *Microbacterium sp.* could obtain the *nif*H gene by horizontal transfer from *P. polymyxa*, while *Bacillus mycoides* received its *nif*H gene from *Klebsiella oxytoca*. The existence of horizontal transfer of the *nif*H gene has been debated among evolutionists over the last three decades [26]. The *nif*H gene is often carried on symbiotic islands or plasmids that can be transferred between different bacterial species within and between genera (horizontal

transfer) [7]. Therefore, *nif*H-based diversity measurements are unlikely to be directly comparable to diversity measurements performed with 16S rRNA genes [26]. Furthermore, Agri et *al.* [1] and Khianngam et *al.* [20], observed that bacterial genomes that have >97% similarity regarding the 16S rRNA gene sequence can have up to 23% differences in their *nif*H sequences. Steenkamp *et al.* [47] and Radl *et al.* [29], concluded that horizontal gene transfer significantly influenced the evolution of the root nodule bacteria *Vigna unguiculata* in South Africa and in northeast Brazil, respectively.

The presence of *nif*H gene indicated the N-fixing potential of the strain [9]. It has been proved that colonization of N-fixing bacterial endophytes increases nitrogen uptake efficiency and endogenous nitrogen content in plants, being important signaling molecules, nitrogen regulates phytohormonal balance, improve the soil nitrogen concentration, rhizobacterial population levels, and soil nitrogenase activities [3, 33]. It was reported by Diale et *al*. [9] and Etesami et *al.* [12] that the endophytic non-rhizobial bacteria belonging to genus *Bacillus*, *Microbacterium*, *Klebsiella* and *Paenibacillus* were tested positive for the presence of the *nif*H gene. When reinoculated into plants, showed positive results for N-fixing activity and improved plant growth on height and dry weight [9, 12].

Abiotic soil stresses such as high temperatures, salinity, acidity, and alkalinity represent increasing challenges for sustainable agriculture [48]. Recent studies report that non-rhizobial endophytic bacteria can show resistance to the above-mentioned abiotic stress factors [24, 50]. We evaluated the tolerance of isolates of this study to stresses related to high temperatures, acidity, alkalinity and salinity. Isolates SVU15 and SVU18 were tolerant to acidic pH, while strain SVU9 was tolerant to alkaline pH. SVU1 and SVU3 tolerated high temperatures and high NaCl concentrations (halotolerant). While SVU18 showed resistance to high salinity conditions (extremophilic). Although SVU18 is also resistant to acidic pH, it lacks the nifH gene and has no nitrogen fixation potential. Although these endophytes have not yet been tested in the field, their potential as bio-inoculants could improve soil tolerance to abiotic stresses. Furthermore, biochemical analysis showed that all strains can use tryptophan. This may suggest the possibility of indole-3-acetic acid (IAA) production, given that this amino acid is its precursor.

Furthermore, numerous studies reported the potential of endophytes as valuable biological resources to enhance crop production by nutrient acquisition, plant protection and growth promotion. In fact, *Bacillus* and *Lysinibacillus* species exhibited antibacterial activity by producing important antimicrobial enzymes against *E. coli* and *Pseudomonas aeruginosa* and showed particularly high amylase, endocellulolytic and esterasic activities [18, 35]. *Paenibacillus polymyxa* were reported to possess antifungal activity against *Fusarium oxysporum*, *Pythium ultimum* and *Phytophthora capsici* [15]. Whereas, the endophytic *Microbacterium* showed probiotic solubilization of plant nutrients/minerals, produced hydrolytic enzyme/phytohormones in rice seedlings [17]. While, endophytic *Serratia* and *Paenibacillus* species can enhance phosphate solubilization and promote seed germination [21, 38]. The non-rhizobial endophytic bacteria have been reported to enhance symbiosis between rhizobia and host plants [23].

Overall, this study contributes to the characterization of endophytic bacteria in cowpea nodules and highlights the diversity of these bacteria. The results for the *nif*H gene showed that these bacteria open avenues for further research into the potential uses of endophytic bacteria to promote N-fixation and improve plant growth. These bacteria can interact directly with the rhizobia or the plant and influence various physiological and potentially beneficial processes for the cowpea plant. Further field research could lead to the development of new agricultural practices, such as promising microbial inoculants to increase cowpea productivity and agricultural sustainability under changing environmental conditions and limiting the use of chemical N-fertilizers.

Understanding the diversity and function of endophytic bacteria in cowpea nodules is an active area of research because of its implications for optimizing symbiotic nitrogen fixation, improving plant productivity, and developing sustainable agricultural practices.

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