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Isolation and characterization of endophytes from nodules of *Mimosa pudica* with biotechnological potential

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ABSTRACT

Legumes establish symbiotic relationships with different microorganisms, which could function as plant growth promotion microorganisms (PGPM). The finding of new PGPM strains is important to increase plant production avoiding or diminishing the use of industrial fertilizers. Thus, in this work we evaluated the plant growth promotion traits of ten strains isolated from *Mimosa pudica* root nodules. According to the 16S rDNA sequence, the microorganisms were identified as *Enterobacter* sp. and *Serratia* sp. To the best of our knowledge this is the first report describing and endophytic interaction between *Mimosa pudica* and *Enterobacter* sp. These strains have some plant growth promoting traits such as phosphate solubilization, auxin production and cellulase and chitinase activity. Strains identified as *Serratia* sp. inhibited the growth of the phytopathogenic fungi *Fusarium* sp., and *Alternaria solani* and the oomycete *Phytophthora capsici.* According to their biochemical characteristics, three strains were selected to test their plant growth promoting activity in a medium with an insoluble phosphate source. These bacteria show low specificity for their hosts as endophytes, since they were able to colonize two very different legumes: *Phaseolus vulgaris* and *M. pudica*. Seedlings of *P. vulgaris* were inoculated and grown for fifteen days. *Enterobacter* sp. NOD1 and NOD10, promoted growth as reflected by an increase in shoot height as well as an increase in the size and emergence of the first two trifolia. We could localize NOD5 as an endophyte in roots in *P. vulgaris* by transforming the strain with a Green Fluorescent Protein carrying plasmid. Experiments of co-inoculation with different *Rhizobium etli* strains allowed us to discard that NOD5 can fix nitrogen in the nodules formed by a *R. etli* Fix$^-$ strain. The isolates described in this work show biotechnological potential for plant growth promoting activity and production of indoleacetic acid and siderophores.

1. Introduction

The use of chemical fertilizers is the most widespread source of nutrients for crops. Unfortunately, this practice has several drawbacks since it can provoke water pollution (hypertrophication), soil salinization and it is a costly input for farmers. An alternative to the use of chemical fertilizers is the use of Plant Growth Promoting Microorganisms (PGPM), which are bacteria or fungi that can improve plant growth through a series of mechanisms such as nutrient solubilization and availability (for example, phosphate solubilization, siderophores synthesis) and production of plant hormones (for instance, auxins and gibberelins) (Etesami and Beattie, 2017; Olanrewaju et al., 2017; Yakhin et al., 2017; Jia et al., 2014)

Among the Plant Growth Promoting Bacteria (PGPB), probably the most studied is the genus *Rhizobium*, which fix nitrogen in specialized plant organs called nodules (although some *Burkholderia* species also show this capability) (Andrews and Andrews, 2017; Suárez-Moreno et al., 2012). These are complex relationships that imply colonization of

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the plant tissues by bacteria. Not all PGPB act in such an intimate relation, many of them can be just endophytic (penetrating the plant, but remaining in between cells) or even rhizospheric.

Some endophytic bacteria may also induce resistance to plant pathogens or fix nitrogen (some species of the genus *Azospirillum*, for example) (Kirchhof et al., 1997; Tariq et al., 2014) and can be explored as PGPB. Although internal colonization of the plant tissue is regarded as a major criterion for an isolate to be denoted as an endophyte, several other bacteria whose endophytic nature is yet to be proved remain associated with different plant tissues. While most studies on the occurrence of non-rhizobial bacteria come from cultivated legumes, the vast plethora of uncultivated and wild legumes remain largely unexplored (Selvakumar et al., 2008). In a recent study, the bacterial communities that occur within roots of rice plants by both cultivation-independent and cultivation-dependent approaches were studied (Hardoim et al., 2011). From the directly obtained clone library, 30% of the sequences were assigned to the genus *Enterobacter*. This clearly demonstrated the predominance of *Enterobacter*- related phylotypes in the rice root bacterial community and indicated their potential functional importance. The 16S rRNA sequences also matched a sequence obtained from an *Enterobacter* sp., a rice endophytic bacterium isolated in South Korea that was reported to have plant growth promoting properties (Lee et al., 2006 and Hardoim et al., 2011).

Still, there is a lack of information about the diversity of bacteria that can colonize plants and the mechanisms that can use to promote plant growing. Therefore, the aim of this study was the isolation, characterization, and test the plant growth promotion abilities of non-rhizobial isolates from nodules of *Mimosa pudica*, a wild type legume which was located in a non-perturbed environment in the tropical rain forest in Chiapas, Mexico.

2. Materials and methods

2.1. Isolation of endophytic microorganisms from Mimosa pudica nodules

Nodules were collected from twenty *M. pudica* plants gathered within an extension of twenty hectares located in the Lacandona tropical rain forest in Chiapas México (16°45'0" N; 91°30'0" W). Roots were unearthed and approximately ten nodules per plant were collected. After 10 washes using sterile water, nodules were surface-sterilized using 70% ethanol (10 min) and 2% sodium hypochlorite (20 min). Finally, they were washed 10 times with sterile water. Sterile nodules were crushed in 1 mL of 10 mM $MgSO_4$ and the resulting suspension was streaked on PY agar plates (2% peptone, 1% yeast extract, 2% bacteriological agar), which were incubated at 28 ± 1 °C for 5 days. Axenic cultures were obtained and conserved in 20% glycerol.

2.2. Molecular identification of the bacterial strains

The genomic DNA from ten of the isolates was extracted using the ZR Fungal/Bacterial DNA Kit kit™. The 16S rDNA gene was amplified using the oligonucleotides rD1 and fD1 under the conditions described by Weisburg et al., (1991). The amplification products were purified from the gel using the GeneJET kit (Thermo Scientific) and were sequenced at the sequencing unit of the Instituto de Biotecnología, Universidad Nacional Autónoma de México. The sequences obtained were deposited in the GenBank of the National Centre for Biotechnology Information (NCBI) under accession numbers MH385358 (NOD1); MH385359 (NOD2); MH376910 (NOD3); MH392302 (NOD4); MH392316 (NOD5); MH392321 (NOD6); MH392319 (NOD7); MH392322 (NOD8); MH392323 (NOD9) and MH392324 (NOD10).

The 16S rDNA sequences of the isolates were compared with the 16S rDNA genes in the GenBank database using BlastN and phylogenetic analysis was performed using the program MEGA 6 (Tamura et al., 2013). Briefly, sequences retrieved from the BlastN hits and from known collection strains were used to prepare the phylogeny trees. The phylogenies were constructed using the neighbor-joining method (Saitou and Nei, 1987) based on 800 nucleotides, using the distance matrix of Jukes and Cantor (1969). The tree topology was bootstrapped 1000 times.

2.3. Phosphate solubilization

The ability to solubilize phosphate was qualitatively determined by inoculating the strains in PY liquid medium with 0.14 mM $CaCl_2$ (added after autoclaving) and incubated at 28 °C for 24 h with agitation at 200 rpm to obtain a preinoculum. The bacterial cultures were centrifuged and adjusted to 0.2 OD_{600nm}, were seeded in triplicate in NBRIP culture medium (glucose, 1%; $Ca_3(PO_4)_2$, 0.5%; $(NH_4)_2SO_4$, 0.01%; $MgSO_4{\cdot}7H_2O$, 0.025%; KCl, 0.02%; $MgCl_2{\cdot}6H_2O$, 0.5%; Congo red, 2.5 mg/mL; agar, 1.8%) and incubated at 28 °C for 7 days. After this period, the size of the halos around the colonies were measured (Caballero-Mellado et al., 2007).

Inorganic phosphate concentration was measured quantitatively in liquid culture using NBRIP medium without agar and Congo Red. Strains were inoculated at 0.2 OD_{600nm} and grown for 10 days at 28 °C at 200 rpm. Samples were taken every 48 h, after centrifugation of the samples the phosphate concentration in the supernatant was measured by the method of Rodríguez and Fraga (1999). Phosphate concentration was expressed as μg/mL.

2.4. Determination of auxin production by bacterial strains

To determine the production of the auxin Indoleacetic acid (IAA), strains were grown in liquid NFB medium (composition in g/L: malic acid, 5; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; $FeSO_4$, 0.015; Na_2MoO_4, 0.0025; $MnSO_4$, 0.01; KOH, 4.8; NH_4Cl, 0.2; yeast extract, 0.3; H_3BO_4, 0.01); the bacterial cultures were incubated for 18 h at 28 °C at 200 rpm. Then, the culture was adjusted to an $OD_{600\ nm}$ of 0.2 at and 100 μL of culture were inoculated into Jain and Patriquin medium (composition in g/L: succinic acid, 2.5; fructose, 2.5; K_2HPO_4, 6; KH_2PO_4, 4; NH_4Cl, 1; $MgSO_4$, 0.2, NaCl, 0.1; $CaCl_2$, 0.02; $FeCl_3$, 0.01; $NaMoO_4$, 0.002 and KOH, 2.1) with and without tryptophan (0.1 g/L) and incubated at 28 °C for 24 and 48 h at 200 rpm. One ml aliquots of the cultures were taken, centrifuged for 5 min at 5000 *g* and 0.5 mL of the supernatant were mixed with 0.5 mL of Salkowski reagent (Glickmann and Dessaux, 1995). The mixture was incubated in the dark at room temperature for 20 min and absorbance at 530 nm was measured using a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The presence of IAA in the supernatant was detected according to the modified method by Rahman et al., (2010). IAA concentrations were determined using a IAA (Sigma-Aldrich Corp.) standard curve from 0 to 200 μg/mL.

2.5. Cellulase and chitinase degradation assays

Isolated bacterial colonies were seeded in triplicate in solid NBRIP culture medium supplemented with 1 g/L of colloidal chitin or carboxymethyl cellulose (CMC) (Sigma-Aldrich Corp.) as sole carbon sources. Growth was considered to be positive for cellulose or chitin degradation (and utilization as a carbon source). Plates were incubated at 30 °C for 4 days, and bacteria were transferred to fresh plates containing chitin or CMC as sole carbon source in order to confirm the results. Cellulose degradation was also detected by transferring the colonies to modified Vogel minimal medium (Vogel, 1956) containing 1% w/v CMC and 0.1 w/v glucose instead of sucrose. After 48 h, the plate was flooded with 5 mL of 0.1% w/v Congo red and stained for 15 min at room temperature. After removing the dye, the plate was washed twice with 5 mL 1 M NaCl for 15 min each, then air dried and photographed to assess the halo formation around the colonies, which indicates cellulose degradation (Carder, 1986).

2.6. Antifungal activity

For detection of antifungal activity, *Fusarium* sp., *Phytophthora capsici* and *Alternaria solani* (kindly provided by the herbarium of LANGEBIO, CINVESTAV Irapuato), were grown for 7 days at 28 °C in PDA medium (2% casein peptone, 2% dextrose and 1% yeast extract). Blocks of 7 mm in diameter containing mycelia were cut using a sterile blade and placed in the centre of a fresh PDA plate. Endophytic cultures were streaked at two ends of the plate and incubated at 28 ± 2 °C for 48–96 h to assess the zone of fungal growth inhibition.

2.7. Siderophore production assays

Siderophore production was determined by the method described by Schwyn and Neilands, (1987). CAS-CAA (Chrome azurol (100 mM) and S-casamino acids) agar plates were inoculated with the isolates at 28 °C for 12 days. Orange halos formed around the colonies on blue agar were considered indicative of siderophore production.

2.8. PCR nifH genes amplification

DNA obtained from the ten isolates was subjected to PCR for the detection of the *nifH* gene as an indicator of nitrogen fixation. PolF and PolR oligonucleotides were used for amplification of *nifH* genes, using the PCR conditions described by Poly et al. (2001). The reaction amplifies a 360 bp fragment comprising a fragment of the *nifH1* gene. PCR products were run in a 2% agarose gel using TBE buffer (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA pH 8), stained with an ethidium bromide solution (0.5 µg/mL) and photographed under UV light (264–366 nm) for the record.

2.9. Evaluation of plant growth promotion ability of the bacterial isolates in medium with an inorganic phosphate source ($Ca_3(PO_4)_2$)

Seeds of *Phaseolus vulgaris*, were surface disinfected with a 10% sodium hypochlorite solution for 15 min. After that, seeds were washed with sterile distilled water and incubated for 72 h in sterile trays with humid filter paper. The plantlets were placed in flasks containing modified Fahraeus medium (composition in g/L: $CaCl_2$, 0.1; $MgSO_4$, 0.1; ferric citrate, 0.005 and with 5 g/L $Ca_3(PO4)_2$, instead of KH_2PO_4, and $Na_2HPO_4.2H_2O$). One milliliter of the trace element solution (in g/L: H_3BO_3, 2.86; $MnSO_4$, 2.03; $ZnSO_4$, 0.22; $CuSO_4$, 0.08; NaMoO, 0.14; $NaCl_2$, 0.1; KCl, 0.1) was added for every 100 mL of medium (Fahraeus, 1957). After three days, the plants were inoculated with 1 mL of each of the ten isolates in the growing media adjusted to 0.2 DO_{600nm}. Plants were grown at 28 °C, with a photoperiod of 8 h light /16 h dark and growth was evaluated at day 7 and day 15 by measuring the shoot height with a ruler. Three replicas for each treatment were performed.

2.10. Interaction of NOD1 with P. vulgaris plants with or without R. etli strains

Strain NOD5 was transformed with plasmid pGLO (Bio-Rad) that carries a green fluorescent protein gene inducible by arabinose. Colonies were selected on LB plates with 200 µg/ml of ampicillin.

Bean seeds were disinfected with 25% sodium hypochlorite and 70% ethanol and then thoroughly washed with sterile distilled water. Then they were transferred to sterile trays for three days and when germinated, they were transferred into flasks containing Fahraeus medium. At this moment seeds were inoculated with1 mL of a suspension of cells at 0.2 DO_{600}. The experimental conditions were: a) *R. etli* wild type; b) NOD5/pGLO; *R. etli* wild type + NOD5/pGLO; R. etli Fix^- + NOD5/pGLO and a control without inoculation was also included. Arabinose (10 mM) was added to induce the green fluorescent protein 2 days before the samples were taken. The plants were incubated in a greenhouse and were sacrificed at 14 or 21 days to take the samples. A Zeiss fluorescence microscope was used to evaluate the presence of bacteria expressing GFP in plant tissues.

3. Results

3.1. Isolation of bacterial endophytes from M. pudica nodules

As described in the materials and methods section, we isolated bacterial strains from *M. pudica* nodules and 10 strains were selected, each from different plants nodule isolates. The strains were named NOD1 to NOD10, and were tested for different growth promoting-associated activities described below.

3.2. Molecular identification

The 16S rDNA sequences were analyzed by the BLASTn algorithm and showed high similarity with *Enterobacter* species (NOD1, NOD2, NOD4, NOD5, NOD6, NOD8, NOD9 and NOD10), except NOD3 and NOD7 that revealed high similarity with *Serratia* sp. Phylogenetic trees were constructed using a fragment of the 16S rDNA genes sequences to further confirm the identity of the isolates to the genus level mentioned above (Table 1 and Fig. 1A and B).

The phylogenetic analysis shows that none of the isolates groups were close enough to any reference strain sequence, so we were unable to determine the species for the *Enterobacter* genus isolates. However, it is clear that all of them group in the same branch with several *Enterobacter* species (see Fig. 1A) and in a separate group than those in the *Cronobacter* genera, whose sequences were selected according to the BLAST results, since they also showed high values of similarity (Table 1). A *Burkholderia* sequence was included, since this genus forms nodules in *Mimosa* but it resulted in an outgroup, supporting the identity of the isolates as non-nodulating bacteria. From this analysis we can observe, however that NOD1 and NOD8 are related to *E. koebi*,

Table 1
Blast results using amplified 16 s rDNA sequences form the isolates from *M. pudica* nodules. The first three hits are shown for each sequence.

Code	Possible genus	Related strains (from GenBank sequences)	Query cover (%)	Identity (%)
NOD1	*Enterobacter* sp.	*Enterobacter* sp.	99	99
		Enterobacter asburiae	99	99
		Cronobacter sp.	98	98
NOD2	*Enterobacter* sp.	*Enterobacter* sp.	100	99
		Enterobacter asburiae	100	99
		Cronobacter sp.	99	98
NOD3	*Serratia* sp.	*Serratia marcescens*	100	99
		Serratia sp.	100	99
		Enterobacter sp.	100	99
NOD4	*Enterobacter*	*Enterobacter* sp.	99	99
		Cronobacter sp.	99	95
		Enterobacter asburiae	99	99
NOD5	*Enterobacter*	*Enterobacter* sp.	100	99
		Enterobacter asburiae	100	99
		Enterobacter ludwigii	100	99
NOD6	*Enterobacter*	*Enterobacter* sp.	99	99
		Cronobacter sp.	99	97
		Enterobacter asburiae	99	99
NOD7	*Serratia*	*Serratia* sp.	99	99
		Serratia nematodiphila	99	99
		Serratia marcescens	99	99
NOD8	*Enterobacter*	*Enterobacter* sp.	100	85
		Enterobacter ludwigii	99	85
		Enterobacter aerogenes	99	86
NOD9	*Enterobacter*	*Enterobacter* sp.	100	99
		Enterobacter asburiae	100	99
		Enterobacter ludwigii	99	99
NOD10	*Enterobacter*	*Enterobacter* sp.	100	99
		Enterobacter asburiae	100	99
		Enterobacter ludwigii	99	99

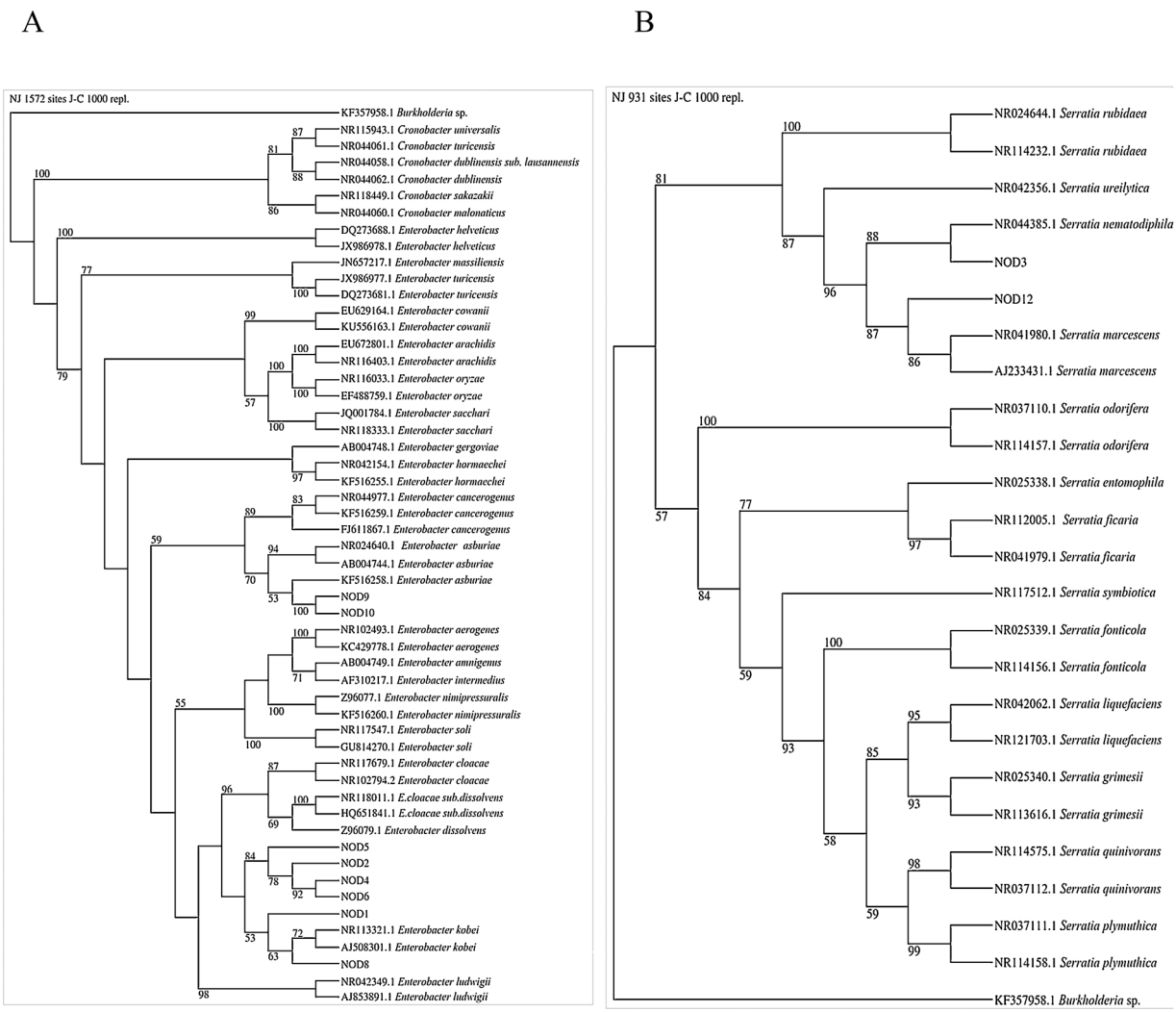

Fig. 1. Phylogenetic analysis of the isolates form *M. pudica*. According to the BLAST results two separate phylogenetic trees were performed. A) *Enterobacter* sp. **B)** *Serratia* sp. Accession numbers of reference sequences are shown before the species name.

but are placed in different branches. Similarly, NOD 9 and NOD10 group together and are close relatives of *E. asburiae*. As well, NOD4 and NOD6 pair together closely to NOD2 but in separate branch, that is related to a more distant branch where we find NOD5, but also sharing a node among the four strains (Fig. 1A).

As for the *Serratia* strains, we found that one of them (NOD3) is related to *S. nematodiphilia*, while NOD7 is closely related to *S. marsescens*.

3.3. Phosphate solubilization

Solubilization of inorganic P is shown in Fig. 2(A and B). This phenomenon is often associated with a lowering in pH by microorganisms, so we decided to measure the pH in the media during the course of the solubilization experiment (Fig. 2B). As can be seen, the pH drops in the first days of the bacterial growth, correlating with phosphate solubilization. However, some strains show an increase of pH at later times (NOD2, NOD5 and NOD9 at 8 and 10 days of growth). With these data in mind, we could organize the ten isolates in different groups according to their solubilization capabilities and their capacity to acidify the medium: those that are very good solubilizers (more than 70 μg/mL; Fig. 2A), decrease the pH in the media to 4 at day four and then increase the pH up to 6 at the end of the experiment (day 8 [pH 5] and day 10 [pH6]; Fig. 2B). These strains are NOD2, NOD5 and NOD9. Other group is formed by strains NOD4, NOD6, NOD8 and NOD10, which are as good solubilizers as the latter group (Fig. 2A), but decrease the medium pH to 4 and maintain this pH along the whole experiment (Fig. 2B). A third group comprises strains NOD3 and NOD7, which are poor solubilizers (only around 20 μg per ml) but still decrease the pH of the medium although more drastically (pH 3; Fig. 2). Finally, strain NOD1 solubilizes a little more phosphate (around 30 μg/mL) than strains in group 3, although statistical analysis (ANOVA, $p < 0.05$) shows that there is no significant difference. This strain is not able to acidify the medium below pH 5 (Fig. 2). Although all strains lower the pH of the media, there is no strict correlation between the best solubilizing strains and the pH in their growth media (see Discussion). After day four, a decrease in the amount of soluble phosphate in the media was noted. This could be due to a regulation of the expression of mineral phosphate solubilizing genes in the presence of (already) solubilized phosphate (Goldstein and Liu, 1987). Similar results were described for *Bacillus* strains by Tejera-Hernandez et al. (2013). Another possibility is the uptake of the solubilized phosphate by the bacteria.

3.4. Indoleacetic acid quantification

Since another important growth promoting factor is the capability to synthesize auxins, we measured the ability of the isolates to produce Indoleacetic Acid (IAA) as it is the most studied auxin (Fig. 3). All strains showed a high amount of IAA in the culture medium except for the *Serratia* sp. isolates NOD3 and NOD7, which produced very small

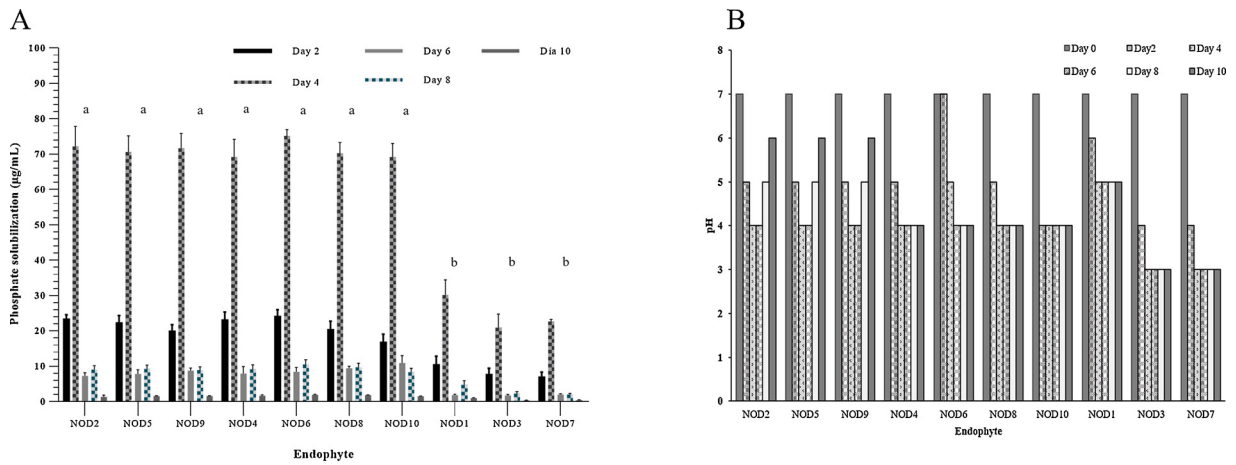

Fig. 2. Solubilization of inorganic phosphate by the 10 isolates from *M. pudica* nodules and its relation with pH. A) Phosphate solubilization B) pH values during the growth of the bacterial isolates. Different letters on the top of the bars indicate a statistically significant difference ($p < 0.05$).

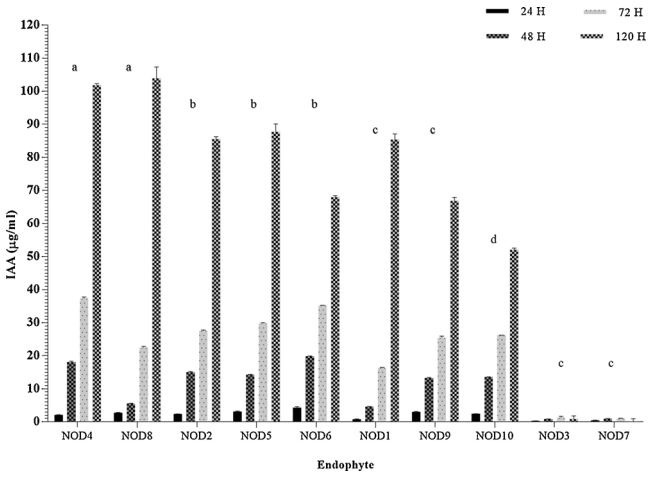

Fig. 3. IAA quantification of the isolates from nodules of *M. pudica*. Media were supplemented with tryptophan. Different letters on the top of the bars indicate a statistically significant difference ($p < 0.05$). Controls in media without tryptophan did not show IAA production (not shown).

amounts of IAA (around 3 µg/mL). It is worth to notice that in the first 48 h the amount of IAA is below 20 µg for all strains (Fig. 3), since the high amounts produced at the end of the experiment could be toxic to plants (see Discussion). Regarding IAA production, we could group the isolates in 5 categories according to the ANOVA ($p < 0.05$) analysis of the amount of IAA produced. NOD4 and NOD 8 were the best producers reaching high concentrations of more than 100 µg/mL; a second group, also produced significant amounts of IAA (on the range of 70–80 µg/ml), these were strains NOD2, NOD5 and NOD6. NOD1 and NOD9 fall into a third group producing around 70 µg/ml, although this is due to the similarity in production during the first days of culture. At day 5, NOD1 produces a similar amount of IAA as the strains belonging to group two. Finally, NOD10 produces also a significant amount of IAA but lower than the other isolates (around 50 µg/mL).

3.5. Siderophore and cellulase production

Colonies from the isolates were placed in CAS-CAA medium and tested for a yellow halo formation, indicative of siderophore production. All strains were positive for this test, including both *Serratia* sp. isolates (Table 3). A representative example is shown in Fig. 4A.

Similarly, the strains were also grown in a minimal medium with CMC as a sole carbon source and the staining was done with Congo red to assess cellulase production. This test resulted positive only for strains NOD2, NOD3, NOD7 and NOD8. A representative example of this test is shown for NOD2 and NO3 (positive); NOD1, NOD6 and NOD5 (negative) (Fig. 4B).

3.6. Chitinase determination

Since chitin is a major component of fungal cell walls and biocontrol of phytopathogens could rely on this activity, we decided to measure chitinase activity of the isolates. The *Serratia* sp. strains (NOD 3 and NOD7) showed chitinase activity (Table 2). In contrast, none of the *Enterobacter* sp. strains showed this activity (data not shown).

3.7. Antagonism against different plant fungal pathogens of the isolates

One of the proposed plant growth promoting mechanisms of endophytes is the control of pathogenic fungi that can have a major effect on crop yield or even kill the plants. With this in mind, we tested the ability of the nodule isolates to inhibit the growth of three well known plant pathogens: *Phytophtora capsici*, *Alternaria solani* and *Fusarium* sp. Only the *Serratia* sp. strains (NOD3 and NOD7) were able to inhibit mycelial growth of these fungi when confronted in the same media (Fig. 5), the *Enterobacter* isolates did not show this behavior, for example see NOD 6, which did not grow and was completely invaded by the fungi (Fig. 5, column 3, all the rows). Isolate NOD 7 was not a good antagonist against *Fusarium* sp. (Fig. 5, column 4, row 3), as compared to NOD3 (Fig. 5, column 2, row 3).

3.8. nifH detection

Since the isolates were selected form nitrogen-fixing nodules form wild type *M. pudica* plants, we decided to explore if the isolates possessed the *nif*H1 gene as a marker for nitrogen fixation, an essential component of the nitrogenase. Of the 10 strains selected, only NOD2, NOD5 and NOD8 (all of them belonging to the *Enterobacter* clade) amplified a PCR product of 360 bp corresponding to the *nif*H1 gene fragment, suggesting that these strains could fix nitrogen (Fig. 6).

3.9. Plant growth promoting activity of the isolates in a phosphorous limited medium

As we detected good values for several plant-growth promoting traits, we developed an assay in a medium containing an insoluble form of phosphate ($Ca_3(PO_4)_2$) to assess the plant growth promoting activities of three *Enterobacter* isolates. We sowed *Phaseolus vulgaris* plantlets with or without the NOD strains. *P. vulgaris* is a legume that forms

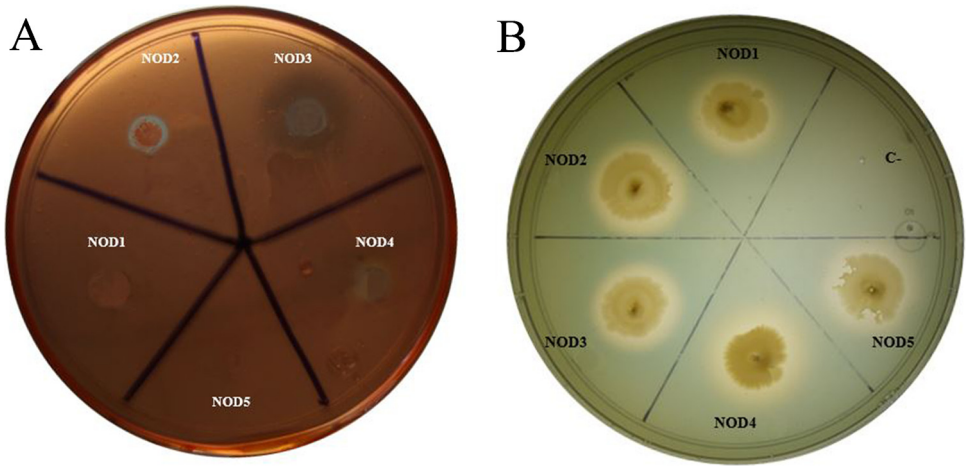

Fig. 4. Determination of siderophore production and cellulase activity by nodule isolates. **A)** Test for siderophore production, the control is a non-inoculated spot. **B)** Cellulase activity using CMC as a substrate. NOD2 y NOD3 show a degradation halo while the other three strains failed to produce it.

nodules in association with *Rhizobium etli, Rhizobium tropici* or *Rhizobium leguminosarum bv. phaseoli* and due to its agronomical importance, we chose it as a model plant for the tests. Only NOD1 and NOD10 were able to statistically increase in a significant manner plant height (Fig. 7). NOD6 showed a small effect at 15 days of growth. Fig. 8 shows the statistical analysis for three independent replicas. Plants inoculated with NOD1 and NOD10 are more developmentally advanced since a second true leave is already emerging compared to the control plants and those inoculated with NOD6 (Fig. 7).

3.10. Interaction of P. vulgaris plants with NOD5

Since NOD5 showed a good phenotype regarding plant growth promoting activities and it carries the *nif*H1 gene, we decided to perform experiments inoculating NOD5 in *P. vulgaris* plants in different situations. For these experiments, we used a NOD5 strain that carries the green fluorescent protein encoded in plasmid pGLO™ in order to visualize its localization and determine if it is an endophyte or a rhizospheric bacterium. First of all, we inoculated bean plants with the NOD5 strain alone to see if it was capable of forming nodules on its own, or if it was capable of fixing nitrogen as an endophyte. As a positive control, we used a wild type *R. etli* strain to assess nodulation and nitrogen fixation. Strain NOD5 was incapable of nodulating *P. vulgaris* plants and no acetylene reduction activity was detected indicating that no nitrogenase activity was present, while the control with *R. etli* was positive for nodule formation and nitrogen fixation (data not shown). However, we could visualize the NOD5/pGLO strain associated to the root at 14 days' post inoculation and inside the root at 21 days' post inoculation (Fig. 9) confirming its endophytic nature.

Another experiment was performed by co-inoculating the NOD5/pGLO strain with *R. etli* (wild type) or with a Fix$^-$ *R. etli* mutant (both copies of the functional *nif*H genes were deleted in this strain). Nodules produced by the mixture of both, *R. etli* wild type and *Enterobacter* NOD5/pGLO, showed a morphological difference when compared to the nodules formed by *R. etli* alone. In the first case, the nodules presented an overlapping amongst them and the borders were thick, whereas when *R. etli* was alone the nodules were clearly individual and no thick borders were observed (Fig. 10). Nitrogen fixation was similar in both cases, suggesting that NOD5/pGLO does not contribute to this process (data not shown). This was corroborated in the mixture of *Enterobacter* sp. NOD5 and *R. etli* Fix$^-$. In this case, although nodules were formed, no nitrogen fixation was detected (data not shown).

Fluorescence microscopy of the nodules formed by the mixture of *R. etli* wild type and NOD5/pGLO allowed us to localize the *Enterobacter* sp. in the borders of the nodule and on the root surface, but no fluorescence was detected inside of the nodules (Fig. 10).

4. Discussion

Nodules collected from wild type *M. pudica* plants from the Lacandona tropical rain forest were explored for the presence of non-rhizobial endophytic bacteria. Ten isolates were characterized for several traits shown to be related to plant growth promoting ability.

Phylogenetic analysis using a fragment of the 16S rDNA, showed that 8 of the isolates belong to the *Enterobacter* genus and two to the *Serratia* genus. This molecular marker did not allow us to further classify the isolates to the species level, so we are looking forward to select a more specific marker for each of the genera (*rpoB*, for example, Fazzeli et al., 2012). However, the cladogram shows that probably each of the isolates belong to a different species, since they do not group closely with any confirmed reference sequences. Most probably the *Enterobacter* species described here are new species, because *Mimosa* nodules are normally screened for Nitrogen fixing Bacteria (*Rhizobium* or *Burkholderia*). Our approach did not bias the screening, allowing us to find undescribed species. This idea is also supported by the fact that the biochemical behaviour of the isolates is unique for each of the strains. For example, the best phosphate solubilizers are strains NOD2, NOD5 and NOD9 which are distantly positioned in the phylogenetic

Table 2
Chitinase activities in the *Serratia* sp. strains NOD· and NOD7. As a negative control, we used *E. coli* DH5α. ND = Non detectable.

	NOD3			NOD7			Negative control	
	volumetric activity U/ml	specific activity U/mg	Standard deviation	volumetric activity U/ml	specific activity U/mg	Standard deviation	volumetric activity U/ml	specific activity U/mg
Day 4	0.063	252.417	0.011	0.062	233.575	0.041	N/D	N/D
Day 5	0.036	144.801	0.010	0.039	154.131	0.007	N/D	N/D
Day 6	0.035	140.742	0.013	0.038	158.596	0.010	N/D	N/D
Day 7	0.033	137.896	0.008	0.039	159.803	0.029	N/D	N/D

Table 3
Biochemical characterization of the different isolates from *M. pudica* nodules.

Strains	Phosphate solubilization mg/l	IaA production µg/mL	Siderophores	Cellulasess	Chitinases U/mg	*nif*H1
NOD1	9.68	26.77	+	–	–	–
NOD2	22.70	32.59	+	–	–	*
NOD3	6.63	0.81	+ + +	+	**252.41**	–
NOD4	22.25	39.85	+ +	+	–	–
NOD5	22.35	33.72	+ +	–	–	*
NOD6	24.09	31.83	+	–	–	–
NOD7	6.87	0.60	+ + +	+	**233.57**	–
NOD8	22.35	33.65	+	+	–	*
NOD9	22.23	27.15	+ +	–	–	–
NOD10	21.39	23.55	+ + +	–	–	–

tree, while the poor solubilizers, NOD1(*Enterobacter* sp.), NOD3 and NOD7 (*Serratia* sp.) even belong to different species. Similar uncorrelated behaviours were observed for all strains regarding different biochemical traits (Table 3), except for the *Serratia* species, that although positioned distantly in the phylogenetic tree show very similar biochemical traits. It is interesting to note the great diversity found within the *Enterobacter* genus, form ten isolates taken at random from nodules of different plants, none of them belongs to the same species and has a different biochemical behavior.

Previous reports have shown that some *Enterobacter* sp. and *Serratia* sp. isolated from legume nodules can fix nitrogen (Muresu et al., 2008; Ibáñez et al., 2009; Selvakumar et al., 2008; Stajković et al., 2009). Moreover, an *Enterobacter* species was reported to fix nitrogen when associated with rice (Hardoim et al., 2013).

In this report, we found strains carrying the *nifH1* gene, an essential component of the nitrogenase (NOD2, NOD5 and NOD8, all belonging to the *Enterobacter* sp.). Although these strains were able to grow in a nitrogen free medium forming a film in the middle section of the culture tube (data not shown), none of them were able to fix nitrogen, as measured by acetylene reduction, when grown in liquid culture but in aerobic conditions (data not shown).

Phosphate solubilization has been considered one important trait that bacteria display to promote plant growth, since phosphate is often found as insoluble salts which are not available to the plant. Usually phosphate solubilization is associated with soil acidification which dissolves the inorganic insoluble phosphate salts. In general, in this study all strains decreased the pH of the media and solubilized phosphate between day 2 and 4. However, we did not find a strict correlation between the amount of phosphate solubilization and pH values in the medium. The most extreme example is NOD6, which shows a similar phosphate solubilization as NOD2, NOD5, NOD9 and NOD4, but does not decrease the pH at day 2 (the medium stays at pH 7). Nonetheless, this strain decreases the pH at day 4 to the same level as the latter strains. The best phosphate solubilizers recovered the pH values up to near the original pH in the media, while a second group maintained the media at pH 4. In contrast, the worst solubilizers (NOD3

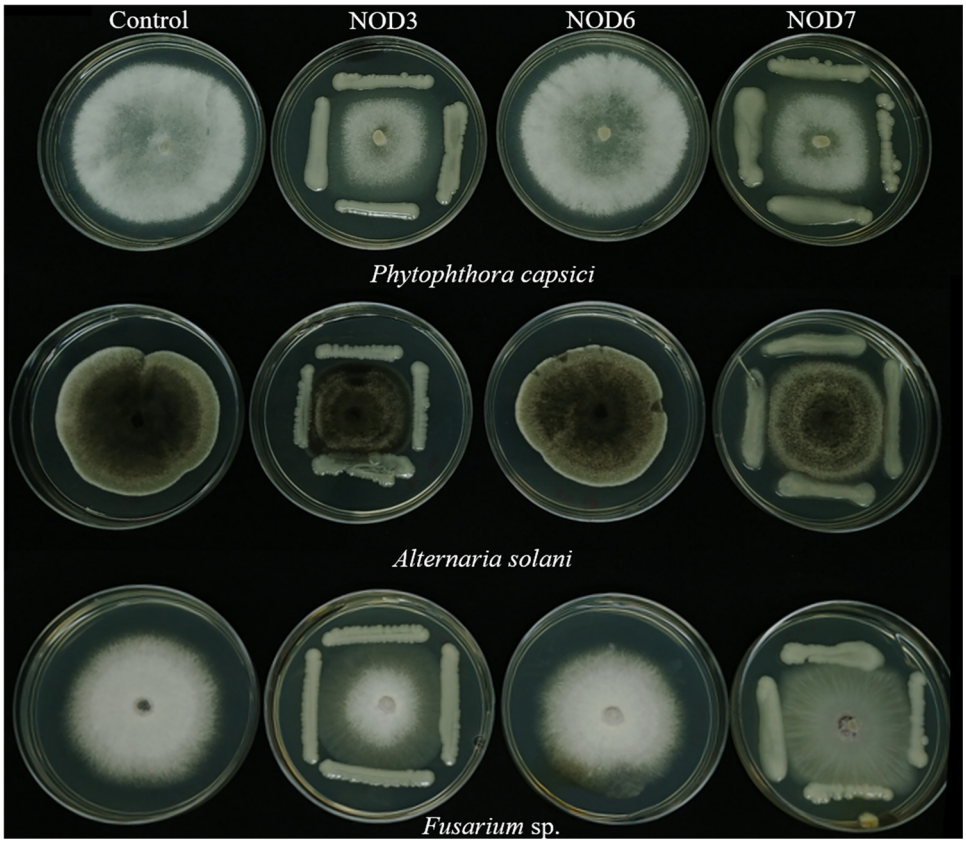

Fig. 5. Antagonism of three isolates against pathogenic fungi. Control columns means that the fungus was inoculated without a phytopathogen. Row1: *Phytophtora capsici*; row 2: *Alternaria solani*; row 3: *Fusarium* sp. NOD6 was not able to control the growth of the pathogenic fungi.

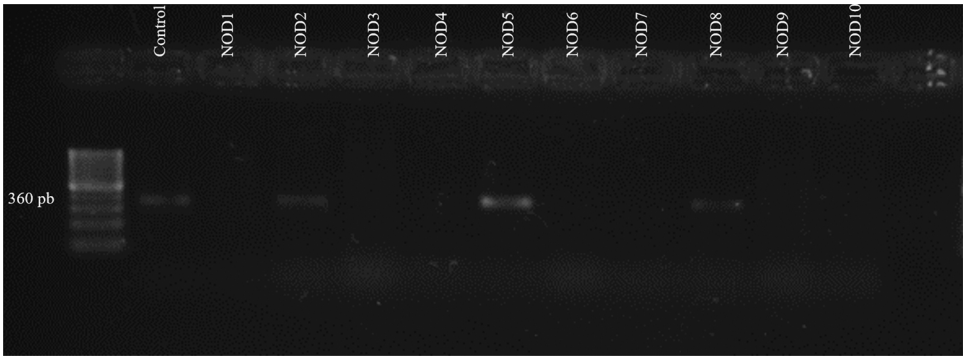

Fig. 6. *nif*H1 amplification of nodule isolates. As a positive control, *Rhizobium etli* DNA was used.

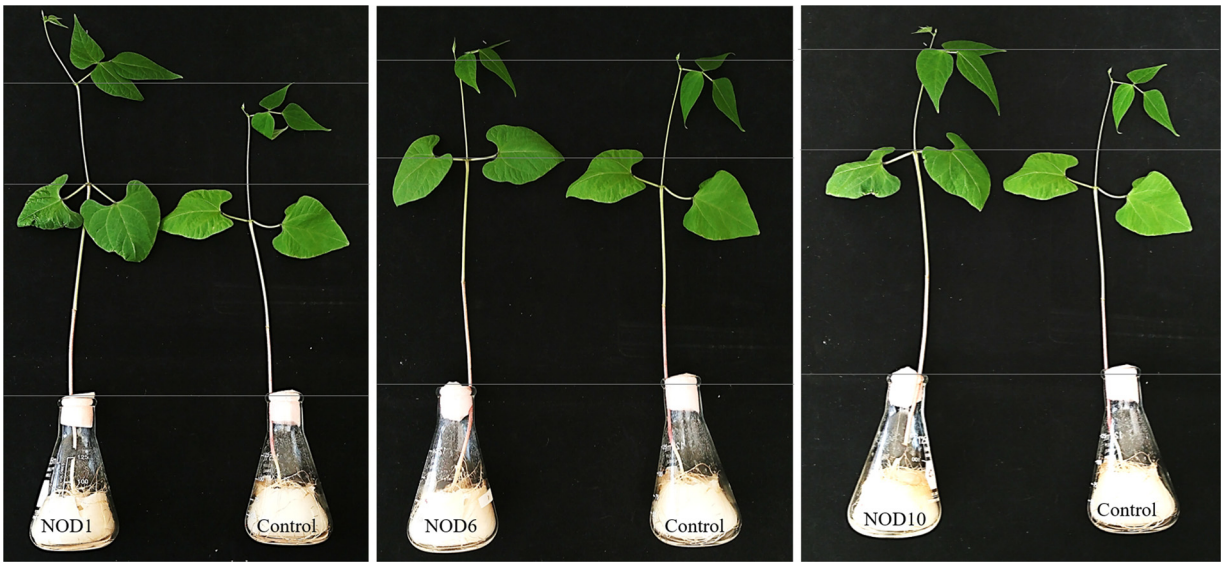

Fig. 7. Plant growth promoting activity of NOD1 and NOD10. *P. vulgaris* plantlets were sowed in phosphorus limited media with (left plants) or without (right plants) NOD1, NOD6 and NOD10. The picture is a representative image of 15 days grown plants.

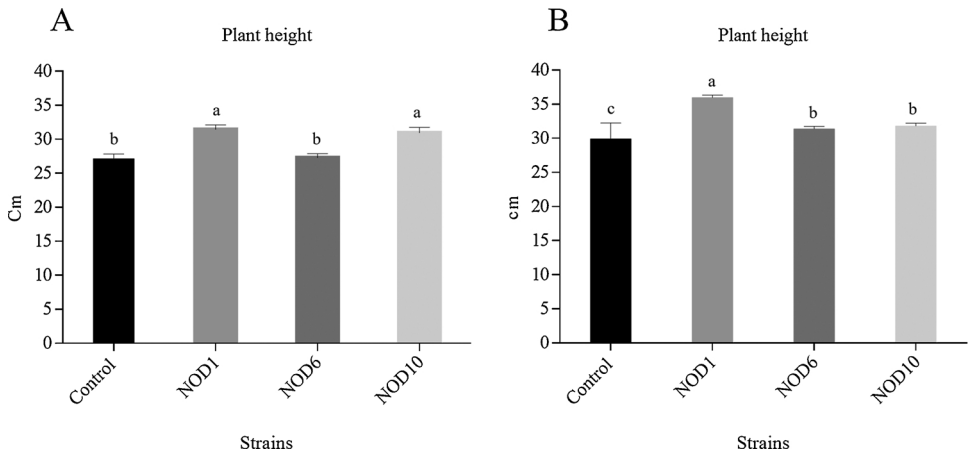

Fig. 8. Effect in the plant growth induction by the nodules isolates in a media with insoluble phosphate Statistical analysis of 7 (left panel) and 15 (right panel) days grown plants with or without NOD strains. Different letters indicate significant.

and NOD7, both *Serratia* sp.), lowered the pH down to 3 and never recovered. This is accordance with findings by other research groups whom have shown that other phosphate solubilization mechanisms could be involved, such as the production of organic acids (Nautiyal et al., 2000; de Oliveira Mendes et al., 2014).

The *Serratia* sp. strains did not produce auxin. On the other hand, all the *Enterobacter* sp. isolates produced high amounts of IAA. However, the amounts of IAA produced by these strains at day five could be deleterious to the plant (even those produced by NOD10) and could act as herbicides (Grossmann, 2003, 2010). Recently an *Enterobacter* sp. that overproduces IAA has been described to act as a bio-herbicide (Park et al., 2015). In this regard, experiments are under way to assess if the isolates described here could act as bio-herbicides for undesirable weeds. Another biotechnological application for our isolates could be

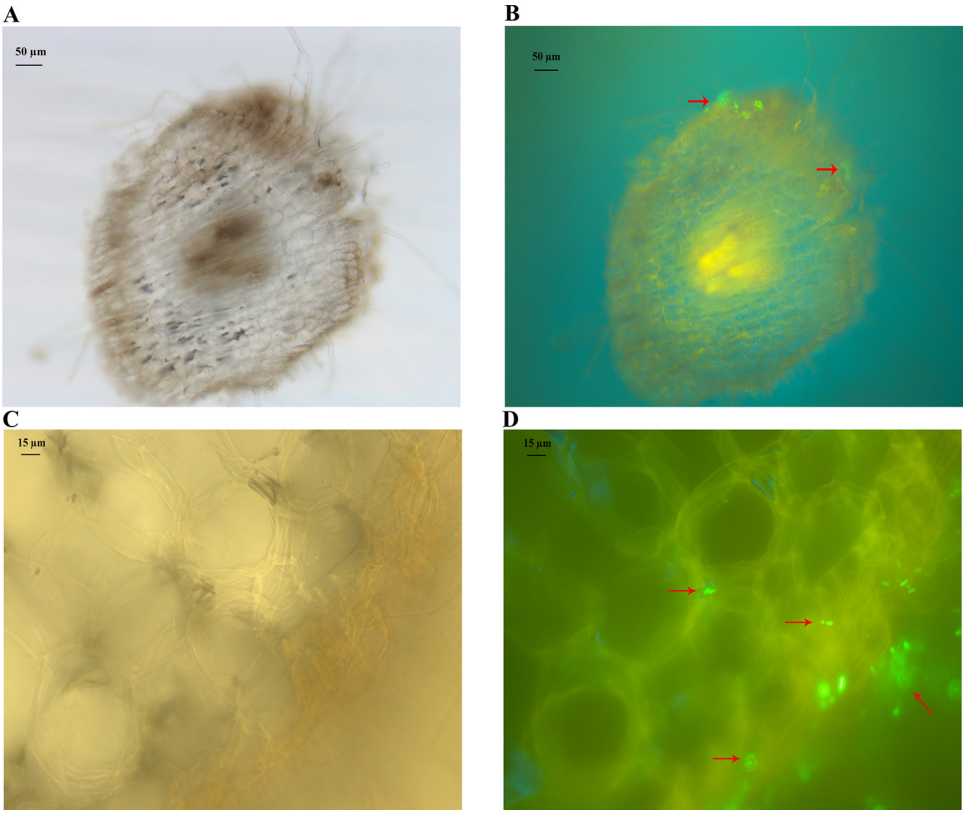

Fig. 9. Panel A and B, representative root section of *P. vulgaris* plants inoculated with NOD5/pGLO. A, visible mode without green filter, 100X zoom. B, UV light green filter 100X zoom of the same section. Red arrows indicate fluorescence by NOD5/pGLO. C and D, 21 days post inoculation. C, visible mode 100X zoom. D UV light green filter, Red arrows indicate fluorescence by NOD1/pGLO.

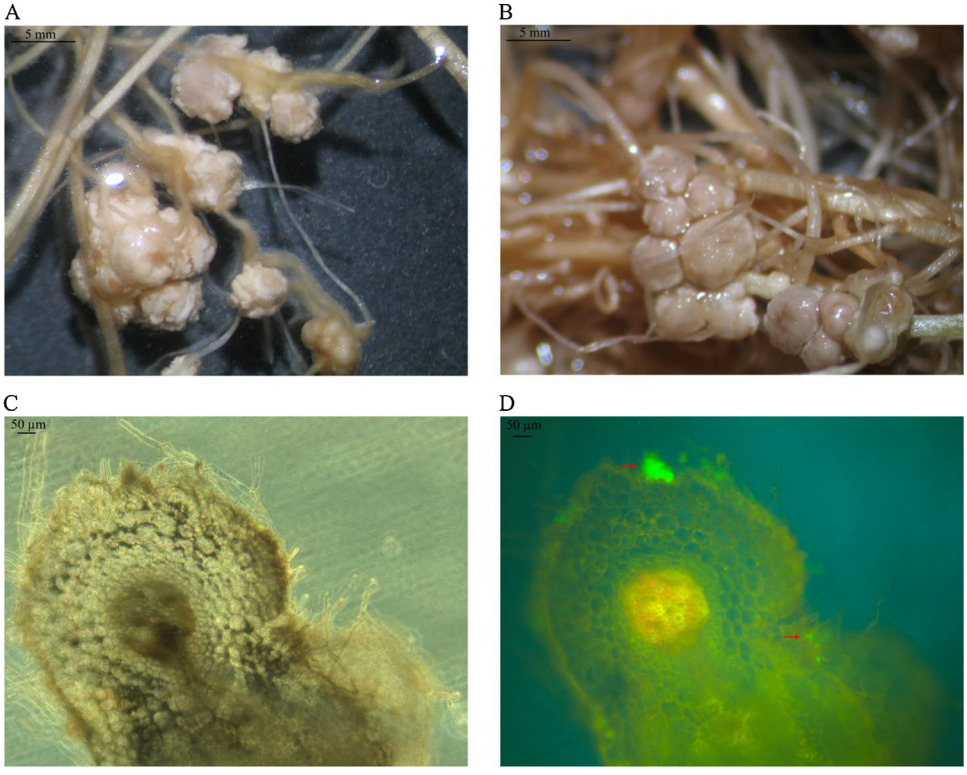

Fig. 10. Nodules formed by mixture of *R. etli* wild type and *Enterobacter* sp. NOD5. A and B, Stereoscopic image (4X) of nodules formed by *R. etli* and NOD5 (A) and *R. etli* alone (B). C and D, Image of the section of a representative nodule (5X zoom) in visible mode (C) and with the UV green filter (D). Red arrows show fluorescent NOD5 cells.

precisely the production of IAA for rooting powders based on auxins. However, it is worth to note that these high IAA concentrations were achieved in a culture medium supplied with tryptophan (although at a much lower concentration than that used by Park et al., 2015; see also Masciarelli et al., 2013); this amino acid is unlikely to be found in large amounts in the soil, so we think that *in vivo*, the production of IAA must be much lower. Also, to be considered is that in the presence of the plant, IAA production by the bacteria could be differently regulated due to compounds secreted by the roots.

Although the *Serratia* sp. strains did not produced IAA and were poor phosphate solubilizers, they might still be used for biological control against phytopathogenic fungi when added to bacterial

consortia for growth promoting purposes (Romero-García et al., 2016).

Siderophore production was also assessed in the isolates described in this work. All strains produced siderophores, an important trait for promoting plant development in iron limiting conditions. Also, siderophores may play a role in biological control of plant pathogens by removing available iron from the media and delivering it to the plant.

Chitinases are important enzymes for fungal cell wall degradation and therefore its production could be an important trait to achieve biological control of phytopathogenic fungi (Berg and Hallmann, 2006; Wang et al., 2013; Gkarmiri et al., 2015). In this work, we found that only the two *Serratia* sp. isolates produced chitinases. This fact correlates entirely with the observation that only these two strains inhibited the growth of three fungal plant pathogens (Table 2 and Fig. 5). The chitinase production of the *Serratia* sp strains could also be useful in biocontrol of insect pests, since the insect exoskeleton is composed mainly of chitin (Gerc et al., 2012; Petersen and Tisa, 2012).

There are some examples in literature in which bacteria isolated from nodules are capable to promote growth of unrelated plants (Selvakumar et al., 2008). Since common bean (*P. vulgaris*) is an important crop and a legume (although distantly related, *M. pudica* belongs to the same family), we decided to test if the phosphate solubilization abilities of the strains isolated in this work could promote growth of this plant in a medium with an insoluble form of phosphate.

Preliminary testing in tubes containing medium with an insoluble phosphate source, pointed to strains NOD1, NOD6 and NOD10 as plant the best growth promoting candidates, so we studied in detail these three strains. Our results show that strains NOD1 and NOD10 (both *Enterobacter* species) clearly promote growth of *P. vulgaris* plants as compared to a control without inoculation. NOD6 shows a small effect at longer periods of growth (Figs. 7 and 8). The three strains are good phosphate solubilizers. It is also worth to note that although the growth promoting effect is not very large, developmentally the plants respond better to the presence of NOD1 and NOD10, since the first trifolium is bigger than those in the uninoculated plants and furthermore, the second trifolium is already appearing in the treated plants but not in the control plants or those in the presence of NOD6.

To try to detect if a strain carrying the *nif*H gene was able to fix nitrogen we performed a series of experiments co-inoculating NOD5/pGLO with *R. etli* wild type or a *R. etli* strain which has both copies of the functional *nif*H genes deleted. These experiments allowed us to conclude that *Enterobacter* sp. NOD5 is a root endophyte in *P. vulgaris*, it was not present in nodule tissues in this plant and did not fix nitrogen in any condition tested. It remains to be explored if NOD5 could fix nitrogen in *M. pudica* plants, since it was isolated from nodules of this legume.

5. Conclusions

In this work, we have shown that it is possible to isolate non-rhizobial endophytic bacteria from *M. pudica* plants from a wild environment. This is the first report of the isolation of *Enterobacter* sp. from *M. pudica* nodules. According to the phylogenetic analysis, some of the isolated *Enterobacter* species are probably new, undescribed species. All of the tested strains showed traits related to growth promoting activities. Although isolated form *M. pudica*, some of the bacteria tested could produce endophytic relations with *P. vulgaris*, a legume belonging to a different subfamily suggesting a broad host range for these *Enterobacter* growth-promoting bacteria. The new *Enterobacter* species described here are also good candidates for further studies more directed to agronomical purposes such as production of plant rooters (IAA), siderophores or plant growth promotion in soils with insoluble phosphate compounds.

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