Asia Pacific Academy of Science Pte. Ltd.

Advances in Analytic Science 2025, 6(1), 3840.
https://doi.org/10.54517/aas3840

Article

Rhizobium pusense associated with chickpea (*Cicer arietinum* L.), in Cuba

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CITATION

Ortega-García M, Ríos-Rocafull Y, Zelaya-Molina L, et al. *Rhizobium pusense* associated with chickpea (*Cicer arietinum* L.), in Cuba. Advances in Analytic Science. 2025; 6(1): 3840. https://doi.org/10.54517/aas3840

ARTICLE INFO

Received: 8 April 2025
Accepted: 28 May 2025
Available online: 4 June 2025

Abstract: Introduction: For many years, chickpeas were considered a restricted host species for nodulation, although recent studies describe greater bacterial diversity associated with the crop, allowing for increased knowledge of this symbiosis to obtain isolates that are efficient in their contribution. **Objective:** To characterize and identify an isolate obtained from chickpea plant nodules and evaluate its effectiveness under field conditions. **Materials and methods:** The research was conducted in the laboratories of two centers: the Instituto de Investigaciones Fundamentales en Agricultura Tropical "Alejandro de Humboldt", Cuba, and the Centro Nacional de Recursos Genéticos, Mexico. The research was also conducted under field conditions in two campaigns during 2018 and 2020 to demonstrate its effectiveness in crop interaction at the first center. Isolate R3 was identified at the taxonomic level by sequencing four genes. A completely randomized design was used for laboratory experiments, while plant trials were conducted using a randomized block design. The results were subjected to an analysis of variance and the means were compared according to the Tukey test ($p < 0.05$). **Results:** Isolate R3 showed variability in its morphophysiological, physiological, and biochemical characteristics. Furthermore, it responded significantly differently than the other treatments in the growth and yield indicators evaluated in chickpeas. **Conclusions:** *Rhizobium pusense* was identified for the first time in Cuba associated with chickpea nodules. The strain showed positive growth at pH levels between 5.5 and 9.0 and temperatures ranging from 29 °C to 38 °C, demonstrating its tolerance to these factors. Its inoculation in chickpeas stimulated nodule formation and increased yield variables.

Keywords: characterization; taxonomy; nodulation; yield

1. Introduction

Chickpea (*Cicer arietinum* L.) was long considered a host species with restricted nodulation capacity, which historically limited studies of plant growth-promoting bacteria (PGPB) associated with this crop primarily to the genus *Mesorhizobium* [1]. Recent studies, however, have documented novel bacterial species associated with chickpea cultivation, including *Rhizobium leguminosarum* [2], *Achromobacter xylosoxidans* [3], *Burkholderia andropogonis*, and *Ochrobactrum ciceri* [4], among others. These findings demonstrate the broader diversity of bacteria capable of forming symbiotic relationships with chickpeas [1].

Research on rhizobia-chickpea symbiosis remains limited due to both the restricted number of beneficial nodulating microorganisms/endosymbionts and chickpea's non-status as a model plant for biological studies [5]. This knowledge gap underscores the need to better understand this symbiotic relationship and its interaction dynamics, particularly as root exudates vary significantly between cultivars in both

chemical composition and concentration. The identification of efficient bacterial isolates capable of associating with higher-nodulating, more productive chickpea cultivars presents a crucial nutritional alternative to reduce mineral fertilizer applications. Consequently, adopting low-input practices like biofertilization emerges as a viable strategy for achieving ecologically sustainable agricultural development [6]. This approach mitigates the negative environmental impacts of excessive agrochemical use while maintaining ecological balance [7].

Chickpea is among the crops that provide benefits to agroecosystems, as it improves soil conditions through biological nitrogen fixation (BNF) in symbiosis with rhizobia, reaching up to 300 kg ha^{-1} of N when cultivated as green manure. It further enhances soil fertility by helping maintain and increase microbial biomass responsible for improving soil structure and making nutrients more accessible, which promotes crop health and increases disease resistance [8]. Additionally, chickpeas stand out as a species of interest for human and animal consumption [9], with a protein content of about 20%, high carbohydrate quantity (40%), high digestibility, richness in unsaturated fatty acids, and low levels of antinutritional elements [10]. Similarly, its consumption is associated with beneficial effects in preventing chronic diseases such as cardiovascular diseases, diabetes, some types of cancer, and digestive problems [11].

The present work aimed to characterize and identify an isolate obtained from chickpea plant nodules and evaluate its effectiveness under field conditions.

2. Materials and methods

2.1. Study site and plant material

The isolation of presumed rhizobia was performed from nodules collected from healthy and vigorous plants of the chickpea (*C. arietinum*) cultivar Nacional-29 in 2012, in the province of Artemisa, Cuba, on an agrogenic Lixiviated Red Ferralitic soil [12], with a pH of 7.36 and organic matter content of 2.09%. A thermometer, rain gauge, and hygrometer were used to evaluate meteorological variables during the November-February period when experiments were conducted. Average temperatures ranged between 13.5 ℃ and 21.8 ℃, with mean annual precipitation from 1016 to 1024 mm and relative humidity between 66% and 85%.

2.2. Morphological, physiological and biochemical characterization

The rhizobium isolate R3 was cultured by exhaustion (successive dilutions of the sample under sterile conditions) in Petri dishes, in triplicate, on yeast mannitol agar (YMA) culture medium with Congo Red [13]. Its morphology was characterized by describing the colonies formed in a 24-h culture at 30 ℃. The cultural aspects considered for characterization included: time of appearance, shape, color, appearance, edge and texture of the colonies, observed under a stereomicroscope (Leica KL 300 LED, 3X magnification). Micromorphological and staining characteristics were described through Gram staining following the procedure described by Madigan et al. [14], and the isolate's response was visualized under an optical microscope (Leica DM300, 1000X magnification). Acid or base production

and ketolactase production were evaluated. For the former, YMA culture medium with bromothymol blue was used, and for the latter, Yeast Lactose Agar (YLA) medium [13]. The plates were incubated for seven days at 28–30 ℃.

For the physiological-biochemical characterization, ten tests were performed that included detection of cytochrome oxidase and catalase enzymes, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, citrate utilization as a carbon source, indole production from tryptophan, glucose fermentation, sulfur utilization, and motility—the latter assessed by the hanging drop method. In all cases, samples were incubated for two to five days at 28–30 ℃, and tests were performed in triplicate.

2.3. Tolerance to different pH and temperature values

pH tolerance was evaluated using Petri dishes containing YMA culture medium adjusted to different pH values (3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, and 9.5) with 1 N HCl or 1 N NaOH [15]. The isolate was inoculated through a central streak and then incubated at 30 ℃. Growth was observed every 24 h for 12 days [16]. Similarly, tolerance to different temperatures was determined, but in this case the isolate was inoculated on YMA medium and the plates were incubated at temperature values (℃): 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, and 40. For both cases, microbial growth on the medium was considered a positive response, while absence of growth was considered a negative result [17].

2.4. Taxonomic identification

The study utilized isolate R3 from Artemisa province, Cuba, with preservation code CM-CNRG 562 at the Centro Nacional de Recursos Genéticos (CNRG) of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, en Tepatitlán de Morelos, Mexico. The isolate was revitalized on YMA culture medium for subsequent identification. For genomic DNA extraction, the isolate was cultured in tryptone yeast extract (TY) medium [18] for 24 h at 28–30 ℃. A single colony was suspended in 100 µL of sterile ultrapure water and centrifuged at 19,000 × g for 2 min. The cells were resuspended in 100 µL of sodium hydroxide (NaOH, 0.05 M) solution, then heated at 100 ℃ for 4 min. After this period, 900 µL of sterile distilled water was added and centrifuged at 19,000× *g* for 2 min. A 700 µL aliquot of supernatant was mixed with an equal volume of 25% (v/v) glycerol and stored at −20 ℃.

Amplification of 16S rRNA, atpD, gltA, and recA genes (**Table 1**) was carried out by Polymerase Chain Reaction (PCR) in a SelectCycler thermocycler. The primers used were 27f (sequence 5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492r (5′-CGG TTA CCT TGT TAC GAC TT-3′) [19]. The reaction mixture consisted of: 2 µL of each primer at 10 µM concentration, 25 µL of DreamTaq Green PCR Master Mix (2X); 4.0 µL of DNA, and 17 µL of ultrapure water. The PCR protocol included: initial denaturation at 95 ℃ for 5 min; 30 cycles of 95 ℃ for 30 s (denaturation); 59 ℃ for 30 s (hybridization or annealing); and 72 ℃ for 90 s (extension), followed by a final extension at 72 ℃ for 7 min.

Table 1. Primers for gene amplification and sequencing. Centro Nacional de Recursos Genéticos (CNRG), Tepatitlán de Morelos, Mexico. 2020.

Gene	Primer	Sequence (5′-3′)	Source
16S rRNA	785F	GGATTAGATACCCTGGTA	Kumar et al. [20]
	907R	CCGTCAATTCMTTTRAGRTT	
atpD	atpD294F	ATCGGCGAGCCGGTCGACGA	Zhao et al. [21]
	atpD771R	GCCGACACTTCCGAACCNGCCTG	
gltA	gltA428F	CSGCCTTCTAYCAYGACTC	Khalid et al. [22]
	gltA1111R	GGGAGCCSAKCGCCTTCAG	
recA	recA6F	CGKCTSGTAGAGGAYAAATCGGTGGA	Zhao et al.[21]
	recA555R	CGRATCTGGTTGATGAAGATCACCAT	

The amplification product was visualized by electrophoresis on a 0.9% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer. The electrophoresis run was performed for 40 min at 90 V. Gels were photographed using a GoodView photodocumentation system (Beijing SBS Genetech Co. Ltd.) with a 1 μL:50 mL (dye: agarose) ratio. The correct amplicon size (1500 base pairs) was confirmed by comparison with the GeneRuler 1 kb DNA Ladder molecular weight marker (Thermo Scientific #SM1332) under ultraviolet light exposure.

The PCR products were sequenced (MACROGEN Inc., Korea) and the *.abi format sequences were converted to FASTA format using BioEdit software version 7.0.9.0. The sequences were analyzed in NCBI databases using MegaBlast version 2.2.22, and a homologous sequence mining from GenBank was performed using Clustal X version 2.0. The alignment was generated and edited with Seaview version 4.0. Using the consensus sequence, a new analysis was conducted in MegaBlast version 2.2.22 to determine the phylogenetic reconstruction of the sequences. The final identity assignment was based on the concordance of sequence clustering with homologous sequences and a percentage identity greater than 99% in the BLAST databases (nucleotide database and 16S database of Bacteria and Archaea) of leBiBi and EzBiocloud platforms.

2.5. Effectiveness on chickpea growth and yield under field conditions

The trial was conducted in agricultural plots at INIFAT, Cuba, on a Compacted Red Ferralitic soil [12], to measure the effectiveness on growth and yield of chickpea variety Nacional-29 under field conditions. Two strains were used: 1) the experimental isolate R3 under study, and 2) the reference strain (*Mesorhizobium ciceri* INIFAT GR-1), employed for chickpea inoculation in Cuba. In both cases, the inoculants were prepared through a submerged fermentation process in an orbital shaker using LM culture medium. For this, an orbital shaker at 200 rpm was used for 48 h at 28–30 °C, until reaching a concentration of 2.4×10^{10} CFU mL^{-1}. These were diluted in water at a 1:10 (v:v) ratio and applied via seed imbibition of variety Nacional-29 for 15 min. For control treatments, seeds were only soaked in water for the same period. In all cases, seeds were shade-dried on a mesh screen before sowing in a Lixiviated Red Ferralitic soil [12].

The trial included four replicates per treatment with a plot size of 30 m^2, consisting of six rows measuring 5 m long by 6 m wide for each treatment. Plant

spacing was 0.20 m between plants and 0.70 m between rows. Standard cultural practices recommended for chickpea cultivation in Cuba were followed [23]. An absolute control treatment without microorganisms or fertilizers was also included.

At 60 days after sowing, ten plants per treatment were extracted, for which nodule number (units) and nodule dry mass per plant (g) were determined, the latter by using an oven at 70 ℃ for 72 h. At harvest time, the borders of each plot were discarded and ten plants per row were used to determine the following indicators for each one: pod number (units), dry mass of 100 grains, and grain dry mass (g), which were determined using a semi-analytical balance (0.01 g error). To quantify grain dry mass, a precision scale (1 g error) was used.

For laboratory experiments, a completely randomized design was used, while plant trials were conducted under a randomized block design. The results were subjected to analysis of variance (ANOVA) and means were compared using Tukey's test ($p < 0.05$). Statistical analysis was performed using InfoStat software [24].

3. Results

3.1. Morphological, physiological and biochemical characterization

The isolate R3 showed cultural, morphological and staining characteristics similar to those of rhizobia, i.e., small cocobacilli or bacilli, Gram negative, non-sporulating. It grew two days after sowing, with whitish-colored colonies of 1 mm diameter, without Congo red absorption, circular, convex and mucilaginous, with a beige coloration. The cells showed a coccobacillary shape, without spores, Gram negative. Growth showed a color change from green to yellow in YMA culture medium with bromothymol blue, indicating its capacity to produce acids and a negative response to ketolactase. Additionally, it showed positive responses to oxidase, catalase, casein hydrolysis, citrate utilization and motility tests. However, the response was negative for starch hydrolysis, gelatin hydrolysis, indole production from tryptophan, sulfur utilization from growth in Kliger's medium, and cellulose degradation.

3.2. Tolerance to different pH and temperature values

The isolate did not grow at pH values between 3 and 5 or at pH 9.5. However, it showed growth at pH values between 5.5 and 9.0. Similarly, it did not grow at temperatures between 25 and 27 ℃, nor at 40 ℃. Nevertheless, it tolerated ranges from 28 to 39 ℃ (**Table 2**).

Table 2. Growth of isolate R3 obtained from nodule collection in Artemisa province, Cuba, at different pH and temperature levels. Cuba. 2020.

pH values															
3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5		
-	-	-	-	-	+	+	+	+	+	+	+	+	-		
Temperature (℃)															
25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-

Note: Growth (+) absence of growth (-).

3.3. Taxonomic identification

In the phylogenetic tree of strain R3 with the four gene sequences (**Figure 1**), the strain is identified as belonging to the species *Rhizobium pusense*. The identity percentages between strains of different *Rhizobium* species are indicated in **Table 3**.

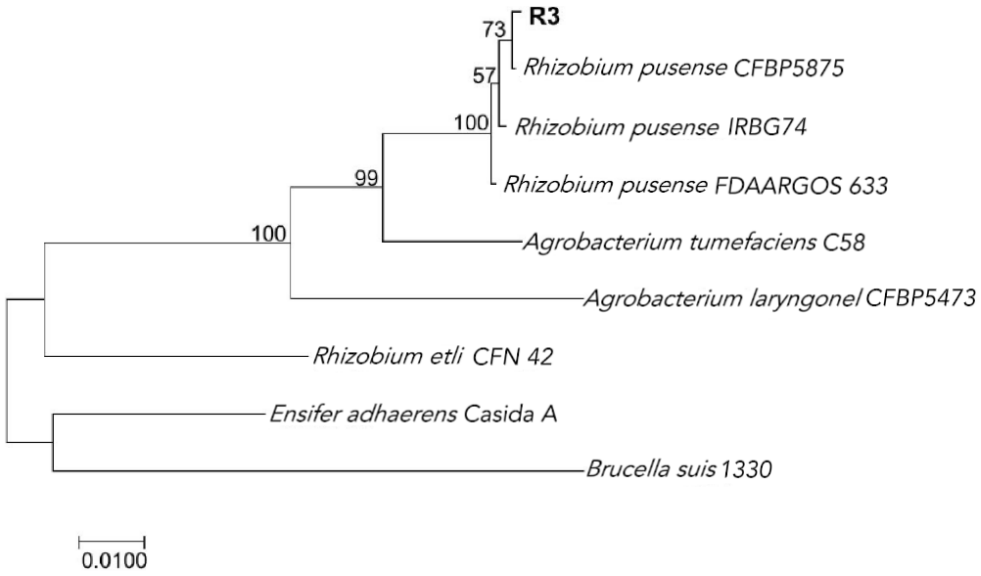

Figure 1. Phylogenetic tree of the R3 strain of *Rhizobium pusense* obtained from nodule collection in the province of Artemisa, Cuba, constructed with the concatenated sequence of the 16S rRNA genes *atpD*, *gltA*, and *recA*, using the maximum likelihood method and the Tamira Nei 93 nucleotide substitution model, with 1000 bootstrap replicates. Centro Nacional de Recursos Genéticos (CNRG), Tepatitlán de Morelos, Mexico. 2020.

Table 3. Percentages of identity with the sequence of strain R3 and related reference strains. Centro Nacional de Recursos Genéticos (CNRG), Tepatitlán de Morelos, Mexico. 2020.

	16S rRNA	***atpD***	***glt*A**	***recA***
Rhizobium pusense FDARGO3_633	99.85	98.19	99.11	99.48
Rhizobium pusense CFBP5875	99.85	99.60	99.25	99.83
Rhizobium pusense IRBG74	99.85	98.79	99.40	99.48
Rhizobium etli CFN_42	94.44	88.58	85.88	88.31

Codes used for the strains: GenBank.16S rRNA: MK108017, *atpD*: OP795820, *gltA*: OP795821, and *recA*: OP795822.

3.4. Effectiveness on chickpea growth and yield under field conditions

The R3 strain showed a better response with significant differences compared to the treatment inoculated with the INIFAT GR-1 strain and the non-inoculated control, with higher nodule number and nodule dry mass (**Figure 2**). Similarly, this result was reflected in the crop yield indicators evaluated, including pod number per plant, 100-grain dry mass, and grain dry mass per plant (**Figure 3**).

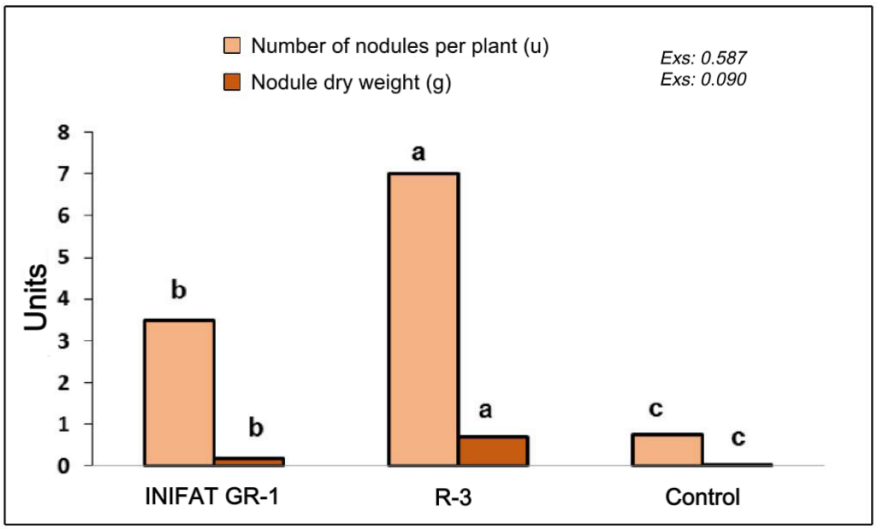

Figure 2. Number and dry mass of nodules of chickpea plants (*Cicer arietinum* L.) of the Nacional-29 variety, inoculated or not (control). Cuba. 2020.

INIFAT GR-1: strain INIFAT GR-1, TA: absolute witness, R3: strain R3. Identical letters do not differ significantly from each other, according to Tukey $\alpha = 0.05$, $n = 10$.

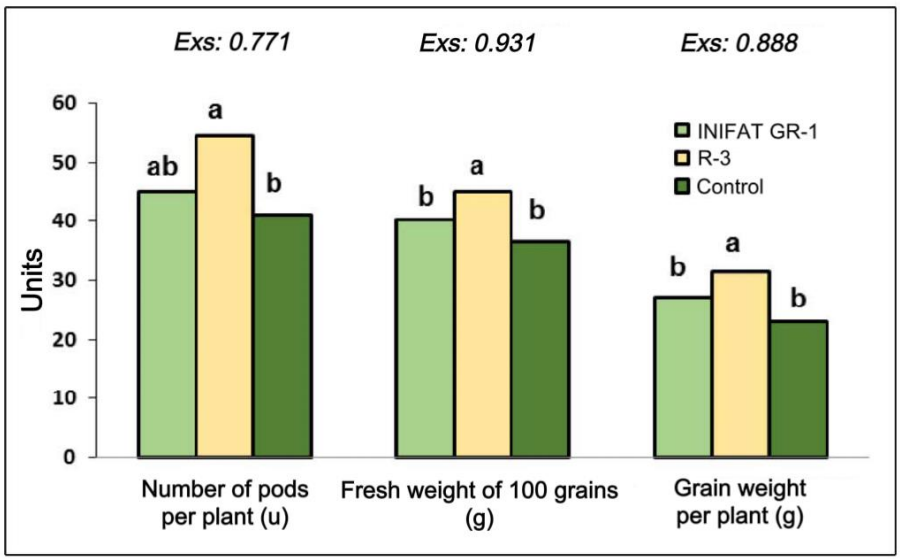

Figure 3. Number of pods per plant, fresh mass (g) of 100 grains, and dry mass (g) of grains per plant in chickpea plants (*Cicer arietinum* L.) of the Nacional-29 variety, inoculated or not (control). Cuba. 2020.

INIFAT GR-1: strain INIFAT GR-1, TA: absolute control, R3: strain R3. Identical letters do not differ significantly from each other, according to Tukey $\alpha = 0.05$, $n = 10$. Cuba. 2020.

4. Discussion

The R3 isolate showed characteristics similar to those described for rhizobia [14]. Notable features included positive catalase and oxidase test results, inability to hydrolyze starch or gelatin (though capable of casein hydrolysis), citrate utilization as a carbon source, and negative responses in Kliger's medium and for indole production from tryptophan [25].

Most rhizobia grow at pH values near neutrality [26]. However, it has been demonstrated that some strains of this family can tolerate pH ranges from 3.5 to 9 [16].

The R3 isolate grew at pH 5.5 and also at pH 9; suggesting the presence of adaptation mechanisms that allow it to survive under these conditions [27]. This characteristic constitutes an advantage for using this microorganism as an active ingredient in biofertilizers to benefit chickpea cultivation, since by maintaining its growth-stimulating effect under different acidity conditions, it could be used in different soil types and agroecosystems.

Rhizobia typically grow at temperatures between 28–30 ℃ [28]. Other studies have reported results similar to or even exceeding those obtained in the present work, with nodule isolates from *Vigna unguiculata* tolerating up to 45 ℃ [16]. This phenomenon of strain tolerance to varying pH and temperature conditions may enhance their multiplication in the rhizosphere and promote greater colonization of host species. Several authors have noted that these adaptive traits increase rhizobia's survival and competitive advantages against other soil bacteria, thereby facilitating legume colonization and atmospheric nitrogen fixation [27].

The phylogenetic analysis revealed that strain R3 belongs to the genus *Rhizobium* and species *pusense*. Originally isolated from chickpea rhizosphere in India, this marks its first reported association with chickpea cultivation in Cuba, demonstrating its potential adaptation to local growing conditions despite being previously undocumented in the country.

The higher number of nodules formed following inoculation with *R. pusense* strain R3 suggests greater affinity between this strain and the chickpea cultivar Nacional-29, resulting in improved root system colonization and bacteroid establishment within nodules, ultimately leading to increased nodule biomass [4]. This enhanced symbiosis translates to greater nitrogen supply to the plant which, combined with other nutrients and water, contributes to increased aerial biomass production. Consequently, this improves photosynthetic capacity and leads to higher pod and grain yields. Supporting these findings, other studies have demonstrated that efficient nodulation provides essential elements for optimal plant growth and development [29]. The R3 strain's performance highlights its potential as an effective biofertilizer candidate for chickpea cultivation systems.

These results represent the first report of *R. pusense* in Cuba associated with chickpea cultivation, suggesting that strain R3 could serve as the active component for a novel bioproduct to enhance crop performance.

5. Conclusions

R. pusense associated with chickpea (*C. arietinum* L.) nodules was identified in Cuba for the first time. The strain showed positive growth at pH 5.5–9.0 and temperatures ranging from 29 to 38 ℃, demonstrating its tolerance to these factors. Its inoculation in chickpea stimulated nodule formation and increased yield parameters. These results provide a foundation for using this strain as an active component in biofertilizers to benefit chickpeas cultivation under Cuban conditions.

Author contributions: All authors contributed equally to the manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: This work was funded by the Institute of Fundamental Research

in Tropical Agriculture “Alejandro de Humboldt”.

Institutional review board statement: Not applicable.

Informed consent statement: Not applicable.

Conflict of interest: The authors declare no conflict of interest.

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