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Isolation, Characterization, and Selection of Bacterial Endophytes from Soybean (*Glycine max*) Nodules and Roots in Some Soils of Cameroon for Promoting Growth of Forage Legume Plants

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Authors' contributions

This work was carried out in collaboration among all authors. Author MB carried out the field and laboratory experiments, processed the data, and wrote the manuscript. Author AMSD provided support during the work, compiled, and revised the manuscript. Authors *AW, RT, HA, and STO collaborated in the laboratory work. Author DN provided the scientific supervision of the work. All authors read and approved the final manuscript.*

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ABSTRACT

Aim: The present study aimed to isolate, characterize and select the most effective bacterial endophytes to enhance soybean growth, biomass, and yield.
Methodology: Seven soil samples were collected from the rhizosphere of forage legumes in fields across three agroecological zones of Cameroon. Bacterial endophytes were isolated from soybean roots and nodules and cultured on nutrient agar. The isolates were screened for the tolerance tests, extracellular enzymatic activity, P-solubilization activity, and IAA production. The best isolates were selected using a two-factor block factorial design. Each treatment was replicated six times.
Results: A total of 85 bacterial endophytes were isolated. Characterization results of 22 preselected isolates revealed an optimal growth temperature of 37°C and a pH range between 6 and 7. Furthermore, the 22 isolates solubilized inorganic phosphate, 7 produced IAA, and 8 exhibited amylase activity. PCR analysis of the *nifH* and *nodC* genes showed that the isolates possessed the *nifH* gene as a nitrogen fixation marker and the *nodC* gene as a nodulation marker. The findings show that, out of the 22 bacterial endophyte isolates, NTT1 and BOSH9 were the most effective in increasing plant height by 26.74% and 31.78%, respectively. Additionally, they resulted in an increase in biomass of 94.24% to 120.48% and an increase of 71.59% to 76.70% in grain yield compared to control treatment.
Conclusion: The selected isolates significantly enhance plant growth, increase biomass, and improve soybean grain yield. However, their potential use as biofertilizers in agriculture will require further investigation under real field conditions.

Keywords: Bacterial endophytes; glycine max; nifH gene; nodC gene; nodules; roots; selection.

1. INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] plays a crucial role in food and nutrition security due to its high nutrient content, while its ability to biologically fix atmospheric nitrogen in symbiosis with nitrogen-fixing endophytic bacteria enhances the productivity of agricultural systems. Soybean production in Cameroon has been increasing since 2010, and it is the second most cultivated legume after peanuts, with the rapid development of cultivated areas from 6,705 ha in 2008 to 15,020 ha in 2018 [1,2,3]. Macroeconomic data show that Cameroon imports an average of 20, 000 tons of soybeans worth approximately CFAF 10 billion a year [1] and GMO soybean meal worth CFAF 14 billion [2]. Hence, there is a challenge to increase domestic supply to meet agro-industrial demand, which is indicative of the enthusiasm of farmers for soybean production. According to Wendt and Atemkeng [4], soybean yield ranged from 448 and 709 kg/ha across the first and second planting seasons, with a significant effect of soil nutrients (especially magnesium content) on soybean yield. In Cameroon, crop production is significantly affected by poor soil fertility, with nitrogen (N) and phosphorus (P) being the main limiting factors [5,6,7]. Soil nutrient deficiencies are typically addressed through the use of chemical NPK fertilizers, which can have harmful effects on the environment and human health when overused. As a result, alternative management practices have been developed to promote crop productivity while maintaining sustainability [8,9,10]. Therefore, a promising alternative to increase crop growth and yield is the use of beneficial microbes [11,12], such as nitrogen-fixing endophytic bacteria to enhance nitrogen that play a crucial role in the growth and development of soybean plants, soil fertility, plant nutrition, and protection. Nitrogen is an indispensable component of amino acids, proteins, chlorophyll, and many essential enzymes critical for photosynthesis and plant growth [13]. It is also necessary to partition photosynthetic waste, stimulate root growth, and improve plant uptake of other nutrients [14]. Bacterial endophytes can be found in various parts of the plant, such as roots, stems, leaves, berries, seeds, and xylem sap [15,16,17,18]. Endophyte population density is higher in roots than in any other plant organ. In the root, the average density is 10^5 cfu per g of fresh weight, while the mean values of 10^4 and 10^3 are indicated for the stem and for the leaf, respectively [19]. Many plants harbour a diverse range of bacterial endophytes in their roots, consisting of hundreds of species (219 in 2006) and almost 100 genera (71 in 2006). The most

common genera are Bacillus, Burkholderia, Enterobacter, and Pseudomonas [15,17,19]. The aim of this study was to characterize bacterial endophyte isolates after isolation and to select the most effective to promote the growth, biomass, and yield of forage legumes.

2. MATERIALS AND METHODS

2.1 Description of the Study Site, Sampling Procedure, and Soil Treatment

The study site was conducted in Garoua (Bocklé), located in the north region of Cameroon. The site's latitude and longitude are 9 ° 18′05 ′ North and 13 ° 24′51 ′ East, respectively, and it has an altitude of 249 m above sea level. Garoua has a savannah climate with a dry winter (Aw) according to the Koppen-Geiger classification and an average annual temperature of 800 mm. The climate is tropical and belongs to the sodano-Sahelian category. We randomly picked seven soils from the rhizosphere of forage legumes (soya, peanuts, and stylosanthes) in fields across three agroecological zones of Cameroon. Zone 1 (Extreme North and North region), Zone II (Adamawa region) and Zone V (Centre region). Using an auger, we collected 30 kg of soil from each sampling point at a depth of 3-20 cm from all fields. After collection, we thoroughly mixed the soil samples to form a composite sample. We mixed the soil samples with coarse sand in a 3:1 ratio to aerate them. Then, we separated particles larger than 2 mm by passing the soil and sand mixture through a coarse sieve with a 2-mm mesh.

2.2 Plant and Microbial Material

Soybean seeds (Houla 1, Docko, and TGX 1910-14F variety) were purchased from the Institute of Agricultural Research for Development (IRAD) and from the National Veterinary Laboratory (LANAVET). A total of 22 bacterial endophytic isolates (BOSH4, BOSH8, BOSH9, BOSD1, BOSD2, BOSD3, BOSD4, BOSD5, BOSD6, BOSD12, BOT1, BOT2, BOS2, SPT1, SPT2, SPS2, SPS3, YAT2, MBT2, MBS2, MBS3 et NTT1) were obtained from soybean roots and nodules. These isolates were selected for their ability to fix nitrogen and promote nodulation in forage legumes. Two reference bacterial strains, *Bradyrhizobium japonicum* and Phosphorus Solubilizing Microorganism (PSM), obtained from the Yaounde I and the National Veterinary Laboratory, were used. Additionally, an Arbuscular Mycorrhizal Fungus (AMF) inoculum was obtained from the GIC Agribiocam and used as microbial material.

2.3 Isolation of bacterial endophytes from soybean roots and nodules

The root fragments, measuring 1-2 cm, were disinfected by soaking them in 2% sodium hypochlorite for 10 minutes, followed by 70% ethanol for 2 minutes. After that, they were rinsed three times in sterile distilled water for 1 minute each. The fragments were then ground separately in sterile mortars under strict aseptic conditions and allowed to release bacterial endophytes for 15-20 minutes. The juice of the ground root materials was then inoculated in Petri dishes containing culture medium [20,21]. A drop of each extract of the ground root material was inoculated in Petri dishes containing culture medium, including Nutrient Agar (Meat extract 1 g. L-1; yeast extract 3 g. L-1; peptone 5g. L-1; mannitol 5 g. L-1; NaCl 5 g. L-1; agar 15 g. L-1; and 1 L dis. H_20) and Yeast Extract Mannitol Agar (Mannitol 10 g. L-1; $MgSO_4$ $7(H_2O)$ 0.2 g. L-1; K_2HPO_4 0.5 g. L-1; NaCl 0.1 g. L-1; yeast extract 0.5 g. L-1; agar 15 g. L-1; and 1 L dis. H_20; pH 6.8). The boxes will be incubated at 35°C until bacterial colonies appear. Then we isolated endophytic bacterial isolates from Glycine max using the method described by Vincent [22]. First, we disinfected the harvested root nodules using Somasegaran et al. [23]. Subsequently, the sterile nodules were individually crushed in distilled water on a sterile Petri dish. The operation was performed under aseptic conditions. Using a platinum loop heated by the Bunsen burner, the juice extracted from the nodule was spread on a Petri dish containing the specific medium: Yeast extract-mannitol agar + Red Congo. The inoculation process was then carried out using the four-quadrant technique to obtain isolated colonies that could be easily characterized.

2.4 Characterization of Bacterial Endophyte Isolates

2.4.1 Morphological characterization

The bacterial endophyte isolates were morphologically characterized to determine their growth rate (slow or fast), pH changes during growth, and other characteristics. The formation of colonies on Yeast-Extract-Mannitol-Agar plates was monitored daily for a 10-day period,

and the pH changes were observed in medium containing 0.25 mg/l bromothymol blue (BTB). Cultures were incubated for 10 days at 28°C and daily monitored for any changes in colour. Isolates that turned the growth medium yellow were classified as fast growers and acid producers, while those that turned it blue were classified as slow growers and alkaline producers. The isolates were then identified based on their dimensions, colour, shape, transparency, borders, and elevation [24], after incubation at 28°C for 2 to 10 days.

2.4.2 Physiological characterization

Effect of Temperature on Growth of Bacterial Endophytes isolates: The isolates' growth was assessed by inoculating them in nutrient broth and incubating them at different temperatures (28°C, 37°C, 47°C, and 52°C) for 48 hours [25]. Subsequently, the optical density at 520 nm was measured.

Effect of pH on Growth of Bacterial Endophytes Isolates: The growth of isolates across a range of pH values was optimized and standardized by inoculating them onto a nutrient broth medium with pH values ranging from 4 to 9. The inoculated isolates were then incubated at 37°C for 48 hours [26]. Growth was evaluated by measuring the optical density at 520 nm.

Effect of NaCl on Growth of Bacterial Endophytes isolates: The bacterial endophytic isolates growth in nutrient broth medium with varying concentrations (1-5%) of NaCl was examined by inoculating them into the medium. After incubation at 37°C for 48 hours [27], the optical density at 520 nm was measured to determine growth.

2.4.3 Biochemical characterization

Catalase test: Catalase activity of bacterial endophyte isolates was detected by transferring the isolated pure colony onto a clean glass slide using a sterile nicrome wire loop. Then, 3% hydrogen peroxide was added to the slide. The presence of oxygen bubbles indicated positive catalase activity, while the absence of gas bubbles indicated negative catalase activity [28].

Urease activity: The bacterial endophyte isolates were streaked on agar slant in test tubes containing Urea Agar Slant (Pancreatic digest of gelatine 1 g. L-1; dextrose 1 g. L-1; K_2HPO_4 2 g. L-1; Nacl 5 g. L-1; urea 20 g. L-1; phenol red 0.012 g. L-1; agar 15 g. L-1; and 1 L dis. H_20) [29] The tubes were incubated at 37°C for 24 to 48 hours. Development of pink in tubes indicated positive urease activity of the isolates [28].

Amylolytic activity: Amylase production was determined by inoculating isolates on Nutrient Agar with 1% starch and incubating at 37°C for 48 hours. The plates were flooded with Gram's iodine to produce a deep blue coloured starch-iodine complex. Isolates that showed a clear zone in starch agar plates were considered to produce amylase [28].

Phosphate solubilization: Bacterial endophyte isolates were tested for their ability to solubilize insoluble calcium phosphate in Pikovskaya agar medium, as described by 30. Pikovskaya R. [30]. A bacterial colony was placed in Pikovskaya agar medium plates using a sterile loop and incubated at 30 ° C for 7 days. The phosphate solubilizing efficiency was measured based on the halo zones around the colonies, as described by Qureshi et al. [31,32].

Indole 3-acetic acid (IAA) production: Indole acetic acid (IAA) production by bacterial endophyte isolates was tested in Trypticase Soy Broth at 35 ± 2 ◦C for 72 hours. A control was also prepared using Trypticase Soy Broth without bacterial inoculation. To assess IAA production, 0.3 mL (3 drops) of kovac's reagent was added to test tubes containing Trypticase Soy Broth liquid medium and inoculated with endophytic bacteria. A change in colour to red indicated IAA production [33]. The optical density at 530 nm was measured using a spectrophotometer [34].

2.5 PCR Analysis of Bacterial Endophytes

2.5.1 DNA isolation of bacterial endophyte isolates

PCR analysis was used to identify the most optimal bacterial endophyte isolates. This method can help detect various microbial communities. Genomic DNA was extracted from each isolate's overnight culture using a modified version of the Weisburg et al. [35] and [36] method and used as a template for PCR amplification.

2.5.2 PCR *nif*H and *nod*C genes amplification

DNA obtained from the twenty-two bacterial endophytes was subjected to PCR for the simultaneous duplex detection of the *nif*H and *nod*C genes as an indicator of nitrogen fixation and nodulation. *NifH* amplification was performed

using the specific nitrogen fixation primers *nif*HF (TACGGNAARGGSGGNATCGGCAA) and *nif*HI (AGCATGTCYTCSAGYTCNTCCA). For amplification of the *nod*C gene, the specific nodulation primers *nod*CF (AYGTHGTYGAYGACGGTTC) and *nod*CI (CGYGACAGCCANTCKCTATTG) were used, according to Laguerre et al. [37]. The composition of the duplex PCR reaction mixture was as follows: reaction buffer 1X Taq Polymerase, $MgCl_2$ (2.5 mM), dNTP (1.2 mM), Taq Polymerase (0,3U), primers (1 µM nifH+ 0.6 µM nodC, template DNA (40 ng). The amplification conditions on the thermal cycler were an initial denaturation cycle (94°C for 5 min); 35 denaturation cycles (1 min at 94 °C), annealing (45 s at 55°C) and extension (1 min at 72°C); and a final extension cycle at 72°C for 1 min [38,39,40]. Migration on 1.5% agarose gel with a marker of 1 Kb.

2.6 Impact of Bacterial Isolates on Soybean Growth in Plastic Bags

2.6.1 Experimental design

The experiment was carried out using a two-factor block factorial design where the main factor was the Houla 1 soybean variety and the secondary factor comprised of treatments (BOSH4, BOSH8, BOSH9, BOSD1, BOSD2, BOSD3, BOSD4, BOSD5, BOSD6, BOSD12, BOT1, BOT2, BOS2, SPT1, SPT2, SPS2, SPS3, YAT2, MBT2, MBS2, MBS3, NTT1, PSM, *Bradyrhizobium japonicum,* and AMF). Each treatment was replicated six times and a control treatment of uninoculated plants was also included.

2.6.2 Culture condition

Soil was collected from an agricultural field in the Bocklé-Garoua, then. air dried, sieved with a 2mm sieve and mixed with sand in a soil to sand ratio of 3:1. The mixture was autoclaved twice for one hour at 121°C. The best isolates of preselected and identified bacterial endophyte isolates were inoculated in nutrient broth and incubated at 35± 2°C for 48 hours on a shaker at 180 rpm. Soybean seeds (*Glycine max*, variety Houla 1) were sterilized by surface treatment with 2.5% sodium hypochlorite for 3 minutes, followed by 5 washes in sterile distilled water. Four of these treated seeds were then seeded in each 6 L plastic pot, which was filled with a 5kg mixture of sterile soil and sand. The plants were grown in a temperature range of 30-37°C and were irrigated with tap water.

2.7 Statistical Analyses

The collected data were subjected to analysis of variance (ANOVA) using SPSS software version 25.0. Mean separation was performed using Duncan's test at a 5% level of significance. Bacterial endophyte colonies were scored numerically based on their morphological and cultural characteristics. The obtained data was then subjected to hierarchical cluster analysis using the squared Euclidean distance similarity and between-groups linkage procedures in SPSS software version 25.0.

3. RESULTS AND DISCUSSION

A collection of 22 bacterial endophyte isolates was compiled after pre-selection tests based on morphological, physiological, biochemical, and PCR analyses of 85 bacterial endophytes isolated from soybean nodules and roots in seven trapping soils from three agroecological zones of Cameroon. Of these isolates, 81.82% displayed fast growth, while 18.18% were slow-growing on YEMA medium with bromothymol blue (BTB) (refer to Table 1). All 22 bacterial isolates showed circular colonies of bacilli, cocci, and coccobacillus type. The pH of the culture medium, as indicated by BTB, became acidic for fast growing isolates within 24 to 48 hours after incubation and the culture medium became yellow, as described by [41].

These results are consistent with those of [42], who also observed fast growing isolates isolated from cowpea plants. The isolates exhibited gram-negative or gram-positive characteristics and had cells that were bacilli, coccobacilli, or cocci in shape. All were cultured on YEMA medium containing Congo red dye. The inability of the isolates to absorb Congo red dye was a distinguishing feature [43].

3.1 Physiological Characterization

All isolates showed optimal growth in the pH range of 6 to 7 in the nutrient broth medium (Table 2). BOSH9 showed the best growth at pH 7 (1.15), while BOSD3 and SPS3 exhibited the lowest growth values among all isolates, with absorbance values of 0.26 and 0.25 at 520 nm, respectively. These results are similar to those obtained by [44] in their study on the isolation of endophytic bacteria from the root and leaf tissues of the *Prosopis cineraria* plant. The strains were found to grow best at a temperature of 37°C and a pH of 7, which confirms earlier research.

Table 1. Collection of bacterial endophyte isolates

Isolates / Characteristics	BOSH4	BOSH8	BOSH9	BOSD1	BOSD2	BOSD3	BOSD4	BOSD5	BOSD6	BOSD12	BOT1	BOT2	BOS2	SPT1	SPT2	SPS2	SPS3	YAT2	MBT2	MBS2	MBS3	NTT1
Gram	-	+	-	+	+	-	+	-	-	-	-	+	-	+	-	+	-	+	+	+	+	+
Colony shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Type of colony	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Coccobacillus	Coccobacillus	Coccobacillus	Bacillus	Bacillus	Coccobacillus	Bacillus	Bacillus	Cocci	Bacillus	Coccobacillus	Bacillus
Colony size	Mean	Small	Mean	Mean	Small	Mean	Mean	Small	Mean	Mean	Small	Mean	Very small	Very small	Mean	Mean	Small	Mean	Small	Small	Very small	Mean
Colony diameter range (mm)	2-4	2	2-4	2-4	2	2-4	2-4	2	2-4	2-4	2	2-4	1	1	2-4	2-4	2	2-4	2	2	1	2-4
Colour	Light pink	White	Light pink	Pink	Pink	Light pink	Light pink	Light pink	Light pink	Light pink	Light pink	Light pink	Light pink	White	Light pink	Light pink	Pink	Light pink	Light pink	Pink	White	Light pink
Colony type of growth	Fast	Fast	Fast	Fast	Fast	Fast	Fast	Fast	Fast	Fast	Slow	Fast	Slow	Fast	Fast	Slow	Fast	Fast	Fast	Fast	Slow	Fast
Colony growth time (hours)	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h	120h	24h	120h	24h	24h	120h	24h	48h	48h	48h	120h	48h
Regrouping	Individualised	In chain	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	Individualised	In clusters	individualised	Individualised	Individualised
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Margin	Regular	Regular	Regular	Regular	Regular	Regular	Regular	Regular	Regular	Regular	Whole	Regular	Whole	Regular	Whole	Regular	Whole	Regular	Regular	Regular	Regular	Regular
Fresh state	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile	Mobile

Table 2. Effects of pH on Growth of bacterial endophyte isolates

	pH				
Isolate Code	**4**	**5**	**6**	**7**	**8**
BOSH4	0.54	0.64	0.24	0.48	0.42
BOSH8	0.19	0.73	0.51	0.52	0.55
BOSH9	0.25	0.58	0.68	1.15	0.47
BOSD1	0.44	0.42	0.30	0.49	0.21
BOSD2	0.36	0.39	0.47	0.29	0.20
BOSD3	0.27	0.33	0.31	0.26	0.13
BOSD4	0.61	0.62	0.82	0.54	0.44
BOSD5	0.25	0.42	0.43	0.51	0.31
BOSD6	0.34	0.41	0.38	0.40	0.26
BOSD12	0.15	0.48	0.43	0.58	0.14
BOT1	0.76	0.87	0.76	0.69	0.26
BOT2	0.20	0.40	0.39	0.44	0.59
BOS2	0.41	0.67	0.63	0.67	0.13
SPT1	0.55	0.36	0.69	0.35	0.28
SPT2	0.78	0.71	0.49	0.30	0.66
SPS2	0.71	0.84	0.85	0.81	0.65
SPS3	0.08	0.66	0.34	0.25	0.15
YAT2	0.54	0.43	0.23	0.43	0.06
MBT2	0.50	0.74	0.30	0.53	0.14
MBS2	0.08	0.25	0.58	0.39	0.34
MBS3	0.50	0.70	0.60	0.46	0.53
NTT1	0.42	0.62	0.71	0.50	0.83

Table 3 shows the growth results of bacterial endophyte isolates at various temperatures. The results indicate that the tolerance to temperature varies between isolates and at different temperatures (28°C, 37°C, 47°C, and 52°C). All 22 isolates exhibited good growth at 28°C, very good growth at 37°C, average growth at 47°C, and reduced growth at 52 ° C. BOSH9 and BOS2 showed the best growth at 37°C, with absorbance values of 1.15 and 0.97 at 520 nm, respectively. Most of the isolates showed an optimal growth temperature of 37°C. These results were in agreement with those of [28].

All the isolates in Table 4 grew at 0.1% NaCl, but only 77% of them were resistant to a salt concentration of 1%. Furthermore, 59% of the isolates were resistant to a salt concentration of 2%, and an additional 23% were resistant to a salt concentration of 3%. BOSD5 and SPT1 were the isolates that exhibited superior growth in salt concentrations higher than 3%, with OD values of 0.31 and 0.39, respectively. These findings contradict the results of [45], who established that 100% of the isolates were able to grow in 2% sodium chloride (w/v), and 65% could do so in 3% NaCl. However, these results are consistent with the findings of [46] for Rhizobium sp. isolated from Phaseolus vulgaris in Morocco, which can tolerate 3% NaCl. According to [47], the microbiological isolates ability to tolerate NaCl could be attributed to the presence of osmoprotective molecules like proline in bacteria.

3.2 Biochemical Characterization

All bacterial endophytes (Table 5) tested positive for catalase and 16 of 22 bacterial isolates were positive for urease. Only 8 isolates (BOSH4, BOSH9, BOSD5, BOSD6, BOSD12, BOT1, BOS2, and MBT2) exhibited amylase activity. Among these, BOSD6 demonstrated the highest amylase activity, measuring 8.75±0.01 mm² in diameter of the clean zone. Bacterial endophytes produce extracellular hydrolytic enzymes that indirectly promote plant growth and protect against pathogens [48, 49]. Catalase activity is crucial for bacteria to reproduce by avoiding cellular toxicity. Urease test was performed to determine the ability of the isolates to break down urea into simple forms of nitrogen that can be rapidly absorbed by plants. The results indicated that 16 isolates had the potential to degrade urea to nitrogen forms, while six isolates had negative urease tests. [44] also reported high urease activity of *Bacillus subtilis* strain isolated from roots of *Prosopis cineraria*. Our results indicate that eight out of 22 tested

bacterial endophyte isolates exhibited amylolytic activity. Furthermore, [49] isolated endophytic *Bacillus* from two mangrove species in Brazil that showed extracellular amylase activity.

The 22 bacterial endophyte isolates demonstrated a marked capability to solubilize inorganic phosphate, resulting in clear zones on the Pikovskaya medium. The width ranged from 0.07 ± 0.01 to 7.87 ± 0.83 mm^3. Seven isolates were shown to be producers of IAA when subjected to Kovac reagent, as indicated in Table 6. SPT1 showed the highest IAA production at 2.21 µg·mL-1.

Indole-3-acetic acid (IAA) is a phytohormone that can be produced by plants and various microorganisms. IAA promotes plant growth and contributes to plant-microorganism interactions [50]. In this study, seven bacterial endophyte isolates were found to have the ability to produce IAA in the presence of Kovac's reagent and in the absence of tryptophan. Although most microorganisms use tryptophan in the synthesis of IAA [51,52], bacterial endophytes have the advantage of producing IAA without the need for tryptophan supplementation. All 22 bacterial endophytes solubilized inorganic phosphate in Petri dishes. These bacteria can help plants obtain insoluble forms of phosphate, such as apatite, by releasing protons and organic acids, mainly gluconic acid, making phosphate available for uptake by plants [53,54]. These bacteria can produce enzymes that mineralize organic phosphorus, making it available to plants [53]. Microorganisms can absorb immobile nutrients, such as P, from soils and transfer them to their host plants, which is one of the main effects of microbial symbiosis.

3.4 *nifH* and *nodC* Genes Detection

The study investigated the presence of the *nifHF* and *nifHI* genes as markers for nitrogen fixation, a crucial part of the nitrogenase system. Furthermore, the *nodCF* and *nodCI* genes were examined as markers of nodulation using isolates (Fig. 1a). PCR analysis revealed that the isolates (BOSH4, BOSH9, BOSD3, BOSD4, BOSD6, BOSD12, BOSH8, BOSD1, BOSD2, BOSD4) were capable of nitrogen fixation and nodule formation. The presence of *nifH*, which confers the ability to fix nitrogen, was confirmed by amplification of a 780-890 bp PCR product corresponding to the *nifH* gene fragment and a 930-1300 bp PCR product corresponding to the *nodC* gene fragment in the duplex PCR.

Table 3. Effects of Temperature on growth of bacterial endophyte isolates

	Temperature (°C)			
Isolate Code	**28**	**37**	**47**	**52**
BOSH4	0.12	0.48	0.33	0
BOSH8	0.17	0.52	0.22	0
BOSH9	0.09	1.15	0.22	0
BOSD1	0.18	0.65	0.16	0
BOSD2	0.18	0.64	0.15	0
BOSD3	0.10	0.29	0.14	0
BOSD4	0.11	0.54	0.30	0
BOSD5	0.15	0.72	0.51	0.19
BOSD6	0.10	0.40	0.07	0
BOSD12	0.14	0.58	0.63	0
BOT1	0.38	0.69	0.48	0.07
BOT2	0.25	0.44	0.51	0.04
BOS2	0.33	0.97	0.95	0.14
SPT1	0.27	0.35	0.54	0.39
SPT2	0.30	0.81	0.66	0
SPS2	0.72	0.95	0.81	0.14
SPS3	0.26	0.68	0.25	0
YAT2	0.31	0.49	0.43	0.14
MBT2	0.32	0.53	0.25	0
MBS2	0.13	0.45	0.39	0.15
MBS3	0.38	0.75	0.46	0.32
NTT1	0.21	0.50	0.45	0

Table 4. Effects of NaCl on the Growth of Bacterial Endophyte Isolates

	NaCl (%)			
Isolate Code	**0.1**	**1**	**2**	**3**
BOSH4	0.48	0	0	0
BOSH8	0.52	0.03	0	0
BOSH9	1.15	0.38	0	0
BOSD1	0.66	0.01	0	0
BOSD2	0.30	0	0	0
BOSD3	0.26	0	0	0
BOSD4	0.54	0	0	0
BOSD5	0,51	0.42	0.32	0.31
BOSD6	0.40	0	0	0
BOSD12	0.58	0.44	0.01	0
BOT1	0.69	0.43	0.19	0
BOT2	0.44	0.43	0.04	0
BOS2	0.67	0.39	0.07	0
SPT1	0.45	0.44	0.44	0.39
SPT2	0.30	0.16	0.12	0.01
SPS2	0.81	0.31	0.23	0
SPS3	0.25	0.09	0.02	0.02
YAT2	0.43	0.04	0	0
MBT2	0.53	0.23	0.16	0
MBS2	0.39	0.36	0.35	0
MBS3	0.46	0.38	0.05	0
NTT1	0.50	0.15	0.13	0.06

Table 5. Extracellular enzymatic activities of bacterial endophyte isolates

Bacterial isolates	**Amylase Diameter of Clean Zone (mm^2)**	**Urease**	**Catalase**
C	0^a	-	-
BOSH4	2.75 ± 0.00^c	+	+
BOSH8	0^a	+	+
BOSH9	2.5 ± 0.01^b	+	+
BOSD1	0^a	+	+
BOSD2	0^a	+	+
BOSD3	0^a	+	+
BOSD4	0^a	+	+
BOSD5	5.75 ± 0.02^f	+	+
BOSD6	8.75 ± 0.01^h	+	+
BOSD12	6.75 ± 0.01^g	+	+
BOT1	2.5 ± 0.01^b	-	+
BOT2	0^a	+	+
BOS2	3.25 ± 0.03^d	+	+
SPT1	0^a	+	+
SPT2	0^a	-	+
SPS2	0^a	-	+
SPS3	0^a	-	+
YAT2	0^a	-	+
MBT2	4.05 ± 0.01^e	+	+
MBS2	0^a	+	+
MBS3	0^a	-	+
NTT1	0^a	+	+

C: control without bacterial inoculation. Different letters between lines in the same column denote that mean values are significantly different ($p \leq 0.05$) by Duncan's test, means ± standard Error. – denotes no enzyme production; + denotes enzyme production.

Table 6. IAA production and phosphate solubilization of bacterial endophyte isolates

Bacterial isolates	IAA production	P Solubilization Diameter of Clean Zone (mm^3)
C	0^{a}	0^{a}
BOSH4	0^{a}	2.36 ± 0.21^{f}
BOSH8	0^{a}	7.87 ± 0.83^{h}
BOSH9	0^{a}	0.33 ± 0.02^{abc}
BOSD1	0^{a}	0.36 ± 0.05^{abc}
BOSD2	0^{a}	1.57 ± 0.25^{de}
BOSD3	0^{a}	0.33 ± 0.03^{abc}
BOSD4	0^{a}	1.43 ± 0.34^{d}
BOSD5	0^{a}	1.93 ± 0.52^{e}
BOSD6	0^{a}	0.42 ± 0.07^{abc}
BOSD12	0^{a}	6.99 ± 0.60^{g}
BOT1	1.93 ± 0.00^{f}	0.07 ± 0.01^{ab}
BOT2	1.41 ± 0.00^{e}	0.28 ± 0.04^{abc}
BOS2	1.09 ± 0.00^{d}	0.07 ± 0.01^{ab}
SPT1	2.21 ± 0.00^{h}	0.36 ± 0.04^{abc}
SPT2	0^{a}	0.07 ± 0.01^{ab}
SPS2	2.10 ± 0.01^{g}	0.42 ± 0.04^{abc}
SPS3	0^{a}	0.34 ± 0.04^{abc}
YAT2	0.16 ± 0.00^{b}	0.14 ± 0.04^{ab}
MBT2	1.09 ± 0.00^{d}	0.14 ± 0.01^{ab}
MBS2	0^{a}	0.36 ± 0.04^{abc}
MBS3	0^{a}	0.49 ± 0.04^{bc}
NTT1	0^{a}	0.16 ± 0.02^{ab}

C: control without bacterial inoculation. Different letters between lines in the same column denote that mean values are significantly different (p ≤ 0.05) by Duncan's test, means ± standard Error

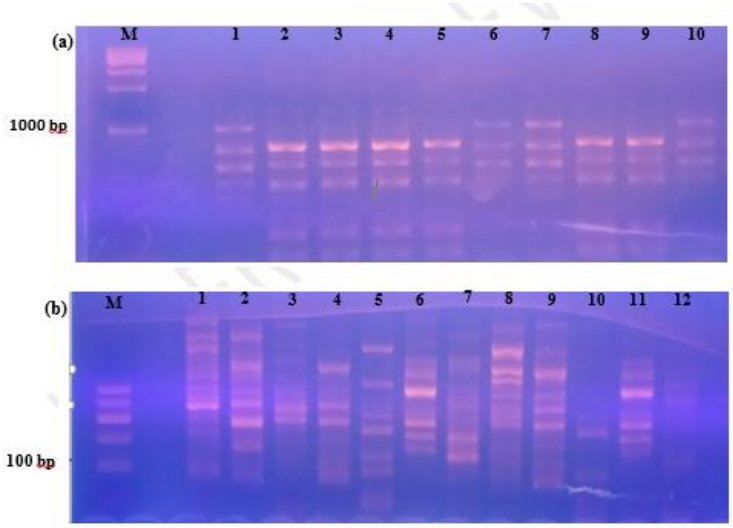

Fig. 1. Duplex-PCR Simultaneous duplex amplification of the *nif*H and *nod*C genes of legume isolates from *G. max* (Houla 1, Docko, and TGX 1910-14F variety)

Caption 1a: Agarose gel electrophoresis of duplex-PCR amplification of nifHF, nifHI, and nodCF, nodCI with conditions of 1.5% agarose gel, 40 ng of DNA template loaded per lane. (Fig 1a) M, 1kb Marker ladder; 1, isolate BOSH4; 2, isolate BOSH8; 3, isolates BOSH9; 4, isolate BOSD1; 5, isolate BOSD2; 6, isolate BOSD3; 7, isolate BOSD4; 8, isolate BOSD5; 9, isolate BOSD6 and 10, isolate BOSD12.

Caption 1b : M, 100 bp Marker ladder ; 1, isolate BOT2 ; 2, isolate SPT2; 3, isolate BOS2; 4, isolate YAT2; 5, isolate BOT1; 6, isolate SPS3; 7, isolate MBS3; 8, isolate NTT1; 9, isolate MBS2; 10, isolate SPS2; 11, isolate MBT2; 12, isolate SPT1.

The 12 isolates, including BOT1, BOT2, BOS2, SPT1, SPT2, SPS2, SPS3, YAT2, MBT2, MBS2, MBS3, and NTT1 (Fig. 1b), produced a 780-890 bp PCR product by duplex PCR. Regarding nodulation, 10 of the 12 isolates tested contain the *nodC* gene fragment, while isolates SPS2 and SPT1 do not.

Multiplex PCR is a commonly used technique in soil and environmental microbiology, especially in rhizobiology [55]. The authors of the study identified a 360 bp amplicon corresponding to the *nifH* gene and a 980 bp amplicon corresponding to the *nodC* gene when amplified alone. However, when amplified by duplex PCR, the amplicon of the *nodC* gene was shown to be significantly greater than 980 bp. According to [37], the size of the *nodC* gene amplicon ranges from 930-1300 bp, while that of the *nifH* gene ranges from 780-890 bp in six bacterial genera.

3.5 Phenotypic Characteristic Analysis of Bacterial Endophyte Isolates

The phenotypic characteristics **(Fig. 2)** were compared using mean distance analysis between the groups in a cluster composed of multiple isolates, subdivided into five groups. Group 1 includes fast-growing isolates (BOSH8, BOSD1, BOSD2, BOSD4, BOT2, SPT1, YAT2, MBS2 and NTT1), with morphological characteristics similar to those of the *Bacillus* sp. reference strain: gram reaction (positive). Group 2 includes isolates (SPT2, SPS3, BOS2 and BOT1) that share similar in both morphological and biochemical characteristics with the *Pseudomonas* sp. reference strain. These characteristics include a negative Gram reaction and rod-shaped colonies. Group 3 contains fast-growing isolates (BOSD6, BOSD12, BOSD3, BOSH9, BOSH4 and BOSD5), which share phenotypic characteristics such as negative gram reaction, colony diameter range of 2-4mm and convex elevation such as *Rhizobium* sp. reference isolate. Group 4 consists of isolates (SPS2 and MBS3) with morphological characteristics similar to the reference strain of *Paenibacillus* sp. Group 5 represents isolate MBT2 with morphological characteristics similar to the reference strain of *Staphylococcus* sp.

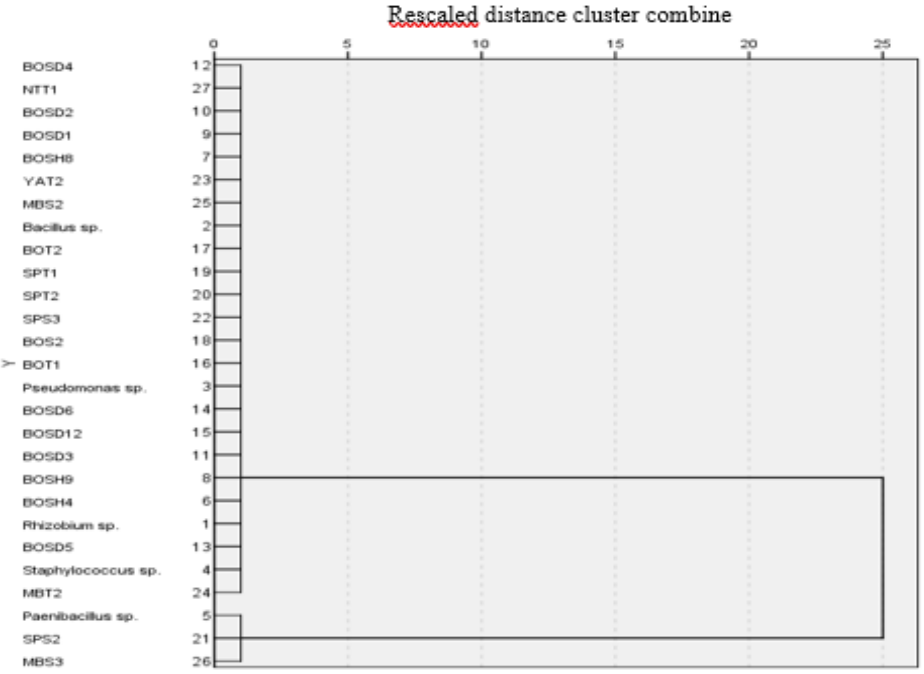

Fig. 2. Dendrogram showing the phenotypic relationships generated between bacterial endophyte isolates from agricultural soils of Bocklé, Jalingo, Mbangassina, Ngaounderé, Ntui, Sanguéré paul, Yagoua in Cameroon

caption 2: Dendrogram of the mean distance analysis between the groups in a cluster consisting of multiple isolates. (Fig. 2), Group 1 (Bacillus spp.); BOSH8, BOSD1, BOSD2, BOSD4, BOT2, SPT1, YAT2, MBS2, and NTT1, Group 2 (Pseudomonas spp.); SPT2, SPS3, BOS2, and BOT1, Group 3 (Rhizobium spp.); BOSD6, BOSD12, BOSD3, BOSH9, BOSH4, and BOSD5, Group 4 (Paenibacillus spp.); SPS2 and MBS3, Group 5 (Staphylococcus sp.); MBT2

Table 7. Effect of bacterial endophytes isolates on the growth, biomass and yield of soybean

Treatments	Number of leaves /plants	Plant height (cm)	Leaf area (cm2)	Flowers number/ plant	Total weight of dried biomass (g/plant)	Seeds weight (g/plant)
C	3.74 ± 0.59a	19.82 ± 0.80a	17.36 ± 0.83ab	6.53 ± 0.80a	8.69 ± 0.46bc	7,04 ± 0.32g
AMF	4.24 ± 0.93a	24.20 ± 1.84bcd	19.61 ± 2.45abcde	12.39 ± 0.07k	12.29 ± 0.97hij	3,81 ± 0.14bc
Brady J	3.54 ± 0.75a	23.53 ± 2.19abcd	17.56 ± 0.79abc	9.36 ± 0.02fh	12.54 ± 0.97hij	2,89 ± 0.08a
PSM	3.78 ± 0.59a	24.50 ± 2.15abcd	21.16 ± 1.59abcdef	9.06 ± 0.09efh	12.26 ± 0.88hij	7,65 ± 0.16h
BOSH 4	4.24 ± 0.95a	23.87 ± 2.43abcd	20.71 ± 1.54cdefg	12.87 ± 0.08k	14.07 ± 0.44kl	7,57 ± 0.21h
BOSH 8	3.83 ± 0.62a	25.29 ± 3.21bcd	21.39 ± 1.25efg	11.16 ± 0.12j	11.00 ± 0.71efghi	4,87 ± 0.09de
BOSH 9	4.87 ± 0.29a	26.12 ± 3.80cd	25.93 ± 2.26h	17.6 ± 0.08o	19.16 ± 1.09o	12,44 ± 0.24l
BOSD 1	4.16 ± 0.95a	23.87 ± 2.23abcd	20.02 ± 1.68bcdef	8 ± 0.12bcd	11.27 ± 1.08fghi	10,66 ± 0.94k
BOSD 2	3.41 ± 0.29a	23.28 ± 1.35abcd	20.25 ± 1.49bcdefg	7.33 ± 0.08abc	7.44 ± 1.12ab	4,96 ± 0.10de
BOSD 3	3.74 ± 0.47a	21.07 ± 2.51abc	17.25 ± 1.53ab	8.16 ± 0.08cde	11.30 ± 0.91fghi	4,08 ± 0.01c
BOSD 4	3.95 ± 0.41a	19.45 ± 2.56a	16.55 ± 1.53a	9.5 ± 0.93h	9.33 ± 0.36cde	6,13 ± 0.12f
BOSD 5	4.16 ± 0.51a	25.87 ± 1.37cd	21.45 ± 1.65efg	12 ± 0.93k	14.56 ± 0.58lm	4,77 ± 0.21de
BOSD 6	3.91 ± 0.99a	24.75 ± 2.38abcd	17.83 ± 2.21abcd	8.85 ± 0.93defh	10.06 ± 0.88cdefg	8,51 ± 0.24i
BOSD 12	3.91 ± 0.89a	25.12 ± 2.77bcd	23.03 ± 0.95fgh	14 ± 0.93l	11.07 ± 0.64efghi	9,59 ± 0.16j
BOT 1	4.37 ± 0.79a	25.33 ± 3.60bcd	23.39 ± 2.37gh	14.33 ± 0.93lm	16.79 ± 0.14n	8,92 ± 0.09i
BOT 2	4.08 ± 0.97a	25.03 ± 3.61bcd	20.32 ± 0.94bcdef	14 ± 0.93l	11.78 ± 0.55ghi	4,44 ± 0.19cd
BOS 2	4.33 ± 0.91a	26.66 ± 2.95d	21.04 ± 2.02defg	12.7 ± 0.93k	17.21 ± 1.29n	8,82 ± 0.10i
SPT 1	4.12 ± 0.97a	25.70 ± 1.87cd	20.96 ± 0.89defg	12.5 ± 0.93k	15.75 ± 0.67mn	6,74 ± 0.18fg
SPT 2	4 ± 0.70a	24.49 ± 2.82abcd	20.30 ± 1.24bcdefg	12.16 ± 0.93k	14.11 ± 0.59lm	6,76 ± 0.17fg
SPS 2	3.54 ± 0.49a	23.24 ± 2.33abcd	19.09 ± 0.69abcde	8.5 ± 0.93def	9.74 ± 0.91cdef	3,39 ± 0.26ab
SPS 3	4.16 ± 0.71a	23.78 ± 2.90abcd	19.45 ± 0.69abcde	10.33 ± 0.93i	10.69 ± 0.27defghi	5,20 ± 0.16de
YAT 2	3.54 ± 0.57a	20.08 ± 3.76ab	18.98 ± 0.69abcdef	7.16 ± 0.93ab	8.90 ± 0.77bcd	3,06 ± 0.04a
MBT 2	4.08 ± 0.69a	24.37 ± 2.79abcd	19.87 ± 0.69abcdef	8.53 ± 0.93def	8.75 ± 0.33bc	8,65 ± 0.92i
MBS 2	3.87 ± 0.53a	21.70 ± 2.62abcd	17.05 ± 0.69a	8.5 ± 0.93def	6.59 ± 0.86a	3,81 ± 0.07bc
MBS 3	3.58 ± 0.62a	23.26 ± 2.21abcd	19.10 ± 0.69acde	6.66 ± 0.93a	10.74 ± 0.42efghi	7,17 ± 0.30gh
NTT 1	4.66 ± 0.78a	25.12 ± 3.27bcd	23.37 ± 0.69gh	15 ± 0.93n	16.88 ± 1.30n	12,08 ± 0.24l

BOSH4, BOSH8, BOSH9, BOSD1, BOSD2, BOSD3, BOSD4, BOSD5, BOSD6, BOSD12, BOT1, BOT2, BOS2, SPT1, SPT2, SPS2, SPS3, YAT2, MBT2, MBS2, MBS3 and NTT1 are potential bacterial endophyte isolates for increasing the growth, biomass and seed yield of the soybeans in plastic bags; C (control without bacterial inoculation); AMF (control with Arbuscular Mycorrhizal Fungus); Brady j (control with Bradyrhizobium japonicum); PSM (control with Phosphorus Solubilising Microorganism). Different letters between lines in the same column denote that means are significantly different (p ≤ 0.05) by Duncan's test, means ± standard Error

In this study, the phenotypic characteristics assigned the 22 isolates to established groups of bacterial endophyte isolates (*Rhizobium* spp.), including microorganisms from the genera: *Bacillus* spp.*, Paenibacillus* spp.*, Staphylococcus* sp*.* and *Pseudomonas* spp. The latter representative genera are not nitrogen-fixing bacteria in the conventional sense [56]. Their ability to fix nitrogen could possibly be attributed to lateral gene transfer. However, the evolutionary impact of the mechanisms of lateral gene transfer remains poorly understood. The PCR analysis results were confirmed by morphological and biochemical data.

3.6 Evaluation of the Impact of Bacterial Endophyte Isolate Application on the Growth, Biomass, and Yield of Soybeans Cultivated in Plastic Bags

Table 7 presents the results that demonstrate the effects of bacterial endophyte isolates on soybean growth, biomass, and grain yield in plastic bags. Out of the 22 isolates inoculated into soybean plants, 6 isolates (BOSH9, BOSD5, BOT1, BOS2, SPT1, and NTT1) had a greater impact than the others. The plant treated with the BOSH9 isolate showed a 30.21% increase in the number of leaves, 169.52% in the number of flowers and 49.36% in the leaf area compared to the control treatment. Isolates NTT1 and BOSH9 significantly increased plant height by +26.74% and +31.78% compared to the control, respectively. Furthermore, BOSH 9 increases plant height by +7.93% compared to (AMF), by +11% compared to (Brady j) and by +6.61% compared to the treatment (MSP). The highest performing isolate, BOSH9, increased plant biomass by 120.48% and grain yield by 76.70% compared to the control treatment. Inoculating soybean plants with bacterial endophyte isolates in this study significantly increased the number of leaves, plant height, leaf area, number of flowers, biomass, and grain yield, as reported by [12-57]. The increase in soybean growth and yield is consistent with the role of inoculated Plant Growth-Promoting Bacteria (PGPB). Additionally, inoculated bacteria may have released ammonia or produced nitrogenase through their *nifH* genes to fix N_2 and uptake by plant roots to improve growth and yield, as reported for *Bacillus* spp. [58,59,60]. Microbial activity also plays a role in the rhizosphere, as indicated by the presence of acid phosphatase and supported by research carried out by [61]. The superior performance of soybean plants that were inoculated with the BOSH9 isolate is supported by [62], they reported improved soybean growth and N_2 fixation, and grain yield after inoculation with bacterial endophytic strains such as *Bradyrhizobium*. [63] also showed that bacterial endophyte isolates can increase the yield of turmeric rhizomes on a sterile substrate after inoculation, with yields ranging from 42 to 105% higher than the control.

4. CONCLUSION

In this study, we obtained 85 bacterial endophyte isolates from nodules and roots of three soybean varieties grown in seven different soils in Cameroon. Characterization of the bacterial endophyte isolates enabled us to preselect 22 out of 85. The characterization focused on tolerance tests for pH, temperature, and NaCl, as well as extracellular enzymatic activity, P-solubilization activity, IAA production, and detection of nitrogen fixation and nodulation genes. The two best isolates, BOSH9 and NTT1, significantly promoted plant growth, increasing plant height, number of leaves, number of flowers, biomass, and soybean grain yield. However, to demonstrate the beneficial role of bacterial endophytes (BOSH9 and NTT1) in promoting plant growth, especially under real field conditions, further investigation is required to determine their potential use as biofertilizers in agriculture.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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