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Sesbania herbacea–Rhizobium huautlense Nodulation in Flooded Soils and Comparative Characterization of *S. herbacea*-Nodulating Rhizobia in Different Environments

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ABSTRACT

The nodulation of *S. herbacea* was compared under flooded and non-flooded conditions in two different soils. One soil was from a flooded field in Sierra de Huautla, the native habitat of this legume, while the other soil was from a well-drained field in Cuernavaca, where rhizobia were found to nodulate the introduced *S. herbacea* plants. Nodulation of the plants was completely eliminated by flooding in the Cuernavaca soil, whereas nodules were obtained in the same soil under non-flooded conditions. In contrast, nodules were formed in Huautla soil under both flooded and non-flooded conditions. Most isolates, except isolate HS2, from Huautla soil and water were identified as *R. huautlense* by colony morphology, growth rate, PCR-RFLP of 16S rRNA genes, MLEE, cellular plasmid contents, and RFLP of *nifH* and *nodDAB* genes. Isolate HS2 was identified as *Mesorhizobium* sp. Isolates from Cuernavaca soil were different from *R. huautlense* in many aspects and were classified into five rDNA types within the genera *Mesorhizobium, Rhizobium,* and *Sinorhizobium* by PCR-RFLP of 16S rRNA genes. *R. huautlense* is a water *Rhizobium* species. Growth by denitrification under oxygen limitation or with ethanol was observed for *R. huautlense* bacteria but not for the isolates from Cuernavaca. In an interstrain nodulation competitive assay under both flooded and non-flooded conditions, *R. huautlense* strain S02 completely inhibited the nodulation of *Mesorhizobium* sp. Sn2, an isolate from Cuernavaca. From these results, we conclude that *R. huautlense* has the unique ability to nodulate *S. herbacea* not only in flooded soils, but in non-flooded soils as well.

Introduction

Nitrogen-fixing root and/or stem nodules are formed in symbioses between leguminous plants and rhizobia; various legume–rhizobia symbioses exist in different ecosystems. Both stem and root nodules are formed in some aquatic legume species within the genera *Aeschynomene* and *Sesbania* [32, 34], whereas only root nodules are found in other aquatic legumes within the genus *Neptunia* [21, 33]. Strains of *Bradyrhizobium* [31, 36], *Allorhizobium undicola* [10], *Azorhizobium caulinodans* [11] *Rhizobium huautlense* [38],

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Sinorhizobium saheli, and *S. terangae* [9] have been isolated from root or stem nodules of these aquatic legumes. Waterlogged soils are considered as microaerobic or anaerobic environments. This anoxic condition is a stress factor for both the survival and nodulation of rhizobia since these bacteria are aerobic organisms. Earlier reports on the effects of flooding on nodulation and survival of rhizobia have been reviewed by Eaglesham and Ayanaba [12] and Lowendorf [26], and these studies indicate that oxygen deficiency inhibits root nodulation and nodule development [15, 25, 27], even for the aquatic legume *Aeschynomene scabra* [12]. However, the formation and activity of stem nodules of *Aeschynomene* and *Sesbania* are not affected by water-logging [1, 12] and are even promoted by high humidity [2, 3].

Sesbania herbacea is an aquatic legume native to the Sierra de Huautla, Morelos, Mexico. The rhizobial populations associated with *S. herbacea* in flooded fields were found to have very limited diversity and were classified as *Rhizobium huautlense* [38], a species phylogenetically related to *R. galegae* [24]. Nodules of *S. herbacea* were also formed by *Mesorhizobium* and by fast-growing rhizobia related to *R. tropici* and *R. etli* in well-drained soils from Cuernavaca, which is located 70 km in the north of Huautla [38]. These observations led us to ask whether flooded or anoxic conditions were a factor for selection of rhizobia by this host plant and if strains of *R. huautlense* were adapted for nodulation of *S. herbacea* in flooded soils. In order to answer these questions, we performed nodulation tests in soils under flooded and non-flooded conditions and carried out phenotypic and genetic characterization of the isolates nodulating *S. herbacea* in different environments.

Materials and Methods

Nodulation of S. herbacea *Grown in Natural Soils and Water*

Two kinds of soils were used in this experiment. Soils from a flooded field were obtained from a lake in Sierra de Huautla, Morelos, the native habitat of *S. herbacea* and *R. huautlense* [38]. Sierra de Huautla is about 140 km South of Mexico City and is at the border of three states (Morelos, Puebla, and Guerrero). Wet brown-sandy loam soils (pH 6.9, with 0.074% N, and 1.5% organic matter) from Cuernavaca, Morelos, where diverse rhizobia nodulating this plant were obtained [38], were used for the non-flooded conditions. Soil from Huautla was a clay; pH 8.0, 0.06% N, and 0.7% organic matter.

Twenty *S. herbacea* plants were grown in two pots (diameter, 25 cm; depth, 21 cm) filled with soil (15 cm in depth) for each of the four treatments: two soil samples × flooded and non-flooded conditions. In order to create a flooded condition, the pots were immersed in tap water to a level of 3 cm above the top. Pots for the non-flooded condition were kept open and watered from the bottom by putting them in water baths with tap water up to 3 cm. For checking the existence of rhizobia in the lake water, autoclaved cotton (20 g) placed in brown glass bottles (1 L volume) was immersed in 800 ml of non-sterilized water from the lake in Huautla. Double concentrated nitrogen-free plant nutrient solution [14] was added to the lake water (1:1 vol/vol). Six seedlings were sown in each of the five bottles and the bottles were opened to the air. For all the treatments, surface-sterilized, germinated seeds of *S. herbacea* were used and the seedlings were grown under sunlight. All of the plants were harvested after 6 weeks. Number of nodules, height, and fresh weight of the plants were recorded and were statistically analyzed by the *t*-test.

Isolation and Characterization of S. herbacea*-Nodulating Rhizobia in Different Environments*

Nodules were isolated as described by Vincent [37] on PY medium (peptone of casein 5 g, yeast extract 3 g, $CaCl_2$ 0.6 g, water 1000 ml). Isolates and reference strains used are listed in Table 1. The characteristics assayed were growth rate; colony morphology; acid production on YMA [37]; PCR-based RFLP of 16S rRNA genes [38]; multilocus enzyme electrophoresis [30] using aconitase, adenylate kinase, esterase, glucose-6-phosphate dehydrogenase, hexokinase, indophenol oxidase, isocitrate dehydrogenase, malaic enzyme, malate dehydrogenase, and phosphoglucomutase; plasmid content [19]; use of ethanol as sole carbon source at the final concentration of 0.1%, 1.0%, 5.0%, 10.0%, and 15% (vol/vol) as described previously [38]; oxygen requirement for growth and reduction of sulfate in AITS (agar of iron and triple sugars) (BIOXON); fermentation of the isolates in semisolid YM [37] medium covered by a mixture of Vaseline and liquid paraffin; anaerobic growth in YM broth supplied with 6 mM of sodium sulfate or potassium nitrate [8]. The nitrate in the bacterial cultures was detected using the NIT 1 and NIT 2 reagents in the api 20 E kit (bioMérieux) as specified by the manufacturer. Southern blot of total DNA digested with *Eco*RI and *Bam*HI [39] was hybridized to a 600 bp PCR-amplified *nifH* internal fragment from *S. meliloti* USDA1002 [13] and a cloned DNA fragment containing *nodDAB* genes from *R. tropici* CFN299 [39].

Nodulation Tests Using Pure Cultures as Inoculants

These tests were performed in Cuernavaca soils under flooded and non-flooded conditions as described above. Twenty seedlings were grown in two pots (10 in each) for each of the treatments. Bacteria were grown in 40 ml of PY broth to $OD_{260} = 0.8$ (about 10^{10} CFU ml^{-1}). Surface-sterilized, pregerminated seeds were inoculated by immersion in fresh bacterial cultures for 10 min and were further inoculated by adding 0.5 ml of the cultures per seed after they were sown in pots. The inoculants were a mixture of strains *R. huautlense* S02 and *Mesorhizobium* sp. Sn2 (1:1, vol/vol) and a mixture of isolates Sn2, Sn1, Sn12, Sn13, and CS3 (1:1:1:1:1, vol/vol), repre-

Table 1. Isolates from root-nodules of *Sesbania herbacea*, reference strains, and their relevant characteristics[a]

Isolate	16S rRNA gene RFLP	ET	Plasmids (kb)	Aerobic growth on AITS with production of	NO_3^- anaerobic growth with production of	Ethanol as sole carbon source	Nodulation in soils	
							Non-flooded	Flooded
Isolates from Huautla soils (HS) and water (HW)								
HS 23, HW1-1, HW1-2	ICAF	1	900, 400, 80	—	Alkali	ND	+	+
HS3, HS5, HS8, HS13, HS15, HS18, HS25, HS33, HW2-1, HW2-4, HW3-2, HW3-9, HW4-2, HW4-10, HW5-2, HW5-10	ICAF		900, 400	—	Alkali	ND	+	+
HS28		2			Alkali	ND	+	+
HS2	FFFF		400	—	—	ND	+	ND
Isolates from Cuernavaca soils								
CS3, CS6	ADAA		1200, 220	Alkali, H_2S	—	—	+	—
CS11	ADAA		1200, 220	Alkali, H_2S	—	—	+	—
CS8, CS10, CS14	ADAA		1200, 220	Alkali	—	—	+	—
Sn1, Sn4, Sn22	ACBH		900, 630	—	—	—	+	—
Sn12	ACBC		—	—	—	—	+	—
Sn15	ACBC		700, 630	—	—	—	+	—
Sn13, Sn14	CBEC		560	—	Acid	—	+	—
Reference strains								
Mesorhizobium sp. Sn2	FFFF		—	—	—	—	ND	ND
M. plurifarium USDA4413	FFFF		ND	ND	ND	ND	ND	ND
R. etli CFN42	CJAC		630, 510, 390, 270, 750					
R. huautlense S02	ICAF	1	900, 400	—	Alkali	±	+	+
S172	ICAF	1	900, 400, 220	—	Alkali	ND	+	+
S02a	ICAF		900	—	Alkali	+	—	—
S41	ICAF		900	—	Alkali	—	—	—
S32	ICAF		—	—	Alkali	+	+	+
S182	ICAF		—	—	Alkali	—	+	+
R. tropici Type B CIAT899	ACBH		ND	ND	ND	ND	ND	ND
S. meliloti USDA1002	ADAA		ND	ND	ND	ND	ND	ND
S. saheli LMG7837,	DDAB		ND	ND	—	ND	ND	ND
LMG8310,	ND		ND	ND	—	ND	ND	ND
LMG11864	ND		ND	ND	—	ND	ND	ND
S. terangae LMG7834	DDAF		ND	ND	Alkali	—	ND	ND
Rhizobium sp. Lc37	CBEC		ND	ND	ND	ND	ND	ND
Allorhizobium undicola LMG11876, LMG11879	ND		ND	ND	—	ND	ND	ND
Azorhizobium caulinodans ORS571	ND		ND	ND	—	ND	ND	ND

[a] Sizes of plasmids were estimated against their migration in 0.75% Eckhardt gel using the plasmids of *R. etli* CFN42 [29] and of *S. meliloti* 1021 [17] as molecular weight standards; NO_3^- anaerobic growth was assayed in YM broth containing 6 mM KNO_3 covered with liquid paraffin [8]; use of ethanol as sole carbon source was tested in basal medium [38] supplied with 0.1 and 1.0% ethanol (vol/vol).
+, Nodules were formed or growth was observed.
—, No growth, or no plasmids, or no nodules were observed.
ND, not done.

senting different rhizobial groups of isolates from Cuernavaca soils. Non-inoculated plants grown in the same soil under flooded and non-flooded conditions were included as control. The bacteria recovered from the nodules were identified according to their growth rate and colony appearance. Each of the strains *R. huautlense* S02 and *Mesorhizobium* sp. Sn2 was inoculated individually to *L. leucocephala* seedlings grown under flooding.

Rhizobia Persistance in Flooded and Non-flooded Soils

Three grams of air-dried soils from Cuernavaca were put in tubes (12 × 150), moistened with 1.5 ml water, and sterilized by autoclaving. Each tube was inoculated with 0.1 ml of PY culture (OD_{600} = 0.8, about 10^{10} CFU per ml) of *R. huautlense* strain S02 or isolates Sn1, Sn2, Sn12, Sn13, and CS3, representing different rhizobial groups. Then, 3.5 ml of sterilized water was added to the tubes to create a flooded condition. Non-flooded soils were also inoculated with the same bacteria. The number of CFU in the soils were counted after 7, 14, 21, and 28 days of incubation at 30°C. Every 3 or 5 days, 0.3 ml sterilized water was added to each tube. Before isolation, 3.5 ml of sterilized water was added to the non-flooded tubes, which were then vortexed for 1 min and their contents allowed to settle for 10 min. A 10 µl sample of the supernatant was diluted 10^4- to 10^6-fold, and 0.1 ml of the diluted solution was

Table 2. Nodulation and growth of *S. herbacea* in different conditions and soils[a]

Treatment[b]	Height of plant (mm)		Weight of plant (mg/plant)		Nodule numbers (per plant)	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
HSF (pH8.0)	133a	5.6	830a	92.0	6.3a	0.60
HS (pH8.0)	95b	2.1	479b	29.6	3.4b	0.27
CSF (pH6.8)	93b	1.7	794a	31.8	0.0c	0.00
CS (pH6.8)	90b	3.0	762a	44.5	1.0d	0.27

[a] Plants were grown 6 weeks under natural sunlight. Soils were not sterilized. Data were obtained from 16 randomly selected plants.
[b] HSF: Huautla soil flooded; HS: Huautla soil without flooding; CSF: Cuernavaca soil flooded; CS: Cuernavaca soil without flooding. pH values were from soil suspensions (10 g of air dried soils in 100 ml of distilled water and disturbed 10 min at room temperature).

spread on PY plate. Triplicate samples were taken for each treatment, and depending on the bacterial type, colonies were counted after 2 to 7 days.

Results

Nodulation of S. herbacea in Soils and Lake Water

The plants were harvested following 6 weeks of growth. Since only 16 plants were obtained from the 20 seeds sown for some of the treatments, only 16 plants were used for the statistical analysis. Most of the nodules were similar in size (2–3 mm in diameter) and were pink or purple inside, indicating that they were effective for nitrogen fixation. We found that nodule number in *S. herbacea* was significantly lower ($P \leq 0.05$ by *t*-test) under the non-flooded condition than under the flooded condition in the Huautla soil (Table 2). Nodulation in Cuernavaca soils under flooded conditions was almost eliminated. There were no nodules on the 16 randomly selected plants, and only one nodule was obtained when all the 20 plants were examined. In contrast, 10 of the 16 plants were nodulated in the Cuernavaca soil under non-flooded conditions. We found that the height of plants was significantly greater ($P \leq 0.05$) in flooded Huautla soils than in other treatments. This result might be related to the nitrogen fixating ability of the nodules in flooded soils. From 29 plants derived from sterilized seeds in gnotobiotic conditions grown in 4 L of the lake water from Huautla, 37 nodules were obtained that evidenced the presence of *S. herbacea*-nodulating bacteria in the water.

Isolation of Bacteria from Nodules

A total of 88 isolates were obtained from *S. herbacea* plants grown in Huautla soil under flooded or non-flooded conditions, and from the lake water in Huautla. Most of the isolates were fast-growing, acid-producing rhizobia with colonies typical of *R. huautlense.* Only one isolate, HS2, grew more slowly, with colonies around 1 mm after a 5-day incubation on PY plates, and produced acid on YMA. The 14 isolates from Cuernavaca soils were slow or moderately slow-growing, acid-producing rhizobia or fast-growing, acid-producing rhizobia with colony morphology different from *R. huautlense.* Fourteen isolates from Cuernavaca soils, the slow-growing isolate HS2, and 20 isolates randomly chosen from the 87 *R. huautlense*-like isolates from Huautla soils and water were further characterized (Table 1).

Comparative Characterization of Isolates and Strains

Most of the isolates from Huautla soils and all from the lake water had 16S rRNA gene RFLP patterns identical to the type strain of *R. huautlense,* and harbored two plasmids (900 and 400 kbp) (Table 1); plasmids were common in *R. huautlense* strains [43]. Three isolates, HS23, HW1-1, and HW1-2, had an extra 80-kbp plasmid. The only exception was isolate HS2, which had 16S rRNA gene RFLP patterns identical to those of *M. plurifarium* and *Mesorhizobium* sp. Sn2. This isolate had a 400-kbp plasmid which was similar in size to the symbiotic plasmid of *R. huautlense.* The fourteen rhizobial isolates from Cuernavaca soils were classified into five rDNA types. They had 16S rRNA gene RFLP patterns identical or very similar to *S. meliloti* USDA1002, *R. tropici* CIAT899, a novel group (type 10) in the genus *Rhizobium* [39], or *M. plurifarium,* respectively. These 14 isolates also had different plasmid patterns (Table 1).

Based on the 10 enzymes tested in the MLEE analysis, the 20 *R. huautlense*-related isolates identified by PCR-RFLP of 16S rRNA genes were divided into two electrophoretic types (ETs) (Table 1). ET1 contained 20 isolates including S02, the type strain of *R. huautlense.* ET2 was composed of only one isolate, HS28 which was different from ET1 only with regard to the patterns of esterase.

None of the isolates or reference strains could grow anaerobically in semisolid YMA or AITS. Vigorous growth with alkali production on the surface of AITS was obtained for the CS isolates (*Sinorhizobium*) (Table 1); isolates CS3, CS6, and CS11 also produced H_2S. Anaerobic growth with alkaline reaction was obtained among all the *R. huautlense* isolates and strains after a 3-day incubation in YM broth supplied with 6 mM of nitrate. Bubbles were observed between the medium and paraffin phases among *R. huautlense* isolates and strains, indicating gas production; no nitrate or

Table 3. Hybridization results of restricted DNAs to *nifH* and *nodDAB* probes presented in fragment sizes (kbp)[a]

Strain or isolate	*nifH* probe hybridized to		*nodDAB* probe hybridized to	
	*Eco*RI-digested DNAs	*Bam*HI-digested DNAs	*Eco*RI-digested DNAs	*Bam*HI-digested DNAs
R. huautlense S32, S182	9.0,7.3	1.2,1.1	3.8	7.2
R. huautlense S41, S02a	—[b]	—	—	—
R. huautlense S02, S11	9.0,7.3	1.2,1.1	9.2	7.2
R. huautlense HS13	9.0,7.3	1.2,1.1	9.2	7.2
Mesorhizobium sp. HS2	2.9	19.0	—	9.4
Mesorhizobium sp. Sn2	11.5	23.1	15.5	11.4
Rhizobium sp. Sn1	5.5,2.5	13.9	15.5,5.1	7.2,4.3,2.9
Rhizobium sp. Sn12	2.5	13.9	15.5,5.1,3.8,2.0	9.4
Rhizobium sp. Sn15	2.5	13.9	15.5,5.1,2.0	7.2,4.3,2.9
Rhizobium sp. Sn13	6.5	18.3	23.2,1.0	9.4,4.3
Sinorhizobium sp. CS3	4.6,4.5	18.3	Very faint	Very faint

[a] Hybridization was performed in high stringent conditions. Probes used were a 600 bp PCR-fragment of *S. meliloti nifH* gene and a 2 kbp cloned fragment containing *nodAB* genes and partial *nodD* gene of *R. tropici* CFN299. The sizes of the hybridized bands were estimated using *Bst*EI-digested Lambda DNA as molecular weight standards.
[b] No hybridization detected

nitrite was detected in these cultures by the NIT 1 and NIT 2 reagents. The remaining isolates, which did not reduce nitrate either, did not grow or grew only on the interface between the medium and paraffin phase and produced acid; this growth may be ascribed to microaerobic growth because nitrate was detected by the NIT 1 and NIT 2 reagents. No growth or weak growth on the interface of medium and paraffin with acid production was obtained for all isolates and strains in YM-sulfate broth, indicating that they did not reduce sulfate. Growth was observed for *R. huautlense* strains S02, S02a, and S32 on basal medium with 0.1% to 1.0% ethanol as sole carbon source, but not among other *S. herbacea*-nodulating isolates (Table 1).

The *R. huautlense* strains and isolates had identical *nif* DNA profiles with two *nifH* hybridization bands for either *Eco*RI- or *Bam*HI-digested DNAs, whereas the remaining *S. herbacea*-nodulating rhizobia contained one or two *nifH* gene hybridizing fragments of different sizes (Table 3). Single *nodDAB* hybridization bands were observed for the symbiotic *R. huautlense* isolates, strains, and for the *Mesorhizobium* isolates HS2 and Sn2 in both *Eco*RI- and *Bam*HI-digested DNAs. In the *Eco*RI-digested DNAs, two plasmid-free *R. huautlense* strains S32 and S182 had a 3.8 kbp hybridizing fragment to *nodDAB* that was smaller than those (9.2 kbp) obtained with the plasmid-harboring strains S02, S11, and isolate HS13; multiple bands were characteristic for the remaining *Rhizobium* isolates. The *Sinorhizobium* isolate CS3 had very weak hybridization signals, indicating low similarity with the *nodDAB* probe from *R. tropici* CFN299. No hybridization bands to either *nifH* or *nodDAB* probe were obtained in the non-symbiotic *R. huautlense* strains S02a and S41.

Interstrain Competitive Nodulation of R. huautlense S02 and *Mesorhizobium* sp. Sn2

Thirty-nine and 35 nodules, respectively, were obtained from 16 *S. herbacea* plants inoculated with a mixture of *R. huautlense* S02 and *Mesorhizobium* sp. Sn2 grown in Cuernavaca soils under flooded and non-flooded conditions. No significant differences in nodule numbers between the two treatments were observed, and the recovered isolates from the nodules were characterized as *R. huautlense* based on their growth rate and colony morphology.

Nodulation of S. herbacea and *L. leucocephala* Inoculated with Different Isolates in Flooded Soils.

After 4 weeks of growth, 11 plants inoculated with the mixture of isolates Sn2, Sn1, Sn12, Sn13, and CS3 did not form nodules in flooded soil. Sixty-one nodules were obtained from 8 inoculated plants grown in non-flooded soil. Three nodules were obtained from one of the three non-inoculated plants grown in non-flooded soils, indicating a background nodulation by native rhizobia of one nodule per plant. No nodules were observed on S02- or Sn2-inoculated *L. leucocephala* plants grown in flooded soil. The roots of *L. leucocephala* plants in flooded soil were black and damaged by flooding, in contrast to *S. herbacea,* which showed healthy root development.

Recovery of Inoculated Bacteria in Flooded or Non-flooded Soils.

After keeping inoculated bacteria for 4 weeks in sterilized soils, approximately 10^8 to 10^9 CFU per g dry weight of soil were recovered from the non-flooded condition from all isolates S02, Sn2, Sn1, Sn12, Sn13, and CS3. In flooded soils, the CFU per g dry weight of soil was 7.5×10^8 for *R. huautlense* S02 and $1.9–9.3 \times 10^7$ for the other isolates tested.

Discussion

The results of nodulation tests using natural soils or pure cultures of rhizobia showed that *S. herbacea* nodulated with *R. huautlense* in both flooded and non-flooded soils, but nodulated with the other rhizobia only in non-flooded soils. Identity of isolates was confirmed by the results of PCR-based RFLP of 16S rRNA genes, MLEE analysis, plasmid content, *nifH* and *nodDAB* gene RFLP, growth rate, and colony morphology. It has been reported that flooding inhibits root nodulation and nodule development [12]. This inhibitory effect was suggested to be due to (1) the deficiency of energy production caused by oxygen limitation [18, 32] and (2) the accumulation of ethylene [16] and ethanol [32]. It may be concluded therefore that *R. huautlense* strains possess mechanisms to overcome these constraints in order to nodulate plants grown in flooded soils.

Growth tests indicated that none of the rhizobia analyzed could grow anaerobically via fermentation or by sulfate reduction. However, the *R. huautlense* strains and isolates could grow by denitrification under oxygen-limited conditions, while the other *S. herbacea*-associated rhizobia could not. These results suggest that *R. huautlense* possess the ability to obtain energy via denitrification [42]. Rhizobia associated with non-aquatic legumes were reported to be capable of denitrification. These rhizobia include *S. terangae* (Table 1), *B. japonicum* [41], *Bradyrhizobium* sp. (*Lupinus*) and *S. meliloti* [8], *R. leguminosarum* from *Hedysarum* species [6], *R. leguminosarum* [5], and *S. fredii* [20, 35]. In this study, no denitrification was obtained for *S. saheli, Azorhizobium caulinodans,* and *Allorhizobium undicola* (Table 1), three rhizobial species associated with the aquatic legumes *Sesbania* spp. [9, 11] and *Neptunia natans* [10]. The relationship between growth using nitrate and the nodulation of *R. huautlense* in flooded soils requires further investigation.

Ethanol accumulation may have inhibited nodulation for some rhizobia in flooded soils [32], but it may be not the case for *R. huautlense.* Unlike the other isolates, some *R. huautlense* strains could use ethanol as sole carbon source, and it is possible that this capability may have helped them to survive and nodulate the legume host in flooded soils.

The soil in the flooded fields of Huautla was moderately alkaline (pH 8.0); we previously found that all the *R. huautlense* strains could grow even at pH 9.0 [38] in PY medium. This capability may allow this bacteria to colonize and nodulate in Huautla.

In this study, from 10^7 to 10^8 CFU g^{-1} of soils were obtained in flooded conditions for different *S. herbacea*-nodulating rhizobia. These numbers appear to be lower than the actual number in the soil due to bacteria absorption by soil particles [40]. Although these tests were performed in autoclaved soils, an environment which is different from the natural soils, it may be concluded that a large proportion of all the introduced rhizobia could survive in 4 weeks of flooding in the test soil. The numbers of rhizobia surviving in the soil after 4 weeks of flooding were high enough to form a maximum number of nodules, as reported in other cases [12], indicating that the nodulation differences among *R. huautlense* and other rhizobia were not directly attributable either to their survival in flooded soil or to the characteristics of Cuernavaca soil (with the low levels of nitrogen described before).

Rhizobia are considered to be soil bacteria; we show here that rhizobia such as *R. huautlense* are also aquatic bacteria based on the positive nodulation results with lake water as inocula of sterilized seeds in gnotobiotic conditions. It has been reported that *Sesbania* seeds may carry *A. caulinodans,* thus constituting a mechanism of spread of the bacteria [22, 23]. The existence of *R. huautlense* in water may suggest that dissemination of rhizobia by the river running to the lake could be a natural mechanism for the spread of this bacterium.

Based upon the results presented in this paper and on a previous report [38], we found that the distribution of *R. huautlense* was related to the distribution of its host, *S. herbacea.* Similar results were reported for *A. caulinodans* [28], a stem-nodulating bacterium of *Sesbania rostrata.* These results also confirmed that the genetic diversity of *R. huautlense* was rather limited as reported previously [38]. Among 84 strains and isolates tested in this research and in a previous study [38], 73 were identified as one ET represented by strain S02. The other 11 strains or isolates were divided into three ETs with one or two enzymes different from S02. All of the bacteria have similar plasmid profiles and symbiotic gene organization based on RFLP of *nifH* and *nodDAB* (Table 3). In contrast, five rDNA types belonging to the genera *Mesorhizobium, Rhizobium,* and *Sinorhizobium* were obtained

from the host plants grown in Cuernavaca soils under non-flooded condition. Among 26 *Mesorhizobium* isolates from *S. herbacea* grown in Cuernavaca soils, 13 ETs were identified (unpublished data). It has been reported that ecological factors can affect the genetic diversity of rhizobial populations in the soils [4, 39]. In our case, the limited diversity of *R. huautlense* might be related to its restricted geographic distribution and to the nodulation in flooded soils. In such an environment, other rhizobia might be hampered in nodulating by the flooding, despite the fact that *S. herbacea* was a non-selective host for nodulation under non-flooded conditions.

Complete denitrification is effected by three enzymes: nitrate, nitrite, and nitrous oxide reductases. The structural gene of nitrous oxide reductase has been located on the *nod* megaplasmid in *S. meliloti* [7]. Similar growth with nitrate reduction was obtained for the *R. huautlense* strains regardless of the presence of plasmids (Table 1), indicating that the genes encoding proteins responsible for anaerobic respiration are not located on plasmids.

Symbiotic genes have been located on the 400-kbp plasmids in *R. huautlense* strains [38]. The similar RFLP patterns among the plasmidless strains S32 and S182 and the other *R. huautlense* strains (Table 3) may be explained if the symbiotic gene cluster(s) or the symbiotic plasmid were located (maybe by transposition) in the chromosome or on megaplasmid not electrophoretically resolved in strains S32 and S182. The DNA sequences leading to the integration of symbiotic gene cluster might be linked to the *nodDAB* genes since different *nodDAB* hybridization bands among S32, S182, and other strains were obtained in *Eco*RI-digested DNAs. The finding of two pSym-cured strains indicates the instability of pSym and may suggest the existence of many non-symbiotic *R. huautlense* in Huautla soils.

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