

Full Length Research Paper

# Bacterial community in the roots and rhizosphere of *Hypericum silenoides* Juss. 1804

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*Hypericum silenoides* Juss. 1804 is an important medicinal plant used in Mexico, but the bacterial community associated with it is unknown. The bacterial communities in the rhizosphere and roots of *H. silenoides* were isolated and identified by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and ribosomal types were determined by amplified ribosomal rDNA restriction analysis (ARDRA) and 16S rRNA gene sequences. Sixty-six strains were obtained from roots of *H. silenoides* and 37 were isolated from the rhizosphere. ERIC fingerprinting distinguished 63 genetic patterns with 44 being unique and the remaining 19 containing 59 strains. Eighteen representative strains were distinguished with ARDRA analysis. 16S rRNA gene sequence analysis revealed that the bacteria isolated from the roots and rhizosphere of *H. silenoides* belonged to the genera *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Sphingobium*, *Stenotrophomonas*, *Agrobacterium*, *Pantoea* and *Serratia*. The largest number of isolates (30) belonged to the genus *Agrobacterium*. Richness and diversity of bacteria was higher on the roots than in the rhizosphere of *H. silenoides*.

**Key words:** *Agrobacterium*, endophytic bacteria, 16S rDNA, amplified ribosomal rDNA restriction analysis (ARDRA), enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), phylogeny.

## INTRODUCTION

Plant diversity in Mexico is among the largest in the world and much of this diversity is found in Chiapas (Heinrich, 2009). Some of these plants have been used in traditional medicine and have even been cultivated for local use (González-Espinosa et al., 2004). The genus *Hypericum* L. or St. John's wort is composed of approximately 450 species of trees, shrubs and herbs found in different temperate regions of the world (Percifield et al., 2007). St. John's wort can be used as bactericide and for wound-healing, and has anti-inflammatory, anti-depressive, diuretic and sedative capacities (Charrois et al., 2007; Adams et al., 2011). In particular *Hypericum silenoides* Juss. 1804 is used by

indigenous communities in Chiapas, such as the Tzotziles and Tzeltals, to treat gastrointestinal diseases (Meckes et al., 1995). There has been a growing interest to cultivate this plant commercially, but its growth is slow and consequently production is low. Plant growth promoting rhizobacteria (PGPR), such as nitrogen-fixing microorganisms, have been used as biofertilizer to stimulate plant growth (Mia et al., 2010). Many are known to produce phytohormones, components that solubilize inorganic phosphates or suppress phytopathogens (Hayat et al., 2010). Plant growth promoting rhizobacteria may be adapted to their host plants so it is important to isolate them from the rhizosphere of the target plant.

The soil microbial communities play an integral and often unique role in ecosystem functions and are among the most complex and diverse communities in the biosphere (Zhou et al., 2003). The study of plant-associated microorganisms is of great importance for

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biotechnological applications, such as biological control of plant pathogens, plant growth promotion, or isolation of active compounds (Bloemberg and Lugtenberg, 2001). Plant-bacterial interactions in the rhizosphere are determinants of plant health and soil fertility (Hayat et al., 2010). Most studies on rhizospheric and endophytic bacteria and their community structure have been done using culture dependent techniques (Axelrood et al., 2002). The aim of this study was to isolate and identify bacteria from the rhizosphere and roots of *H. silenoides*.

## MATERIALS AND METHODS

### Media, culture conditions and bacterial isolation

Plants were collected in two regions of the state of Chiapas (Mexico) (that is, the northern region and the central highlands). The climate in Chenalhó (highland area, Latitude 16° 53', Longitude 92° 38') was humid temperate and the clay-loam soil had a pH of 5.8, while the climate in Jitotol (Northern region, Latitude 17° 04', Longitude 92° 52') was humid and the sandy loam soil had a pH of 6.5. The vegetation in both areas was pine-oak forest (Wolf, 2005). Bacteria were isolated from roots and rhizosphere of *H. silenoides* (Plantae, Magnoliophyta, Magnoliopsida, Malpighiales, Hypericaceae). Sixty eight plants were sampled at Chenalhó (central highlands) and 35 at Jitotol (Northern region). The roots were surface-sterilized by submerging them in 70% (v/v) ethanol for 5 min and in 6.25% sodium hypochlorite for 10 min. The roots were washed with sterile distilled water and three times with 0.1 M MgSO<sub>4</sub>. Three g of the sampled roots was used for bacterial isolation. Plant material was suspended in 0.1 M MgSO<sub>4</sub> and ground with a mortar and pestle (Bashan et al., 1993). A 500 µl aliquot was added to 5 ml nitrogen free broth (NFB) semisolid selective medium with the following composition (g l<sup>-1</sup> distilled water): DL-malic acid, 5; yeast extract, 0.3; K<sub>2</sub>HPO<sub>4</sub>, 0.5; FeSO<sub>4</sub>, 0.04; MnSO<sub>4</sub>.H<sub>2</sub>O, 0.1; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.2; NaCl, 0.1; Na<sub>2</sub>MoO<sub>4</sub>.7H<sub>2</sub>O, 0.002; KOH, 4.8; NH<sub>4</sub>Cl, 0.2; 0.5% alcoholic solution of bromothymol blue, 2 ml; and agar, 2.33.

A sub-sample of 10 g soil was taken from the rhizosphere of *H. silenoides*, and mixed with 100 ml phosphate-buffered saline (PBS) medium [10 mM K<sub>2</sub>PO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub>, 0.14 M NaCl, pH 7.2] (Martínez et al., 2003). The sample was serially diluted 10-fold in sterile water to 10<sup>-6</sup>. A 500 µl aliquot was taken to inoculate tubes with 10 ml NFB medium. The tubes with root fragments and soil were incubated at 28°C for 5 days.

Tubes that showed a thin white pellicle 0.5 and 1.5 mm below the surface and had turned the medium from green to blue were selected (Hartmann and Baldani, 2006). A loopful was taken from this pellicle, streaked on solid NFB and incubated at 28°C for three days. The single colonies obtained on solid NFB medium were streaked on Congo red and incubated at 28°C for 72 h. Bacteria were conserved in 30% glycerol-Congo red broth and stored at -20°C.

### DNA extraction

Total genomic DNA was extracted from colonies suspended in Congo red with glycerol 30% using the DNA isolation kit for cells and tissues (Roche<sup>TM</sup>, Switzerland). The DNA extraction was done according to the manufacturer specifications.

### Fingerprinting (ERIC-PCR)

Enterobacterial repetitive intergenic consensus (ERIC) genomic

fingerprinting was obtained by PCR using primers ERIC1R and ERIC2 as described by Versalovic et al. (1991). Eight µl of the PCR reaction was loaded on 1.5% agarose gels and patterns were analyzed visually.

### PCR amplification of 16S rDNA

The PCR of the 16S rDNA gene was done with primers fd1 and rD1 (Weisburg et al., 1991) on an Applied Biosystems model 2720 thermocycler (Ca, USA). PCR conditions consisted of an initial denaturing step at 94°C for 5 min, 35 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 2 min) and an additional final chain elongation step at 72°C for 7 min. The size of the amplification products was verified by electrophoresis in 1% agarose gels. PCR products were purified using the PCR product purification system kit (Roche<sup>TM</sup>, Switzerland).

### Amplified ribosomal rDNA restriction analysis (ARDRA)

Aliquots (1.5 µl) of the PCR products were digested with a restriction endonuclease *Rsa* I (Promega, Wi, USA) in a total volume of 10 µl as specified by the manufacturer. The restriction fragments were separated by electrophoresis in 3% agarose gels.

### Sequencing and phylogenetic analysis

Purified PCR products of the almost full-length 16S rDNA gene (~1,500 bp) were sequenced by Macrogen Inc. (Seoul, Korea). Sequences were edited with the BioEdit Sequence Alignment Editor v.7.0.0 (Hall, 1999). The basic local alignment tool (BLAST) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and sequence match of the Ribosomal Database Project (<http://rdp.cme.msu.edu>) were used to search for similar known sequences. These sequences were aligned with CLUSTAL\_X version 1.8 (Thompson et al., 1997). Phylogenetic and molecular evolutionary analyses were done with MEGA version 3.1 (Kumar et al., 2004). The phylogenetic tree with the 16S rDNA gene sequences from type strains of *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Sphingobium*, *Stenotrophomonas*, *Agrobacterium*, *Pantoea* and *Serratia* was constructed by neighbour-joining and a bootstrap test using 1000 pseudoreplicates with the Tamura-Nei model (Kimura, 1980). The sequences generated were deposited in the GenBank public database and the accession numbers for the eighteen 16S rDNA gene sequences determined in this study were JF681282 to JF681299, which were included in the phylogenetic tree of 16S rDNA gene.

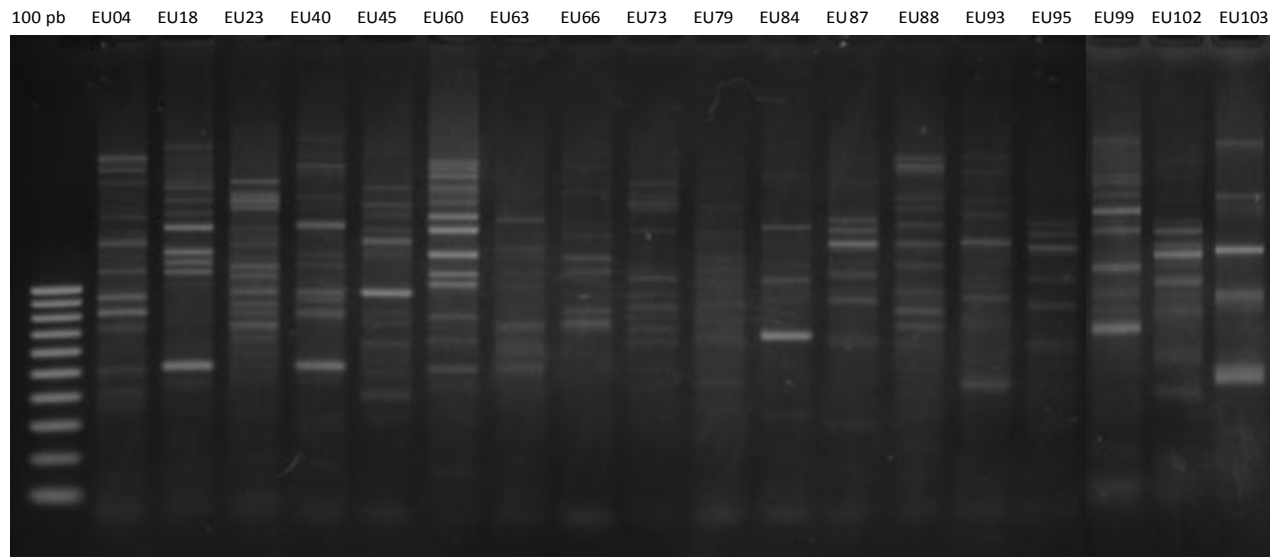
### Richness and diversity indexes

The richness (d) and diversity (H) indexes (Shannon-Weaver index) were calculated based on the total number of bacteria isolated from the rhizosphere and roots of *H. silenoides* (Pereira et al., 2009).

## RESULTS

### Isolates

A total of 103 strains were obtained of which 66 were obtained from the roots of *H. silenoides* and 37 were isolated from the rhizosphere. Richness and diversity of culturable bacteria was slightly higher in the roots



**Figure 1.** ERIC-PCR profiles obtained with the primers fD1 y rD1, from isolates of the medicinal plant *H. silenoides*.

(d index: 3.28, H index: 1.58) than in the rhizosphere (d index: 3.19, H index: 1.48).

#### Diversity revealed by genomic fingerprinting (ERIC-PCR)

Sixty-three different genomic patterns were found among the 103 isolates of *H. silenoides*. Of these 63 genomic patterns, 43 were unique and the remaining grouped 60 strains (Figure 1).

#### Amplified ribosomal rDNA restriction analysis (ARDRA)

Amplified 16S rDNA gene fragments had the expected single size band of approximately 1500 bp. Electrophoresis of ARDRA revealed 18 different patterns of which 9 were unique and the remaining grouped 54 strains (Figure 2).

#### Phylogenetic analysis

Analysis of the 16S rRNA gene sequences of the 18 representative strains revealed that the isolates belonged to eight bacterial genera and a phylogenetic tree was constructed for each one (data not shown). Strains EU04, EU18, EU40, EU60, EU88 and EU99 belonged to the genus *Acinetobacter*, with EU18 having a 99% and EU60 a 97% sequence similarity with *A. johnsonii*. The EU40 strain had a 98 to 99% sequence similarity with *Acinetobacter* species, such as *A. johnsonii* and

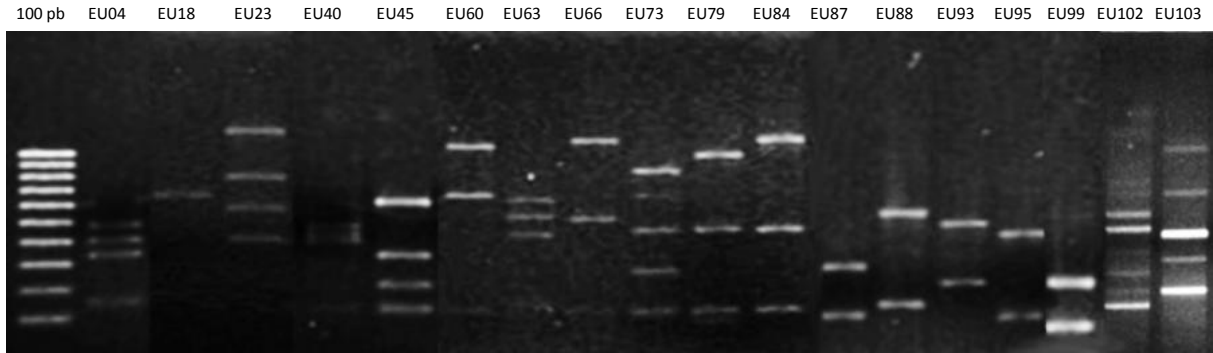
*A. haemolyticus*. The strain was clustered with *A. bouvetii* 4B02<sup>T</sup> (AF509827) in the constructed phylogenetic tree (Figure 3). In the sequence analysis, strains EU04, EU88 and EU99 had a 99% sequence similarity with *A. calcoaceticus*.

Strains EU45, EU63 and EU102 belonged to the genus *Pseudomonas*. The EU45 strain had a 99% sequence similarity with *P. monteilli*, *P. putida*, *P. plecoglossicida* and *P. aeruginosa*. Isolate EU63 had a sequence similarity of 99% with *P. putida*. Strain EU102 was most closely related to *P. putida*, *P. plecoglossicida* and *P. monteilli* with a sequence similarity of 94%.

Isolate EU84 belonged to the genus *Stenotrophomonas* and had a 99% sequence similarity with *S. maltophilia* with a bootstrap support of 100% in the phylogenetic tree. The EU66 strain belonged to the genus *Sphingobium* and had a sequence similarity of 99% with *S. yanoikuyae* and *S. paucimobilis*. Isolates EU79 and EU95 had a 99% sequence similarity with *A. tumefaciens* and the isolate EU87 a 100% similarity. The three isolates were supported with bootstrap values > 80%.

The EU93 strain belonged to the genus *Serratia* with a sequence similarity of 99% with *S. marcescens* and *S. nematodiphilia*. Isolate EU103 was related to *Pantoea agglomerans*, but with only 93% sequence similarity. Strains EU23 and EU73 belonged to the genus *Enterobacter* and had both a 98 to 99% sequence similarity with *E. cancerogenus*, *E. aerogenes* and *E. asburiae*. The largest number of isolates (30) belonged to the genus *Agrobacterium*, followed by *Acinetobacter* with 25 and *Pseudomonas* with 21 isolates (Table 1).

Of the 18 strains described, the following showed unique patterns in both analysis: EU40, EU63, EU79, EU84, EU87, EU88, EU102 and EU103. The strain EU45



**Figure 2.** ARDRA patterns, digested with *Rsa* I and separated by electrophoresis in 3% agarose gel.

had a unique pattern using the ERIC technique and strain EU04 when using ARDRA.

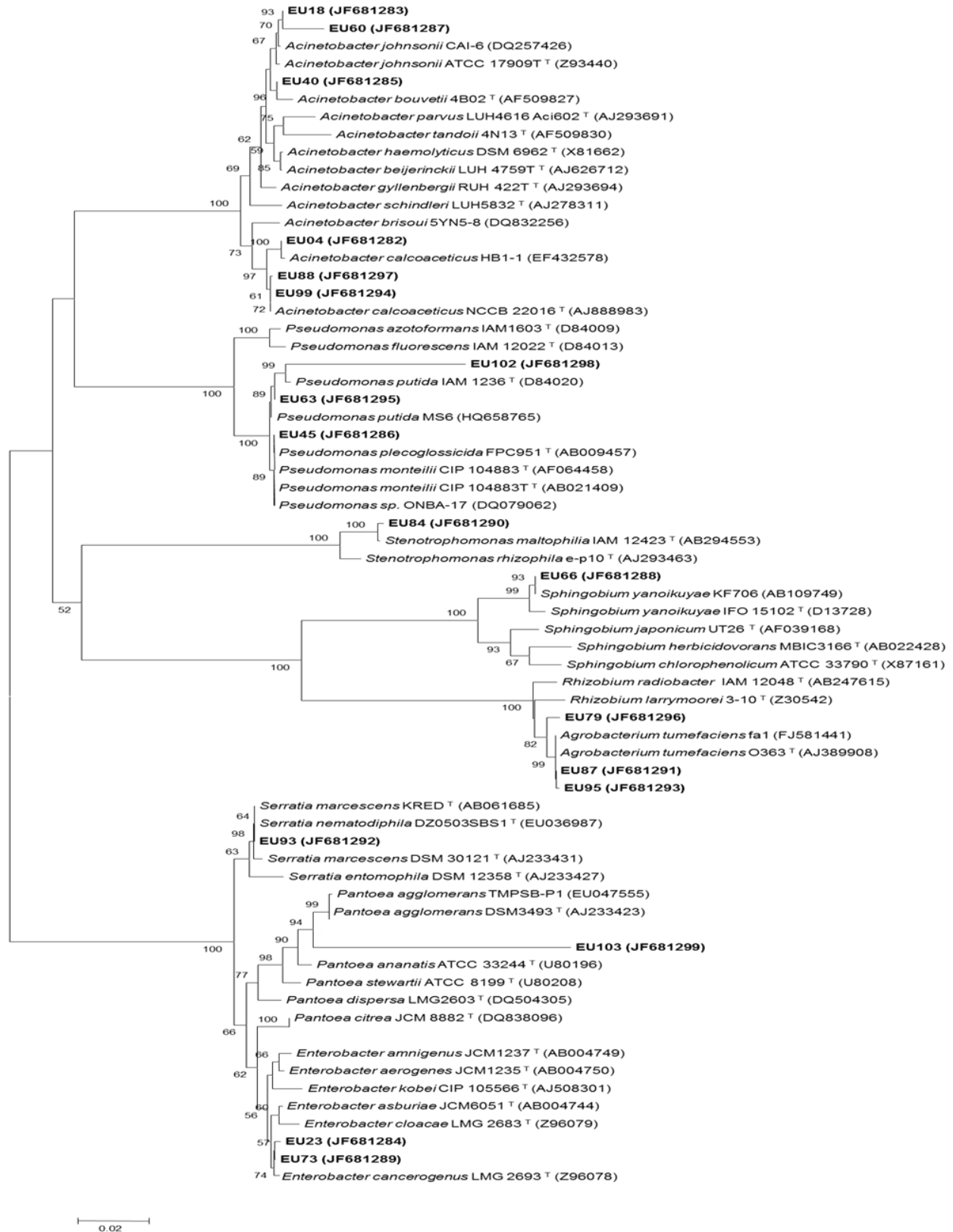
## DISCUSSION

The present study employed cultivation-dependent methods to assess the diversity of bacteria in the rhizosphere and in the roots of the medicinal plant *H. silenoides* collected in Chiapas. In total, 103 strains were cultured of which two are new phylotypes based on <98% 16S rDNA gene sequence similarity with any previously cultured isolate. The strain EU102 showed 94% 16S rDNA gene similarity with *Pseudomonas putida*, *P. plecoglossicida* and *P. monteilli*, and the strain EU103 93% with *Pantoea agglomerans*. If strain EU102 has the same characteristics as *P. putida* then it might aid the growth of *Hypericum silenoides* in nutrient poor soils. *Pseudomonas putida* has the capacity to fix N<sub>2</sub> and synthesize phytohormones, for example, IAA. In the same way, phylotype EU103 might also stimulate growth of *H. silenoides*. These results indicated that a diverse microbial population can be cultured from this medicinal plant and reinforces the concept that relatively simple cultivation techniques can be used successfully to isolate as-yet-undescribed taxa (Janssen et al., 2002).

In other studies, a higher diversity of bacteria has been found in the rhizosphere than in the inner root tissues (Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2004). The presence of different endophytic species in some plants, however, depends on plant genotype, plant age, tissue sampled and also on the season of isolation (Rosenblueth and Martínez-Romero, 2006). In our study, the density of bacterial endophytes in the roots was higher than in the rhizosphere of *H. silenoides* (Table 1). This distribution pattern was reported previously for corn (*Zea mays* L.) (Fisher et al., 1992), pea (*Pisum sativum* L.) (Elvira-Recuenco and van Vuurde, 2000) and soybean (*Glycine max* (L.) Merr.) plants (Kuklinsky-Sobral et al., 2004). Endophytic populations, like rhizospheric populations, are

conditioned by biotic and abiotic factors (Hallmann et al., 1997, 1999; Fuentes-Ramírez et al., 1999; Seghers et al., 2004), but endophytic bacteria might be better protected from biotic and abiotic stresses than rhizospheric bacteria (Hallmann et al., 1997).

Phylogenetic analysis using the 16S rDNA gene revealed that most of the isolates belonged to the genus *Agrobacterium*. The other isolates were classified as *Pantoea*, *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Sphingobium*, *Stenotrophomonas* and *Serratia*. Previous studies have shown that isolates belonging to these bacterial genera promote the growth of different crops and are able to control specific plant diseases. Many of these genera are growth promoters or PGPR (Podile and Kishore, 2006). For example, *Agrobacterium*, which is found as an endophyte (Wang et al., 2006) and commonly isolated from plants of temperate regions, has the ability to solubilize inorganic phosphate (Rodríguez et al., 2006) and in rare cases is able to fix nitrogen (Mhamdi et al., 2005). It is possible then, that this plant-bacterium association is an alternative to obtain this nutrient because soils where *H. silenoides* grows are nitrogen deficient (Montañez et al., 2009). *Pantoea* has also been found as endophyte in many plants (Loiret et al., 2004; Feng et al., 2006). For example, the rice endophyte *Pantoea agglomerans* YS19 showed nitrogen-fixing activity *in vitro*, produced four different phytohormones, including IAA, and promoted plant growth (Feng et al., 2006). Recently, a new endophytic nitrogen-fixing *Pantoea* sp. was isolated from sugarcane plants (*Saccharum* sp. L.) in Cuba (Loiret et al., 2004), although its role in plant growth promotion has not been established. *Pantoea* and *Enterobacter* strains are also endophytes of cotton (*Gossypium* sp. L.) (Sturz et al., 1997) and alfalfa (Phillips et al., 1999). Members of the genus *Enterobacter* were isolated from soil and roots of *H. silenoides*. Previous studies isolated *Enterobacter* strains from stems of wild and cultivated rice (*Oryza* sp. L.) (Elbeltagy et al., 2001) and from sugarcane (Mirza et al., 2001). Rice development and nitrogen fixation in rice and in sugar cane was stimulated when inoculated with



**Figure 3.** Phylogenetic tree based on partial sequences of the 16S rDNA gene for the isolates obtained in this study determined by the neighbor-joining method using the Tamura Nei model. Values at branching points indicate bootstrap support higher than 50% (1000 resamplings). *Hypericum silenoides* isolates are shown in bold with their accession numbers indicated within brackets.

**Table 1.** Representative isolates from *Hypericum silenoides*.

| Genus                   | Number of isolates | Isolation from |             |
|-------------------------|--------------------|----------------|-------------|
|                         |                    | Roots          | Rhizosphere |
| <i>Acinetobacter</i>    | 25                 | 20             | 5           |
| <i>Agrobacterium</i>    | 30                 | 24             | 6           |
| <i>Enterobacter</i>     | 16                 | 6              | 10          |
| <i>Pantoea</i>          | 1                  | 1              | 0           |
| <i>Pseudomonas</i>      | 21                 | 7              | 14          |
| <i>Serratia</i>         | 5                  | 5              | 1           |
| <i>Sphingobium</i>      | 4                  | 3              | 1           |
| <i>Stenotrophomonas</i> | 1                  | 0              | 1           |
| Total                   | 103                | 66             | 37          |

*E. cloacae* isolates (Elbeltagy et al., 2001; Mirza et al., 2001). Our results showed that *H. silenoides* was associated with species of the genus *Pseudomonas*, which were isolated mainly from the rhizosphere. They might have contributed to the uptake of nutrients and facilitated the colonization of the roots. In addition, *Stenotrophomonas* species were also found in this study. Denton and Kerr (1998) reported that this genus is ubiquitous in a wide range of environments and geographical regions, and occupies various ecological niches. *Stenotrophomonas* is often associated with plants and has been isolated from different rhizospheres (Rosenblueth and Martínez-Romero, 2006). Investigations have indicated a potential role for this species in biotechnology as a biological control agent of fungal plant pathogens in agriculture (Kobayashi et al., 1995; Nakayama et al., 1999) and in bioremediation (Binks et al., 1995). *Serratia sp.* is widely distributed in nature, and frequently associated with plants. In our study, it was found in the roots of *H. silenoides*. This organism has been isolated from the rhizosphere and the phyllosphere of various plants, e.g., as an endophyte from the endorrhiza of potato (*Solanum tuberosum* L.) (Breed et al., 1948; van Overbeek and van Elsas, 2008). There is an increasing interest in developing their biotechnological potential (that is, to improve phytoremediation and the sustainable production of non-food crops for biomass and biofuel production (Ryan et al., 2008).

Bacteria of the genus *Acinetobacter* are characterized by their potential for degradation of organic compounds (for example, phenols, tannins). *Acinetobacter* has been isolated from banana plants (*Musa sp.*) (Pious and Thyvalappil, 2009), tree species of coniferous (Hironari et al., 2008) and sugarcane (Velázquez et al., 2008). It has bioremediators activity in these plants. The *Sphingobium* genus is characterized for their potential to degrade organic compounds. The strain *Sphingobium yanoikuyae* B1 is able to degrade a range of polycyclic aromatic hydrocarbons (PAHs).

Endophytic bacteria, just like rhizosphere bacteria, can

be plant growth-promoting due to the production of phytohormones, enzymes involved in growth metabolism, such as ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, auxins, indole-3-acetic acid (IAA), acetoin, 2,3-butanediol, cytokinins, or combinations thereof (Kuklinsky-Sobral et al., 2004).

It can be assumed that some bacterial species will establish positive interactions with other organisms, such as mycorrhizae (Jaderlund et al., 2008). The bacteria *Serratia* was isolated from the rhizosphere of *Hypericum silenoides* and it forms a synergism with mycorrhizae (Villegas and Fortin, 2002). This synergism might increase the role that bacteria have stimulating plant growth. Mycorrhizae will improve the solubilization of P and bacteria might provide components that help the growth of the fungi. The bacteria might also excrete chemical components that stimulate plant growth (Bianciotto et al., 1996).

It was concluded that there is a great genetic diversity of bacteria associated with the plant *H. silenoides* as it was found at two sites in the State of Chiapas, Mexico. From a total of 103 isolates, 63 different genomic fingerprint patterns were obtained with ERIC-PCR tests. Subsequent analysis with ARDRA revealed 18 different restriction patterns. Phylogenetic analysis of the 16S rDNA gene of 18 representative strains showed that they belonged to eight different bacterial genera (that is, *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Sphingobium*, *Agrobacterium*, *Serratia*, *Pantoea* and *Stenotrophomonas*). The largest numbers of isolates (30 strains) belonged to the genus *Agrobacterium* (*Rhizobium*). Isolates can now be characterized, while their physiology, ecology, and biofertilizer potential can be determined.

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