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RESEARCH ARTICLE

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Isolation and evaluation of endophytic bacteria from root nodules of *Glycine max* L. (Merr.) and their potential use as biofertilizers

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Abstract

Aim of study: To isolate and characterize endophytic bacteria inhabiting soybean root nodules collected from two tropical cropping systems in Mexico, and to evaluate the bacterial effects in soybean plants under controlled conditions.

Area of study: The study was carried out at two locations (San Antonio Cayal and Nuevo Progreso municipalities) of Campeche State, Mexico.

Material and methods: Two experimental stages were performed: 1) isolation, morphological and biochemical characterization, and molecular identification of endophytic bacteria from root-nodules of four soybean varieties grown at field conditions; and 2) evaluation of the effects of endophytic isolates on soybean growth and nodule development, and the effects of bacterial co-inoculation on soybean plants, under controlled conditions.

Main results: Twenty-three endophytic bacteria were isolated from root nodules, and identified as *Agrobacterium, Bradyrhizobium, Rhizobium, Ensifer, Massilia, Chryseobacterium, Enterobacter, Microbacterium, Serratia*, and *Xanthomonas*. Under controlled conditions, *Rhizobium* sp. CPO4.13C or *Agrobacterium tumefaciens* CPO4.15C significantly increased the plant height (46% and 41%, respectively), whereas *Bradyrhizobium* sp. CPO4.24C promoted the nodule formation (36 nodules/plant). The co-inoculation of *B. japonicum* USDA110 and *Bradyrhizobium* sp. CPO4.24C enhanced plant growth, height (33.87 cm), root nodulation (69 nodules/plant) and N-fixation (3.10 µmol C_2H_4 h^{-1} $plant^{-1}$) in comparison to the negative control.

Research highlights: Results suggest that the native *Bradyrhizobium* sp. CPO4.24C may be used as a biofertilizer directed to developing sustainable soybean cropping at tropical regions.

Additional keywords: *Bradyrhizobium*; co-inoculation; free-living endophytic bacteria; endophytic symbiotic bacteria; plant growth promotion; soybean; symbiosis.

Abbreviations used: ARA (acetylene reduction assay); BNF (biological N-fixation); CTAB (cetyltrimethylammonium bromide); GPA (glucose-peptone agar); GPA-BP (glucose-peptone agar with bromocresol purple); NDW (nodule dry weight); NN (number of nodules); PCR (polymerase chain reaction); PH (plant height); RDW (root dry weight); RR (Roundup Ready); SDW (shoot dry weight); YMA (yeast extract-mannitol agar medium); YMA-BTB (yeast extract-mannitol agar medium containing bromothymol blue).

Authors' contributions: AAVD performed the experiment and drafted the manuscript; RFC made critical revision of the manuscript for important intellectual content; HVSR acquired, analyzed and interpreted data of bacteria identification; AA supervised the work. All authors read and approved the final manuscript.

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Introduction

Soybean [*Glycine max* (L.) Merr.], is one of the most cultivated legume worldwide due to its high protein content and important industrial by-products (Stacey *et al.*, 2004; Masuda & Goldsmith, 2009). Nitrogen is one of the macro-nutrients required for growth and development of plants, so this nutrient must be supplied

via chemical fertilization and/or biological N fixation (BNF) (de Carvalho *et al.*, 2013; Gai *et al.*, 2017). For soybean, the BNF is carried out in root nodules by symbiotic bacteria known as rhizobia. Rhizobia obtain carbon sources from plants and in return, the bacterial nitrogenase enzyme activity provides ammonia to plants (Santi *et al.*, 2013). The symbiosis between soybean and rhizobia is a complex process involving activities of several genes (de Carvalho *et al.*, 2013; Lira *et al.*, 2015). Many legumes form root nodules that may stimulate plant growth when nodules are formed by compatible and functional rhizobial strains. In this regard, the bacterial establishment is highly specific, but strongly affected by genetic bacterial/plant interactions that determine an efficient symbiotic relationship (Boonkerd & Singleton, 2002). This symbiosis is also largely dependent on environmental conditions (Valencia *et al.*, 2010).

Most soybean varieties fail to nodulate efficiently in tropical soils even though plants are inoculated with competitive rhizobial strains (Kueneman *et al.*, 1984). In this sense, native rhizobia most likely promote greater plant growth since they are more adapted to environmental conditions (Waluyo *et al.*, 2005; Soe & Yamakawa, 2013). Thus, the isolation and re-introduction of highly competitive and effective native rhizobia are important for increasing soybean production.

Besides rhizobia, root nodules may host other endophytic non-symbiotic bacteria (Li *et al.*, 2008; Saïdi *et al.*, 2011; Aserse *et al.*, 2013), and these bacteria are unable to form nodules nor perform N-fixation. However, these endophytic non-symbiotic bacteria may favor plant growth and nutrition, or assist the solubilization of insoluble forms of phosphates in the rhizosphere (Bai *et al.*, 2002; Li *et al.*, 2008; Liu *et al.*, 2010; Stajkovic *et al.*, 2011; Aserse *et al.*, 2013). Co-inoculation of endophytic non-symbiotic bacteria along rhizobia has gained special interest as part of sustainable agriculture, since both bacteria may act synergistically for enhancing legume growth and performance, in comparison to the single inoculation of rhizobia (Bai *et al.*, 2002). For instance, *Rhizobium phaseoli* co-inoculated with either *Pseudomonas* sp. or *Bacillus* sp. Bx, resulted in significant increase in the stem dry weight of beans (Stajkovic *et al.*, 2011). Similarly, the co-inoculation of *Mesorhizobium gobiense* with *Bacillus pumilus* B402 resulted in increased number of nodules and growth of *Sphaerophysa salsula* (Pall.) DC. (Deng *et al.*, 2011). Also, an increased root nodulation in *Medicago sativa* L. was reported due to the co-inoculation of *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* (Wang *et al.*, 2006). Similarly, an increase in nodulation of *Wisteria sinensis* (Sims) DC. was observed by combining *S. meliloti* and *Agrobacterium* sp. II CCBAU 21244 (Liu *et al.*, 2010). In the case of soybeans, the co-inoculation of *Bradyrhizobium japonicum* with *Bacillus subtilis* or *B. thuringiensis* increased both plant weight and nodulation (Bai *et al.*, 2002, 2003). In contrast, Camacho *et al.* (2001) reported decreases in root nodulation of soybean due to the co-inoculation of *B. japonicum* USDA110 and *Bacillus* sp. CECT450. The contrasting effects of bacterial co-inoculation on soybean indicate the necessity for identifying efficient combinations of rhizobia and other endophytic strains to promote plant growth and yield, thus, reducing the application of high doses of chemical fertilizers. So, it is important to explore endophytic bacterial strains cohabiting soybean nodules, as they may contribute to growth promotion in legumes. Consequently, the latter allows the reduction of environmental pollution and promotes sustainable agriculture of soybean in tropical regions.

The objectives of this work were to: 1) isolate and characterize symbiotic and endophytic non-symbiotic bacteria from root nodules of *Glycine max* collected from two tropical cropping systems at Campeche, Mexico, and 2) evaluate the effects of the most prominent endophytic bacterial strains when co-inoculated with referential or native rhizobial strains on the growth of soybean plants. The results are expected to contribute on selecting an efficient combination of rhizobia and endophytic bacteria for being used as biofertilizers for soybean cultivation in tropical conditions.

Material and methods

Sites, cultivars and nodule sampling

Soybean roots with nodules were collected during August 2015 from two soybean fields at Campeche state, Mexico, at the locations of San Antonio Cayal (19°39' N, 19°40' W) (municipality of Campeche), and Nuevo Progreso (19°40' N, 89°43' W) (municipality of Hopelchén). At San Antonio Cayal, three varieties of soybean already registered by the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) were collected: 'Huasteca 100' (SOY-014-251104), 'Huasteca 200' (SOY-015-251104), and 'Huasteca 400' (SOY-022-291105). At Nuevo Progreso, the 'Huasteca 200' variety, and the transgenic soybean resistant to herbicide-glyphosate (also known as Roundup Ready®, GR, or RR) were collected. In each location, the root system of five randomly selected healthy plants of each variety was harvested after 50 days after of sowing. Roots were transported in plastic sterile bags to the laboratory.

Bacteria isolation from root nodules

Nodules were dissected for each root system, especially those in which the presence of pink coloration in the cortex was observed, indicating their viability and potential N-fixation. Root nodules were kept at 4 °C and surface disinfected with 70% ethanol for 10 s, and with 0.4% sodium hypochlorite for 1 to 3 min, and then rinsed 5 to 6 times with sterile distilled water. Nodules were immediately placed in sterile test tubes containing 1 mL of sterile distilled water, and crushed for obtaining a bacterial suspension. An aliquot (100 µL) of the bacterial suspension of each soybean variety was spread on the surface of Petri dishes containing yeast extract-mannitol agar (YMA) medium (Vincent, 1970) modified to contain 5 g/L of mannitol and 0.00125% Congo red (w/v); then, incubated at 28°C for five days. The purification of bacteria was performed as indicated by Vincent (1970), and single bacterial colonies were selected by color and shape, and re-streaked for assuring the purification.

Morphological and biochemical characterization of bacterial isolates

Bacterial isolates were morphologically characterized by distinguishing form, color, margins, surface, and size of colonies. In addition, bacterial cells were stained with the Gram technique and microscopically examined (Leica CME) with a 100X objective lens.

All bacterial isolates were grown on glucose-peptone agar with bromocresol purple (GPA-BP) (Somasegaran & Hoben, 2012), and in litmus milk liquid medium (Litmus Milk®) (Ferrera-Cerrato *et al.*, 1993). The isolates that did not show growth in these two culture media were considered as potential rhizobia. The isolates were also grown on YMA medium containing bromothymol blue (YMA-BTB) as indicator for determining their ability to produce alkaline (blue color) or acidic (yellow color) reactions (Ferrera-Cerrato *et al.*, 1993), and tested for identifying the ability to solubilize $Ca_3(PO_4)_2$ by streaking on Pikovskaya agar medium. The presence of a clear zone around the bacterial colony indicated a potential release of organic acids for inducing the phosphate solubilization (Sundara & Sinha, 1963).

Molecular identification of the isolates

The isolates were identified by partial sequencing of their 16S rRNA gene. The isolates were grown in YMA medium for 24 to 72 h, depending on the isolate. Total DNA was extracted using cetyl-trimethylammonium bromide 2% (CTAB) (Doyle & Doyle, 1990). Partial sequence of 16S rRNA gene was amplified by PCR using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3') (Eden *et al.*, 1991), using the following conditions: one cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 2 min, 59°C for 1 min, 72°C for 1.5 min, and finally at 72°C for 5 min.

The PCR products of approximately 1500 bp were purified with EXO-SAP (Affymetrix, USA) following the manufacturer instructions. The fragments were sequenced in a Genetic Analyzer® Model 3130 (Applied Biosystems, USA) but the consensus sequence was generated from forward and reverse sequence data using BioEdit v7.2.5 (Hall, 1999). It is important to note that all of the consensus sequences were analyzed with the Blastn algorithm from the BLAST/NCBI software (Altschul *et al.*, 1997) and the Ribosomal Database Project release 11 (https://rdp.cme.msu.edu/). Sequences obtained in this study were compiled in a FASTA format along with sequences belonging to type strain (http://www.bacterio.net/). Sequences were aligned using the muscle option included in Mega X software (Kumar *et al.*, 2018). In addition, they were trimmed at the ends for analyzing fragments with the same length. The phylogenetic reconstruction of all sequences was performed with Bayesian inference (BI) in MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), mega file was converted to nexus file for BI and the INVGAMMA substitution model was used with 1,000,000 generations and sampled every 1000 generations. The first 25% of generated trees were discarded as the burn-in phase option of each analysis and posterior probabilities were determined for the remaining trees. The construction of the phylogenetic tree considered the strain NR114653 of *Thermococcus marinus* as outgroup.

Effects of the endophytic isolates on soybean growth and nodule development

The bacterial isolates were evaluated for inducing nodule formation on roots of soybean 'Huasteca 200' plants, following a bioassay procedure (Ferrera-Cerrato *et al.*, 1993). Seed surface was sterilized twice by using 0.2% sodium hypochlorite for 1 min, followed by 70% ethanol for 1 min (2 times), and 5 rinses with sterile distilled water. Seeds were placed on sterile filter paper in Petri dishes. After germination, seedlings were transplanted to 500 mL-pots with autoclaved perlite (121°C for 2 h).

Each isolate was grown in YMA for five days and then, adjusted to a concentration of 10^9 CFU/mL. One milliliter was used for inoculating each plant in accordance to the corresponding bacterial treatment,

and ten individual plants were used as replicates per treatment. In total, 25 treatments were evaluated (corresponding to the 23 bacterial isolates) including a negative control (uninoculated plants), and a positive control (inoculated with the reference strain *B. japonicum* USDA110) (Abou-Shanab *et al.*, 2017).

Plants were kept in growth chamber under light intensity of 136 μmol m^{-2} s^{-1} with a 12 h photoperiod, temperature of 27 ± 2°C, and 45% of relative humidity, and irrigated with sterile distilled water as needed and with 50 mL of the N-free Jensen's nutrient solution every week (Vincent, 1970). Plants were harvested after 31 days of inoculation, and the plant height (PH), root dry weight (RDW), shoot dry weight (SDW), number of nodules (NN), and nodule dry weight (NDW) were evaluated. The RDW, SDW and NDW were determined after drying plant tissues at 70 ± 2°C for 48 h.

Effects of the bacterial co-inoculation on soybean growth and nodule development

In a second trial, three prominent bacteria, having been selected from the previous experiment, were used for the co-inoculation study. A total of 16 different inoculation treatments were applied to soybean plants var. 'Huasteca 200'. The inocula were prepared with isolates CPO4.24C (*Bradyrhizobium* sp.), CPO4.13C (*Rhizobium* sp.) and CPO4.15C (*Agrobacterium tumefaciens* CPO4.15C), individually or in binary combinations with two referential strains as follows: *Bradyrhizobium japonicum* USDA110, and the plant growth promoting rhizobacteria *Pseudomonas tolaasii* P61 (Angulo-Castro *et al.*, 2018). The referential strains were also applied individually and in combination. An uninoculated control group was also in place. The binary inoculum (1 mL) was prepared using a ratio 1:1 (v/v) of the constituent strains.

Soybean plants were kept in a growth chamber at light intensity of 138 μmol m^{-2} s^{-1}, temperature: 26 ± 2°C, and 44% relative humidity, and irrigated as previously described. Plants were harvested after 32 days of inoculation for determining the NN, and their total dry biomass. The N fixation was measured using the acetylene reduction assay (ARA) as described by Ferrera-Cerrato *et al.* (1993). Roots were placed in 1000 mL hermetically sealed flasks, and 100 mL of air (10% volume of the flask) was pulled out with a syringe and replaced with 100 mL of acetylene. After 1 h of incubation, 5 mL of the gaseous mixture of each flask were recovered and placed in Vacutainer® tubes for further analysis in gas chromatograph (Chrompack, model 5890, series II, USA) using an Agilent J&W Capillary Poraplot Q column® (25 m/0.32 mm) (Agilent Technologies, Santa Clara, CA, USA). In addition, PH, NN, RDW, SDW and NDW were also recorded. The relative chlorophyll content was measured by taking SPAD readings (Minolta SPAD-502) from 15 plants randomly chosen. Readings were taken from full developed leaves (3rd and 4th position leaves) of each plant. For this determination an average value from 15 measurements per plant were taken.

Statistical analysis

A completely randomized design was applied in both experimental assays. Collected data were subjected to an analysis of variance (ANOVA), and to a Least Significant Difference test (LSD, $\alpha = 0.05$) with the assistance of the Statistical Analysis System (SAS) v. 9.

Results

Morphological and biochemical characterization of endophytic bacterial colonies obtained from soybean nodules

A total of 23 culturable isolates were obtained from field grown soybean nodules collected from the test site at Nuevo Progreso, Hopelchén (7 from the 'Huasteca 200' variety, and 5 from the transgenic variety), and from San Antonio Cayal (6 from 'Huasteca 100', 4 from 'Huasteca 400', and 1 from 'Huasteca 200') (Table 1); all bacterial isolates represented bacilli based on microscopic examinations. The colonial characterization showed that 59% of them had circular shape, 33% were pointed, and only 8% had an amoeboid shape.

The isolates labelled as CPO4.24C, CPO4.13C, CPO4.17TA and CPO4.18T showed scarce growth on GPA-BP medium and no growth on litmus milk, with slight acidification on the YMA-BTB medium, indicating that these four strains may correspond to symbiotic bacteria. The isolates CPO4.1T and CPO4.19T produced alkaline reactions, and the remaining bacterial isolates showed strongly acidic reactions in culture medium. Furthermore, the CPO4.10C, CPO4.7CA, CPO4.8CA, CPO4.9CA, CPO4.11C, CPO4.12C, CPO4.14C, CPO-4.15C, CPO4.35C, CPO4.45, CPO4.22S and CPO-4.2TA were able to solubilize tricalcium phosphate (Table 1). Isolates were identified as gram-negative bacteria, and only the isolate CPO4.23C was identified as gram-positive.

Molecular identification of the isolates

All of the nucleotide sequences of the bacterial isolates from this study were deposited in the GeneBank, NCBI

Table 1. Biochemical characterization of bacterial strains isolated from nodules of *Glycine max* (L.) Merr., collected from two trial sites in the southern state of Campeche, Mexico, and sourced from different soybean varieties.

Location/Soybean varieties used for bacterial isolation	Strain	GPA-BP[1]	Litmus milk®[2]	YMA-BTB[3,*]	Solubilization of tricalcium phosphate[4,**]
'Huasteca 100'	CPO4.10C	A	A	+	+
	CPO4.7CA	A	A	+	+
	CPO4.9CA	A	A	+	+
	CPO4.8CA	A	A	+	+
	CPO4.23C	A	A	+	−
	CPO4.11C	A	A	+	+
'Huasteca 200'	CPO4.24C	N	N	−	−
'Huasteca 400'	CPO4.13C	E	N	+	−
	CPO4.12C	A	A	+	+
	CPO4.14C	A	A	+	+
	CPO4.15C	A	A	+	+
'Huasteca 200'	CPO4.35	A	A	+	+
	CPO4.20S	A	A	−	−
	CPO4.58	A	A	+	−
	CPO4.45	A	A	+	+
	CPO4.21S	A	A	+	−
	CPO4.65			+	−
	CPO4.22S	A	A	+	+
Transgenic	CPO4.2TA	A	A	+	+
	CPO4.17TA	E	E	−	−
	CPO4.1T	A	A	++	−
	CPO4.18T	E	E	−	−
	CPO4.19T	A	A	++	−
Referential strain	USDA110	A	A	−	−

[1]GPA-BP: glucose-peptone agar with bromocresol purple; [2]Litmus Milk: litmus milk liquid medium; [3]YMA-BTB: yeast extract-mannitol agar medium containing bromothymol blue; [4]Pikovskaya agar growth medium. E = low growth; A= abundant growth; N = null growth. *For the YMA-BTB growth medium: + = change of coloration to yellow (acidic), ++ = strong blue color change (alkaline). **For the modified Pikovskaya agar growth medium: − = no coloration change (without phosphorus solubilization halo), + = phosphorus solubilization halo.

(USA) to obtain the corresponding accession numbers (Table 2). Bacterial isolates were identified by using 16S rRNA gene sequence analysis. Using the BLAST analysis, the bacterial sequences showed a maximum identity of 99-100% with genera like *Agrobacterium* (CPO4.15C), *Enterobacter* (CPO4.10C, CPO4.7CA, CPO4.9CA, CPO4.8CA, CPO4.12C, CPO4.14C, CPO-4.35, CPO4.58, CPO4.45, CPO4.65, and CPO4.22S), *Bradyrhizobium* (CPO4.24C), *Ensifer* (CPO4.17TA and CPO4.18T), *Chryseobacterium* (CPO4.20S and CPO4.21S), *Massilia* (CPO4.1T), *Microbacterium* (CPO-4.23C), *Rhizobium* (CPO4.13C), *Serratia* (CPO4.11C, CPO4.2TA), and *Xanthomonas* (CPO4.19T) (Table 2).

The consensus sequences were pooled by phylogenetic analysis to determine the identity at the species level. The clustering of the 16S rRNA sequences (Fig. 1) showed that *Enterobacter* was the most predominant clade in which the strains CPO4.7CA, CPO4.8CA, CPO4.12C, CPO4.14C, CPO4.35, and CPO4.22S were placed in the group of *E. cloacae*, whereas the strains CPO4.10C and CPO4.9CA were in the group of *E. ludwigii,* and the strain CPO4.45 to *E. hormaechei*. On the other hand, the strains CPO4.17TA and CPO4.18T corresponded to *Ensifer adhaerens*, whereas strains CPO4.11C and CPO4.2TA had a maximum identity with *Serratia marcescens*, and the strain CPO4.15C belonged to *Agrobacterium tumefaciens*. The remaining strains were identified only at genus level because they belonged to a cohort of undescribed species most likely to the genera *Bradyrhizobium, Chryseobacterium,*

Table 2. Molecular identification of bacterial strains isolated from nodules of *Glycine max* (L.) Merr.

Location/Soybean varieties used for bacterial isolation	Strain	Taxa identified	GenBank Accession numbers	Ribosomal Database Project/Closest related accession number	RDP Database Similarity
San Antonio Cayal, Campeche					
'Huasteca 100'	CPO4.10C	*Enterobacter ludwigii*	MF666750	*Enterobacter ludwigii* / KR476387	1.000
	CPO4.7CA	*Enterobacter cloacae*	MF666747	*Enterobacter cloacae* / JF772071	0.995
	CPO4.9CA	*Enterobacter ludwigii*	MF666749	*Enterobacter cloacae* / JQ659564	1.000
	CPO4.8CA	*Enterobacter cloacae*	MF666748	*Enterobacter cloacae* / KF956588	0.995
	CPO4.23C	*Microbacterium* sp.	MF666752	*Microbacterium oleivorans* /JQ342859	0.997
	CPO4.11C	*Serratia marcescens*	MF666751	*Serratia marcescens* / JN896750	1.000
'Huasteca 200'	CPO4.24C	*Bradyrhizobium* sp.	MF666757	*Bradyrhizobium* sp. / AF363136	1.000
'Huasteca 400'	CPO4.13C	*Rhizobium* sp.	MF666754	*Rhizobium* sp. / GQ483459	1.000
	CPO4.12C	*Enterobacter cloacae*	MF666753	*Enterobacter cloacae* / JQ904624	0.998
	CPO4.14C	*Enterobacter cloacae*	MF666755	*Enterobacter cloacae* / JQ904624	0.998
	CPO4.15C	*Agrobacterium tumefaciens*	MF666756	*Agrobacterium* sp. / GQ849306	1.000
Nuevo Progreso, Hopelchén					
'Huasteca 200'	CPO4.35	*Enterobacter cloacae*	MF666743	*Enterobacter cloacae* / JF772071	1.000
	CPO4.20S	*Chryseobacterium* sp.	MF666740	*Chryseobacterium* sp. / JN585683	1.000
	CPO4.58	*Enterobacter* sp.	MF666745	*Enterobacter* sp. / EU855204	1.000
	CPO4.45	*Enterobacter hormaechei*	MF666744	*Enterobacter hormaechei* /KF516241	0.991
	CPO4.21S	*Chryseobacterium* sp.	MF666741	*Chryseobacterium* sp. / JN585683	1.000
	CPO4.65	*Enterobacter* sp.	MF666746	*Enterobacter* sp. / KF956573	0.990
	CPO4.22S	*Enterobacter cloacae*	MF666742	*Enterobacter cloacae* / CP010377	1.000
Transgenic	CPO4.2TA	*Serratia marcescens*	MF666736	*Serratia marcescens* / EU031439	1.000
	CPO4.17TA	*Ensifer adhaerens*	MF666737	*Ensifer adhaerens* / JX971519	1.000
	CPO4.1T	*Massilia* sp.	MF666735	*Massilia* sp. /LC065173	0.964
	CPO4.18T	*Ensifer adhaerens*	MF666738	*Ensifer adhaerens* / JX971519	1.000
	CPO4.19T	*Ensifer adhaerens*	MF666739	*Ensifer adhaerens* / AB016762	1.000
Referential strain	USDA110	*Bradyrhizobium japonicum*	-	–	–

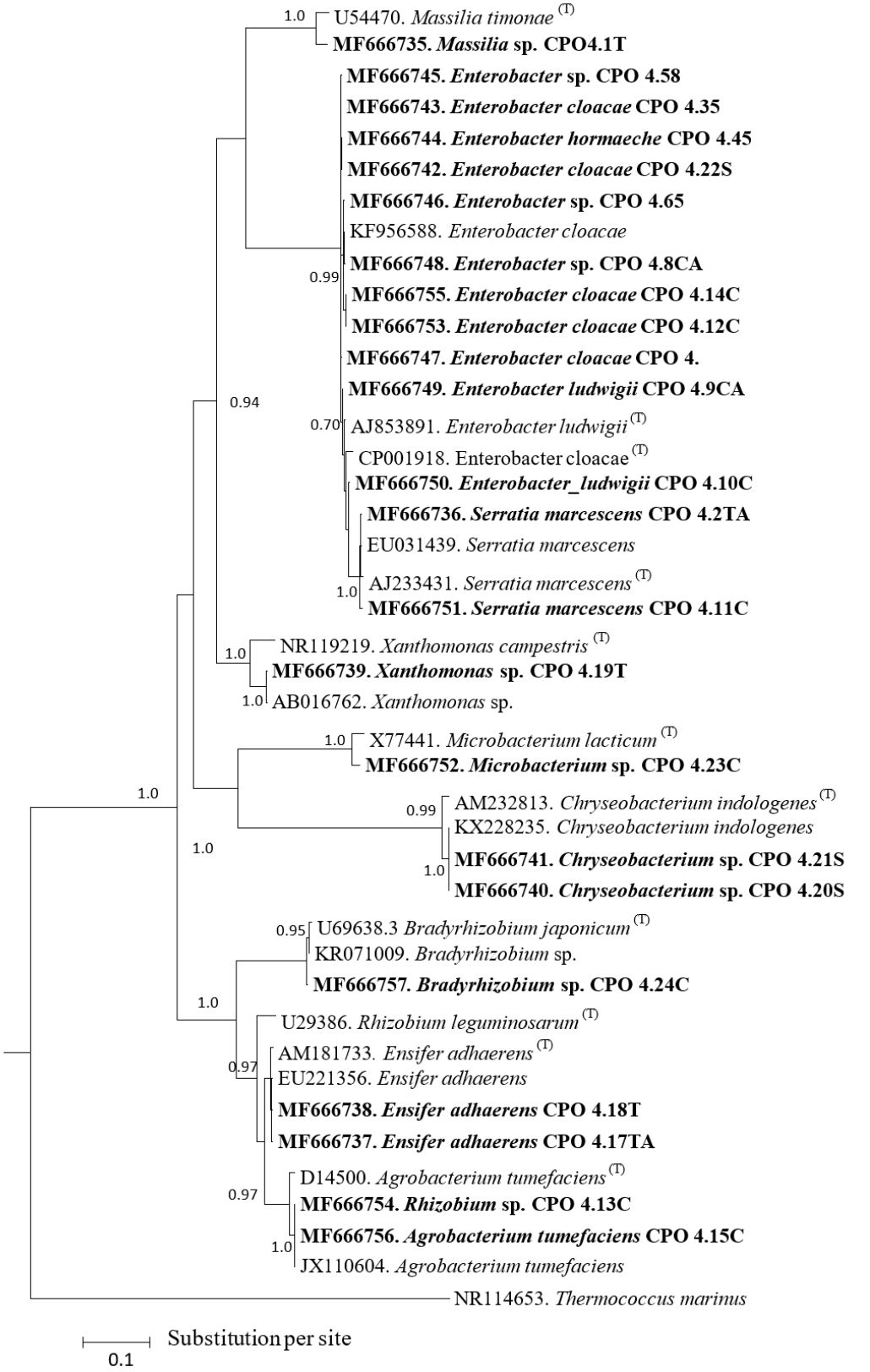

Figure 1. Phylogenetic tree constructed with Bayesian inference using 1,000,000 generations. The sequences correspond to the amplification of the 16S rRNA gene of endophytic and symbiotic bacteria (bold names) associated with root nodules of *Glycine max* (L.) Merr. The species *Thermococcus marinus* was used as outgroup. Superscript 'T' indicates type strain. The scale bar indicates the number of substitutions per site.

Enterobacter, Massilia, Microbacterium, Rhizobium, and *Xanthomonas*.

Effects of inoculation produced by the endophytic isolates on soybean growth and nodule development

Out of the 23 strains evaluated, only *Bradyrhizobium* sp. CPO4.24C produced 36 nodules/plant on average, which was significantly higher than those obtained by inoculating the referential strain *B. japonicum* USDA110 (21 nodules/plant) (Table 3). Plants inoculated with *Rhizobium* sp. CPO4.13C or *A. tumefaciens* CPO4.15C resulted in significantly increased PH (51.2 and 49.5 cm, respectively) and SDW (0.47 and 0.41 g/plant, respectively) when compared to the negative control (uninoculated plants) (35.1 cm and 0.26 g/plant, respectively) (Table 3). In addition, the inoculation of *Rhizobium* sp. CPO4.13C resulted in significantly higher RDW (0.17 g/plant) when compared to plants of the negative control (0.10 g/plant).

Based on these results, the strains *Bradyrhizobium* sp. CPO4.24C, *Rhizobium* sp. CPO4.13C, and *A. tumefaciens* CPO4.15C were selected for their application as co-inoculants with the referential strain *B. japonicum* USDA110 or with the plant growth promoting bacterium *P. tolaasii* P61.

Effects of bacterial co-inoculation on soybean growth and nodule development

The co-inoculation of *B. japonicum* USDA110 and the strain *Bradyrhizobium* sp. CPO4.24C (T2) resulted in significantly greater plant height, NN and NDW than the negative control (T16) (Table 4). Moreover, no significant differences (PH, NN and NDW) were observed between plants individually inoculated with each *Bradyrhizobium* strain (T11 and T12). However, the single inoculation of *Bradyrhizobium* sp. CPO4.24C (T11) yielded greater SDW and RDW (0.69 and 0.27 g/plant, respectively) than the negative control (0.30 and 0.15 g/plant), but no significant differences were observed with the remaining treatments (Table 4).

In regards to the relative leaf chlorophyll content, the individual inoculation with the two *Bradyrhizobium* strains (CPO4.24C or USDA110), and the combination of *B. japonicum* USDA110+*P. tolassi* P61 (T1) resulted in the highest chlorophyll content (28.4 SPAD units) which was significantly higher than the negative control (T16) (13.28 SPAD units) (Table 4). In contrast, the co-inoculation of *Bradyrhizobium* sp. CPO4.24C with either *B. japonicum* USDA110 (T2) or *P. tolassii* P61 (T5) resulted in significantly higher activity of acetylene reduction (3.10 and 2.91 μmol C_2H_4 h^{-1} $plant^{-1}$, respectively) than the remaining treatments. Co-inoculation of *Bradyrhizobium* sp. CPO4.24C with either *A tumefaciens* CPO4.24C or *Rhizobium* sp. CPO4.13c also induced significantly higher ARA values than those obtained with the single bacterial inoculation.

Discussion

The legume-rhizobia symbiosis is an important biological process for plant productivity (Bai *et al.*, 2003; Stajkovic *et al.*, 2011). Many studies have shown that the co-inoculation of rhizobia and some endophytic bacteria may contribute on plant growth promotion and yield (Bai *et al.*, 2003; Liu *et al.*, 2010; Deng *et al.*, 2011); thus, such co-inoculation may improve the effectiveness of the symbiotic relationship. In this context, the nodules are colonized by several non-rhizobial endophytes (Bai *et al.*, 2002; Palaniappan *et al.*, 2010; Saïdi *et al.*, 2011; Li *et al.*, 2012; Aserse *et al.*, 2013) which influence the growth and yield of legumes by different mechanisms such as mineral solubilization, or enhanced root nodulation and N fixation activity (Bai *et al.*, 2003; Palaniappan *et al.*, 2010; Deng *et al.*, 2011; Stajkovic *et al.*, 2011; Li *et al.*, 2012). In the present study, 23 strains (predominantly Gram-negative bacteria) were isolated. Of them, 19 were found as non-symbiotic endophytes, and 4 showed symbiotic features. In this respect, Li *et al.* (2008) isolated a high number of Gram-negative endophytes from soybean nodules. In contrast, other studies reported low number of endophytic bacteria with the predominance of Gram-positive bacteria (Bai *et al.*, 2002; Hung & Annapurna, 2004; Aserse *et al.*, 2013). Therefore, our study besides isolating three genera of potentially nodule-forming rhizobia, also reports the proliferation of 7 genera of non-symbiotic bacteria harbored in root nodules.

The analysis of the 16S rRNA gene sequence of the isolated strains (symbiotic and non-symbiotic) indicated that they belonged to 10 different genera (Table 2). Among the isolated bacteria, *Agrobacterium tumefaciens* is known as a soil-borne phytopathogen previously reported from soybean nodules (Li *et al.*, 2008). Same authors also reported the genus *Serratia* in the soybean nodules, and this bacterium has been shown to stimulate the growth and development of soybean (Zhang *et al.*, 1996); however, the two *Serratia* strains isolated in this work did not show significant effects on growth nor on soybean nodulation. Our report revealed that bacterial genera contrast with other findings in which other genera of endophytic bacteria, such as *Acinetobacter, Bacillus, Burkholderia, Deinococcus, Rhodococcus, Pantoea, Staphylococcus*

Table 3. Effect of endophytic and symbiotic bacteria isolated from nodules of *Glycine max* var. 'Huasteca 200' after 31 days of inoculation.

Location/Soybean varieties used for bacterial isolation		NN (Nodule/ plant)	NDW (mg/ plant)	PH (cm)	SDW (g/plant)	RDW (g/plant)
San Antonio Cayal, Campeche						
'Huasteca 100'	*Enterobacter ludwigii* CPO4.10C	–	–	38.37 bcd	0.30 bcde	0.13 abc
	Enterobacter cloacae CPO4.7CA	–	–	41.62 abcd	0.33 bcde	0.11 bc
	Enterobacter ludwigii CPO4.9CA	–	–	42.03 abc	0.32 bcde	0.10 bc
	Enterobacter cloacae CPO4.8CA	–	–	36.70 bcd	0.30 bcde	0.09 bc
	Microbacterium sp. CPO4.23C	–	–	44.58 abc	0.40 abc	0.12 bc
	Serratia marcescens CPO4.11C	–	–	36.60 bcd	0.28 cde	0.11 bc
'Huasteca 200'	*Bradyrhizobium* sp. CPO4.24C	36 a	9.00 a	45.57 ab	0.38 abcd	0.13 ab
'Huasteca 400'	*Rhizobium* sp. CPO4.13C	–	–	51.16 a	0.47 a	0.17 a
	Enterobacter cloacae CPO4.12C	–	–	42.68 abc	0.35 abcde	0.12 bc
	Enterobacter cloacae CPO4.14C	–	–	43.38 abc	0.39 abcd	0.14 ab
	Agrobacterium tumefaciens CPO4.15C	–	–	49.46 a	0.41 abc	0.13 ab
Nuevo Progreso, Hopelchén						
'Huasteca 200'	*Enterobacter cloacae* CPO4.35	–	–	39.50 abcd	0.30 bcde	0.10 bc
	Chryseobacterium sp. CPO4.20S	–	–	41.64 abcd	0.42 ab	0.14 ab
	Enterobacter sp. CPO4.58	–	–	41.67 abcd	0.35 abcde	0.11 bc
	Enterobacter hormaechei CPO4.45	–	–	33.92 d	0.25 e	0.08 c
	Chryseobacterium sp. CPO4.21S	–	–	41.18 abcd	0.33 bcde	0.11 bc
	Enterobacter sp. CPO4.65	–	–	42.72 abc	0.37 abcde	0.10 bc
	Enterobacter cloacae CPO4.22S	–	–	45.65 ab	0.36 abcde	0.13 ab
Transgenic	*Serratia marcescens* CPO4.2TA	–	–	37.70 bcd	0.31 bcde	0.11 bc
	Ensifer adhaerens CPO4.17TA	–	–	40.90 abcd	0.26 de	0.10 bc
	Massilia sp. CPO4.1T	–	–	45.57 abcd	0.38 abcd	0.14 ab
	Ensifer adhaerens CPO4.18T	–	–	41.67 cd	0.38 abcd	0.11 bc
	Xanthomonas sp. CPO4.19T	–	–	38.82 abcd	0.30 bcde	0.11 bc
Referential strain	*Bradyrhizobium japonicum* USDA110	21 b	8.00 a	39.70 abcd	0.35 abcde	0.11 bc
Negative control	Uninoculated plants	–	–	35.15 cd	0.26 f	0.10 bc

NN: nodule number, NDW: nodule dry weight, PH: plant height, SDW: shoot dry weight, RDW: root dry weight. Ten replicates were used per treatment. Values followed by different letters in the same column denote statistical significance according to the *t*-test LSD ($p \leq 0.05$).

and *Tsukamurella*, were found in soybean nodules (Bai *et al.*, 2002, 2003; Hung & Annapurna, 2004; Li *et al.*, 2008; Aserse *et al.*, 2013).

Our study also noted the isolation of other endophytic bacteria not previously described as inhabitants of soybean nodules, such as *Enterobacter, Chryseobacterium, Massilia, Microbacterium*, and *Xanthomonas*. However, these bacteria were reported as nodule inhabitants of other legume species; for example, *Enterobacter* was identified from nodules of *Abrus precatorius* and *Vigna unquiculata* (Ghosh *et al.*, 2005; Leite *et al.*, 2016), *Chryseobacterium* from *V. unquiculata* (Leite *et al.*, 2016), *Massilia* from nodules of *Hedysarum flexuosum* (Ezzakkioui *et al.*, 2015), *Microbacterium* from *Medicago sativa* and *Sphaerophysa salsula* (Stajkovic *et al.*, 2009; Deng *et al.*, 2011), *Serratia* from nodules of *Sphaerophysa salsula* and *Hedysarum flexuosum* (Deng *et al.*, 2011; Ezzakkioui *et al.*, 2015), and *Xanthomonas* was identified in nodules of *Medicago hispida* (Arone *et al.*, 2014). More importantly, the influence of these bacteria on the symbiosis between rhizobia and soybean has been rarely described.

In the present study, the co-inoculation of non-symbiotic endophytic bacteria did not produce significant effects on the growth of soybean plants. In contrast, both dry weight and root nodulation were increased due to the co-inoculation of *B. japonicum* 532C with the endophytic *Bacillus subtilis* and *B. thuringiensis* (Bai *et al.*, 2002). Similarly, the

Table 4. Effect of individual or co-inoculation of referential and native bacterial strains isolated from root nodules of soybean, on the growth and nodulation of soybean plants var. 'Huasteca 200', after 32 days of inoculation.

Treatments[1]	PH[2] (cm)	NN[3] (Nodules/ plant)	NDW[4] (mg/plant)	SDW[5] (g/plant)	RDW[6] (g/plant)	Chlorophyll content (SPAD units)	ARA[7] (µmol C_2H_4 h^{-1} $plant^{-1}$)
T1	26.70 cd	65 a	45.0 abc	0.55 abc	0.18 de	28.23 ab	1.33 d
T2	33.87 a	69 a	55.0 a	0.52 bc	0.20 bcde	25.40 bc	3.10 a
T3	33.28 ab	62 ab	52.0 ab	0.66 ab	0.24 abc	26.98 abc	1.86 c
T4	27.05 bcd	67 a	46.0 abc	0.60 abc	0.20 cde	26.57 abc	2.35 b
T5	22.25 de	58 ab	35.0 c	0.52 bc	0.19 cde	25.02 cd	2.91 a
T6	28.45 abcd	60 ab	52.0 ab	0.56 abc	0.25 abc	26.60 abc	2.58 b
T7	29.83 abc	52 b	41.00 bc	0.57 abc	0.22 abcd	22.13 d	1.84 c
T8	24.82 cd	0 c	0.0 d	0.50 c	0.23 abcd	15.25 fg	0.0 e
T9	26.48 cd	0 c	0.0 d	0.66 ab	0.21 abcde	18.54 e	0.0 e
T10	26.57cd	0 c	0.0 d	0.63 abc	0.26 ab	16.63 ef	0.0 e
T11	30.15 abc	66 a	49.0 ab	0.69 a	0.27 a	28.45 a	1.86 c
T12	30.28 abc	59 ab	42.0 bc	0.67 ab	0.25 abc	28.12 ab	1.73 c
T13	23.38 de	0 c	0.0 d	0.56 abc	0.23 abcd	15.22 fg	0.0 e
T14	31.15 abc	0 c	0.0 d	0.57 abc	0.23 abcd	15.63 efg	0.0 e
T15	31.07 abc	0 c	0.0 d	0.60 abc	0.24 abc	15.18 fg	0.0 e
T16	18.12 e	0 c	0.0 d	0.30 d	0.15 e	13.28 g	0.0 e

[1]T1=*Bradyrhizobium japonicum* USDA110 (reference strain) + *Pseudomonas tolaasii* P61 (reference strain), T2=*B. japonicum* USDA110 + *Bradyrhizobium* sp. CPO4.24C, T3=*B. japonicum* USDA110 + *Rhizobium* sp. CPO4.13C, T4=*B. japonicum* USDA110 + *Agrobacterium tumefaciens* CPO4.15C, T5=*B.* sp. CPO4.24C + *P. tolaasii* P61, T6=*B.* sp. CPO4.24C + *R.* sp. CPO4.13C, T7=*B.* sp. CPO4.24C + *A. tumefaciens* CPO4.15C, T8=*P. tolaasii* P61 + *R.* sp. CPO4.13C, T9=*P. tolaasii* P61 + *A. tumefaciens* CPO4.15C, T10=*R.* sp. CPO4.13C + *A. tumefaciens* CPO4.15C, T11=*B.* sp. CPO4.24C, T12=*B. japonicum* USDA110, T13=*P. tolaasii* P61, T14=*R.* sp. CPO4.13C, T15= *A. tumefaciens* CPO4.15C, T16=Negative control (uninoculated plant). [2]PH: plant height. [3]NN: nodule number. [4]NDW: nodule dry weight. [5]SDW: shoot dry weight. [6]RDW: root dry weight. [7]ARA: acetylene reduction activity. Ten replicates were used per bacterial isolates. Values followed by different letters in the same column denote statistical significance according to the *t*-test LSD ($p \leq 0.05$).

coinoculation of *S. meliloti* with endophytic bacteria like *A. tumefaciens* (Wang *et al.*, 2006) or *Rhizobium* sp. II CCBAU21244 (Liu *et al.*, 2010) resulted in increased nodulation of *Melilotus dentatus* and *Wisteria sinensis*. Nevertheless, in our work, the co-inoculation of *A. tumefaciens* CPO4.15C did not affect root nodulation which is opposite to results obtained by Camacho *et al.* (2001). Overall, the endophytic bacteria isolated in the present study did not influence plant growth, however, they may be involved in creating an ecological micro-niche suitable for both survival and proliferation of symbiotic bacteria, as discussed by Deng *et al.* (2011).

On the other hand, the symbiotic strain *Bradyrhizobium* sp. CPO4.24C was able to form nodules in the soybean plants. On the contrary, the absence of nodules in the 'Huasteca 200' variety inoculated with *Rhizobium* sp. CPO4.13C and *Ensifer adherensis* CPO4.2TA or CPO4.18T can be explained due to the specificity between legumes and rhizobia; in this regard, these two bacterial genera are not soybean symbionts (Wu *et al.*, 2011; Zhang *et al.*, 2011; Yan *et al.*, 2014). Similarly, some non-nodulating *Rhizobium* and *Bradyrhizobium* bacteria were reported in the rhizosphere of legumes (Segovia *et al.*, 1991; Pongsilp *et al.*, 2002; Aserse *et al.*, 2013).

As mentioned, the inoculation of *Bradyrhizobium* sp. CPO4.24C resulted in greater nodulation in comparison to the inoculation of the reference strain *B. japonicum* USDA110 (Table 3). This reference strain has induced abundant nodulation at low temperatures ranging between 17 to 23ºC (Ando & Yokoyama, 1999; Suzuki *et al.*, 2014). Nevertheless, the average temperature recorded in the present study was 27 ± 2ºC by which the growth and infectivity of the reference bacterial strain might have been affected. However, the co-inoculation of both strains resulted in high PH and nitrogenase activity (consequently greater N fixation can be expected) when compared to the single inoculation of each strain. This demonstrates a synergistic effect produced by both of the aforementioned bacteria. In this respect, Htwe &

Yamakawa (2016) reported lower ARA (1.15 C_2H_4 h^{-1} $plant^{-1}$) due to the co-inoculation of soybean plants with *B. japonicum* SAY3-7 and *Streptomyces griseoflavus* P4. In addition, these authors reported that SDW and RDW were significantly higher in co-inoculated plants (0.42 and 0.25 g/plant) than uninoculated controls (0.39 and 0.25 g/plant). Similarly, in this study the shoot and root biomass were enhanced with the co-inoculation of *Bradyrhizobium* sp. CPO4.24C and *B. japonicum* USDA110 in comparison to uninoculated control (Table 4).

Our results show that the native strain *Bradyrhizobium* sp. CPO4.24C has good potential for being introduced as biofertilizer for soybean cultivation in the tropical regions of Mexico. Nevertheless, further research should be conducted for evaluating the effects of this bacterium on the growth and yields of soybean plants under appropriate field conditions.

Overall, this study isolated twenty-three endophytic bacterial strains belonging to ten different genera from nodules of four varieties of *Glycine max* grown at field conditions. Furthermore, co-inoculation of the three prominent bacterial endophytes with either native or referential *Bradyrhizobium* strains did not enhance plant growth nor root nodulation. The native *Bradyrhizobium* sp. CPO4.24C showed high potential for being inoculated alone or combined with the referential strain *B. japonicum* USDA110, since PH, nodulation, dry weight, relative chlorophyll content, and nitrogenase activity were significantly enhanced.

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