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ORIGINAL PAPER

Diverse endophytic bacteria isolated from a leguminous tree *Conzattia multiflora* grown in Mexico

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Abstract *Conzattia multiflora* is a leguminous tree present only in Mexico and Guatemala. There is no record about its symbiotic or pathogenic microbes. In this study, we found that numerous bacteria with 10^4–10^6 individuals per gram of fresh epidermis were distributed in the tissue of this plant. All the bacteria isolated from the *Conzattia* epidermis were Gram-negative, facultative anaerobic rods and formed yellow or colorless colonies. They were identified as endophytes by inoculation tests. Some of the bacteria could significantly promote the growth of *Conzattia* seedlings. Nine different groups were defined by PCR-based RFLP, which were classified as *Pantoea, Erwinia, Salmonella, Enterobacter, Citrobacter* and *Klebsiella* by the phylogenetic analysis of 16S rRNA genes. The existence of plant-borne lineages of *Salmonella* indicates that the unexplored plants may harbor some unknown microbes.

Keywords Diversity · Phylogeny · Endophytic bacteria · *Conzattia* · Epidermis

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Introduction

The endophytes are microorganisms residing in the tissue of living plants and do not visibly harm the plants (Hallmann et al. 1997). These microorganisms are relatively unstudied and are potential sources of novel natural products for exploitation in medicine, agriculture, and industry (Strobel et al. 2004). In nature, each individual plant is the host to one or more kinds of endophyte and only a handful of these plants (grass species) have been studied in regard to their endophytic biology. Thus there is a great opportunity to find new and interesting endophytic microorganisms among myriad plants in different settings and ecosystems (Strobel et al. 2004).

Leguminous plants are important in agriculture and forestry and many of them can form nodules on roots and/or stems in symbiosis with nitrogen-fixing bacteria in the phylum Proteobacteria. Because of their commercial and ecological importance, the rhizobia–legumes symbiosis has been investigated intensively. Meanwhile, the endophytic bacteria of legumes have been poorly studied. Previous studies on pea and soybean plants have demonstrated that the endophytic bacteria can induce the resistance of pea plants to pathogenic fungi (Castejón-Muñoz and Oyarzun1995; Elvira-Recuenco and van Vuurde2000) or improve the growth of soybean (Kuklinsky-Sobral et al. 2004). Based on these and other reports, it seems that *Enterobacter* spp., *Pseudomonas* spp., and *Bacillus* spp. are the most abundant endophytic bacteria in legumes and other plants, such as, potato and maize. Using selective media, many other bacteria such as the free-living nitrogen-fixing bacteria and actinomyces also can be isolated from inside the plants.

Located in the tropical zone, Mexico has been reported to be the center of origin for many leguminous species. Many investigations have been done on the diversity and taxonomy of the nodule bacteria associated with legume plants grown in Mexico, such as *Phaseolus vulgaris*. In a survey of rhizobia in the Sierra de Huautla, Morelos, Mexico, we found nodules on the trunk of *Conzattia multiflora*, a legume tree indigenous to Mexico (Allen and Allen 1981). It is in the tribe *Caesalpinieae* of the subfamily *Caesalpinioideae*. Most of the 47 genera in this tribe appear not to nodulate and there are no reports of it for nearly half of the genera, including *Conzattia* (Allen and Allen 1981). Based on this background, it was of interest to investigate if the nodules fixed nitrogen and what bacteria lived inside the nodules. We therefore decided to isolate the bacteria from the epidermis of nodules and of the trunk of *Conzattia* and characterize the isolates. The aims of this work were to determine the incidence of bacteria in the epidermis and to identify the bacteria, as well as to investigate the impact of the bacteria on *Conzattia* trees.

Materials and methods

The *Conzattia* tree and sampling

The sampling sites were in La Sierra de Huautla, Morelos State, Mexico, where was a natural reserve for plants and animals. We found that the *Conzattia* tree had nodules on its trunk and on the main branches (Fig. 1). Most of the nodules are balls from 1 to 5 cm in diameter with a woody core independent from the xylem of the trunk (Fig. 1), so they are easily separated from the trunk. The epidermis of the nodules and of the trunk contains water-soluble purple pigments with absorption peaks at 430 and 490 nm (not shown). The nodules were only observed on large trees (more than 20 cm trunk diameter) and the number of nodules in each tree varied from 10 to 100. Five 1- to 2-year old *C. multiflora* trees in the fields were checked thoroughly and no nodules were found on their roots or stems. We collected the nodules on June 1999, when the plants grow vigorously, and in the beginning of October, when the leaves have just fallen away, and at the end of November. The nodules were immediately put into 1,000 ml side-arm flasks sealed with rubber plug. After replacing 100 ml of air with the same volume of acetylene in the flasks, they were incubated at environmental temperature for 6–12 h. Gaseous samples were then taken from the flasks to determine the reduction of acetylene by gas chromatography.

Observation, isolation and counting of bacteria in epidermis

The presence of bacteria in epidermis of the nodule was checked under microscope after staining with methyl blue and by scanning electron microscopy. A total of 27 nodules collected from eight trees distributed in two zones 10 km apart were isolated. To isolate the bacteria, epidermis tissues of nodules were surface-sterilized by standard methods (Vincent 1970) and the

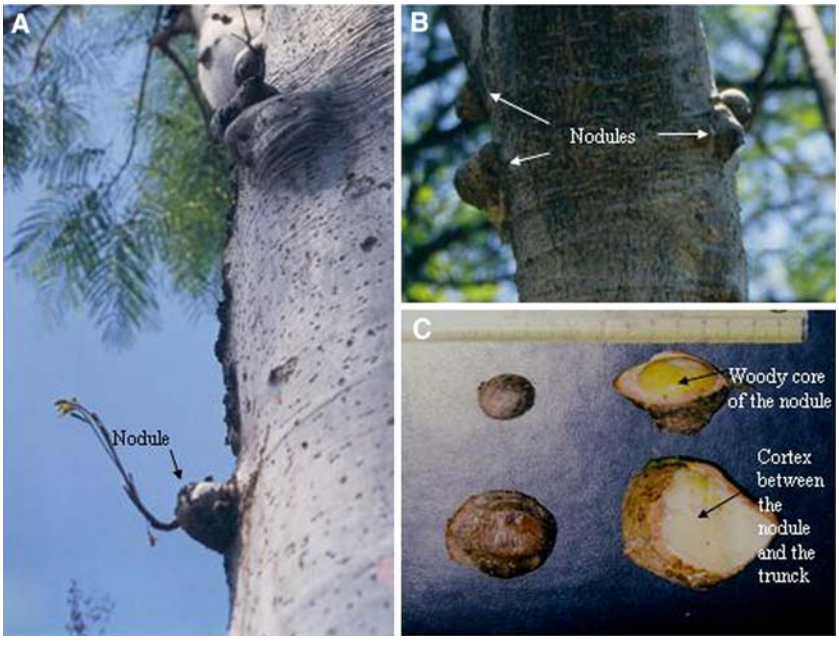

Fig. 1 Nodules of *Conzattia multiflora*. **a**, **b** Nodules grown in the trunk; **c** Nodules separated from the tree

cortex was peeled away to avoid fungal contamination. The purple tissues were cut into small pieces (~2 mm^3) and ground in sterilized 1.5 ml microtubes tubes. The extracted juice was streaked on YMA (Vincent 1970) and PY medium (Yao et al. 2002) or inoculated into semisolid PY medium in tubes. In each medium, 0.025% of BTB (bromothymol blue) was added as pH indicator. An alternative method was to inoculate the small pieces of purple tissue into semisolid N-free medium (Sucrose, 10 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; BTB, 0.0025 g; distilled water 1 l; agar, 2.3 g; pH 7.0). Bacterial numbers inside the plant tissue were estimated from five nodules collected in October (just after the falling of leaves) and five collected in November by using the method of most possible number (MPN). The endophytic bacteria were released by grinding 1 g of the epidermis in 9 ml of sterile $MgSO_4$ solution (10 mM). Serial dilutions (10^{-1} –10^{-6}) were made and an aliquot of 100 µl of the suspension was inoculated into 5 ml of PY broth with 0.0025% BTB (in triplicate). All the inoculated media were incubated at 28°C. The isolates were purified by repeatedly streaking on PY medium and by microscopic observation of the Gram-stained cells.

PCR-RFLP and sequencing of 16S rRNA genes

The methods described previously (Wang et al. 1999) and the primers fD1 and rD1 (Weisburg et al. 1991) were used to amplify the 16S rRNA genes from the cell lysates. The restriction endonucleases *Alu*I, *Cfo*I, *Hinf*I, and *Msp*I were separately used to digest 10 µl of the PCR products. The RFLP patterns were separated by electrophoresis in 3% agarose gels and were visualized by observation under UV light of ethidium bromide stained gels as described (Wang et al. 1999). Different RFLP patterns were marked alphabetically. Isolates were grouped based upon the electrophoretic patterns. Representative isolates were used for sequencing of the 16S rRNA genes by a PCR-based procedure (Hurek et al. 1997). The sequences obtained were compared to those in the GenBank database by Blast searching. A phylogenetic tree was constructed using the Clustal W package (Thompson et al. 1997).

Nitrogen fixation tests for the isolates

Representative isolates were tested for acetylene reduction ability in nitrogen-free semisolid medium by gas chromatography. Reference strains *Burkholderia vietnamensis* LMG11347 as positive control and *Escherichia coli* DH5α as negative control were included. The bacteria were incubated at 28°C for 3 days. Southern hybridization of *nifH* was performed using a 600 bp *nifH* fragment of *R. etli* CFN42 as described previously (Wang et al. 1999). The probe was amplified by using the primers nifH-1 and nifH-2 and the procedures reported by Eardly et al. (1992).

Phenotypic characterization of the isolates

Growth in LB medium at 28 and 37°C, growth in PY at 37°C and growth under anaerobic conditions in semisolid PY medium (covered with a layer of liquid paraffin) at 28°C were tested. The cellular morphology was observed by Gram-staining and microscopy. Generation time of the bacteria was estimated by the spectrophotometric method (Vincent, 1970).

Inoculation of the bacteria on the host plants

The seeds of *C. multiflora* were scarified, surface-sterilized (Vincent 1970), and immersed in PY broth containing streptomycin and tetracycline (100 and 5 µg ml^{-1}, respectively) and incubated at 28°C for 20 h to eliminate any seed-borne bacteria. Then the seeds were washed six times with sterilized water and germinated on semisolid water-agar (Vincent 1970). The seedlings were transferred into 250 ml-flasks containing 7 g of cotton and 100 ml of nitrogen-free plant nutrient solution (Vincent 1970), inoculated with 100 µl of overnight PY culture (OD600 = 0.8–1.0) and grown in green house with natural sunlight. During growth, the shoots of seedlings were exposed to air and the roots were maintained under the aseptic conditions by sealing the flasks with cotton plugs. Sterilized nitrogen-free plant nutrient solution was supplied once a week. Alternatively, some seedlings were grown inside cotton-sealed glass tubes to maintain the entire plant under aseptic conditions. The plants were harvested after two months. After the measurement of height and weight, the whole seedlings were surface-sterilized and ground in sterile 0.1 mM $MgCl_2$ (1:10 w/v). The extracts were used to isolate and to count the endophytic bacteria. Statistical analyses (*t* test) were applied for the inoculation tests using the data of height and weight of the seedlings. Six 1-year-old plants grown in pots and two large trees in the fields were also inoculated by injection of a mixture of isolates and were kept for long-term observation.

Seedlings of common bean (*Phaseolus vulgaris*) were also inoculated with the isolates from *Conzattia*. PCR was performed to amplify the *nodA* gene using the primers nodA-1 and nodA-2 and the procedure

described previously (Haukka et al. 1998) to verify if the isolates contain this nodulation gene.

Results

Observation of the bacteria in *C. multiflora* tissues

The bark of *C. multiflora* is composed of four layers (from outer to inner): cortex (1–2 mm), a thin layer of green tissue (0.5–1 mm), a thicker layer of tender tissue (epidermis, 3–10 mm) containing water-soluble purple pigment, and a white layer of rough tissue (3–10 mm). In the microscopic observation of methyl blue stained bark, bacteria were found in the epidermis with purple color (not shown). Two nodules were checked by scanning electron microscopy and five fields in each nodule were observed. Abundant bacteria were found inside the cells of the purple layer. The bacteria were cocci or rods with different sizes and formed clusters inside the plant cells (Fig. 2).

Isolation of the bacteria in the epidermis

Isolation was not successful using the methods usually used for rhizobial isolation. No bacterial growth occurred when the crushed nodule tissues were streaked directly on the PY or YMA plates after 20 days of incubation. However, growth was observed by inoculating the nodule tissues or extracts into semisolid media or in broth. Bacteria were obtained from all the 27 nodules. The bacteria produced alkali in the upper part and acid in the lower part of the media. After growing in the semisolid or broth medium, these bacteria could grow on PY plates and formed two types of colonies (yellow or colorless). In some nodules, there were both types with different ratios and in others there was only one. The colonies were > 2 mm in diameter, which produced alkali after a two-day incubation on PY plates.

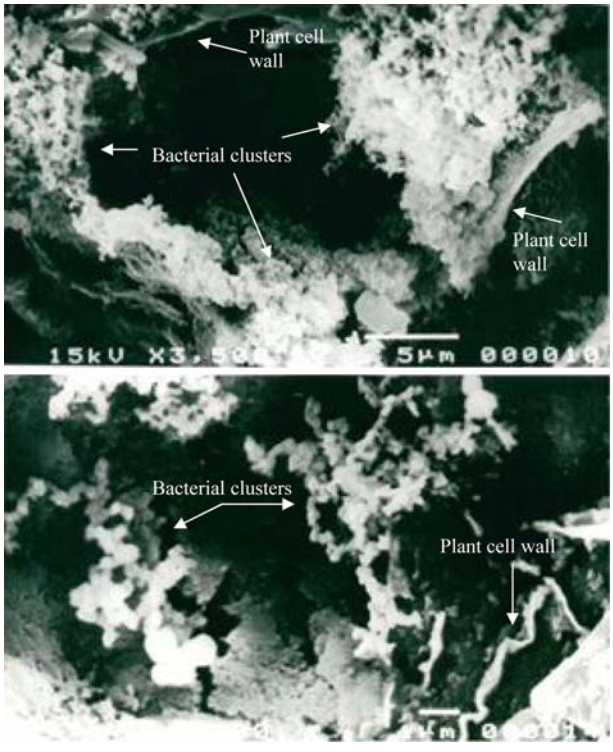

Fig. 2 Photograph showing the endophytic bacteria inside the nodule cells of *Conzattia multiflora*. The photograph was taken by scanning electron microscopy

Counting of the bacteria

The number of bacteria varied from 1.5×10^4 to 4.5×10^6 g^{-1} of fresh tissue with the mean value of 2.29×10^6 in the nodules obtained in October, and 2.28×10^4 in the nodules collected in November (Table 2). The similar number of bacteria (30–45,000 cells g^{-1} of fresh tissue, with mean of 3.64×10^4) was also counted from the epidermis of the trunk collected in November. The isolates obtained from the MPN tubes formed yellow or colorless colonies.

RFLP and sequencing of PCR-amplified 16S rRNA genes

Among the 33-isolates obtained with different methods, nine rDNA types were obtained (Table 1). Isolates obtained in different media and with different methods also showed different RFLP patterns. The results of sequencing analysis indicated that the 9 rDNA types defined by PCR-RFLP were different lineages (Fig. 3) and the isolates in the same rDNA type (Co9926 and Co9927) showed the most close relationship, confirming that the definition of RFLP groups was confident.

From the results of PCR-based RFLP and the sequencing of 16S rDNA, the isolates could be identified as different genera of enterobacteria and they were closely related to *Citrobacter*, *Enterobacter*, *Erwinia*, *Klebsiella, Pantoea* and *Salmonella*. Ten strains representing the 9 rDNA types of *Conzattia* endophytes showed rDNA sequence similarities from 97 to 99% with the reference strains for the corresponding species (Table 1). The main groups are the *Pantoea*-related bacteria (including 8 strains in rDNA types 3, 8 and 9), the *Salmonella*-related bacteria (covering 13 isolates in the rDNA types 1 and 7), *Enterobacter* (including 7 isolates in rDNA type 2), and *Erwinia*

Table 1 Bacterial strains isolated from the nodule-like structure of *Conzattia multifora* and their relevant characteristics

Strains[a]	rRNA type (RFLP pattern)	Isolated from	Medium	Most related bacteria (sequence similarity)
Co9901, Co9907, Co9917, Co9919, Co9923, Co9925	1 (AAAA)[b]	Block of tissue	Semisolid PY	*Salmonella enterica* AE006468[c] (98%)
Co9902, Co9904, Co9908, Co9913, Co9918, Co9921, Co9924	2 (AAAB)	Block of tissue	Semisolid PY	*Enterobacter homaechei* AJ871858 (98%)
Co9926, Co9927, Co9928	*3 (AAAC)*	Block of tissue	Semisolid PY	*Pantoea agglomerans* AJ251466 (99%)
Co9929, Co9930, Co9931, Co9932, Co9933, Co9934	4 (AAAE)	Block of tissue	N-free medium	*Erwinia rhapontici* U80206 (97%)
Co9935	5 (AABE)	Block of tissue	N-free medium	*Klebsiella oxytoca* AJ871858 (99%)
Co9936	6 (AAAF))	Block of tissue	N-free medium	*Citrobacter farmeri* AF025371 (97%)
Co9937, Co9938, Co9939, Co9940	7 (AABA)	Block of tissue	N-free medium	*Salmonella bongori* AF029226 (98%)
Co9945	8 (AACD)	Serial dilution	PY broth	*Pantoea agglomerans* AJ251466 (97%)
Co9941, Co9942, Co9943, Co9944	9 (BABD)	Serial dilution	PY broth	*Pantoea ananatis* Z96081 (99%)

[a] Strains marked with block letters are used in 16S rDNA sequencing

[b] The four letters in the parentheses represented the RFLP patterns of PCR-amplified 16S rDNA digested separately with *Alu*I, *Cfo*I, *Hinf*I and *Msp*I

[c] GenBan accession number

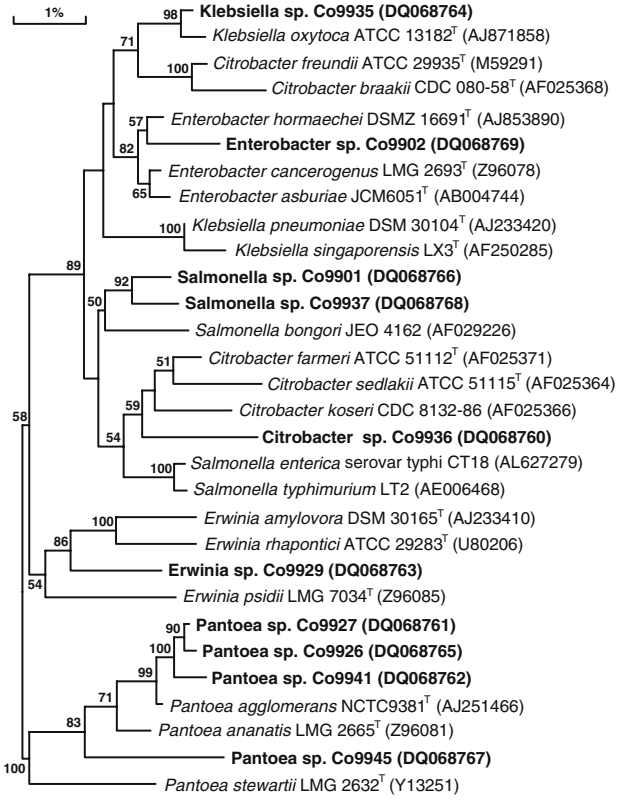

Fig. 3 Phylogenetic tree of 16S rDNA showing the relationships among the isolates (*marked with block letters*) from *Conzattia* nodules and the related bacterial species. The Neighbor-Joining dendrogram was derived from a 16S rDNA sequence distance matrix (Jukes-Cantor). Bootstrap confidence levels greater than 50% are indicated at the internodes. GenBank accession numbers are shown in parentheses. Bar 1%nucleotide divergence

(including 6 isolates in the rDNA type 4). Among all of these bacteria, *Pantoea* may be the most abundant bacteria because the 5 isolates in rDNA types 8 and 9 were isolated by serial dilution (from 10^{-3} to 10^{-6} dilution). We named the isolate Co9935 as *Klebsiella* sp. because the 16S rDNA sequence of this isolate was most similar to that of *Klebsiella pneumoniae* (Fig. 3).

Growth and cellular characteristics of the bacteria

All the isolates (Table 1) could grow aerobically and anaerobically in PY medium, indicating that they were facultative anaerobic bacteria. All of them could grow at 37°C in both PY and LB broth. The generation time of 11 isolates representing the 9 rDNA types was less than 2 h in PY broth. They were Gram-negative rods of 0.4–0.7 × 0.7–3.0 μm.

Nitrogen-fixing ability

No acetylene reduction was detected in the tests with whole nodules. Compared with the reference strain *Burkholderia vietnamensis* LMG11347, all the 11 representative isolates obtained from the nodules grew poorly in the semisolid nitrogen-free media. The isolates *Pantoea* spp. Co9944, Co9945 and Co9926; *Salmonella* spp. Co9937, Co9939 and Co9940; *Erwinia* sp. Co9930, Co9932, and Co9934; and *Klebsiella* sp. Co9935 had very low but stable acetylene reduction ability (the peak of C_2H_4 was less than 1% of the C_2H_2). Under the same conditions, the positive control strain *B. vietnamensis* LMG11347 had very high activity and the negative control strain *E. coli* DH5α had no activity (Figures not shown). In the hybridization of *Eco*RI digested total DNA to *nifH* fragment of *R. etli* CFN42, none of the isolates showed hybridization bands, and there was no *nifH* band obtained by PCR with DNA from these isolates.

Inoculation tests

We found that the surface-sterilized seeds of *C. multiflora* carried bacteria inside. These seed-borne bacteria were morphologically similar to the nodules-borne ones. They were resistant to 100 μg ml^{-1} of streptomycin but could be eliminated by incubation of the surface-sterilized seeds in PY broth supplied with the mixture of 100 μg ml^{-1} of streptomycin and 5 μg ml^{-1} of tetracycline. This treatment was used prior to the inoculation tests.

In the inoculation tests, no nodules were found on the inoculated seedlings after two months, and the plants looked healthy although they were grown in N-free plant nutrient solution. The injection of bacteria did not cause the formation of nodules in the five young trees grown in pots and two large trees of *C. multiflora* grown in the fields after one year. No amplified fragments of *nodA* were obtained from the tested isolates. Bacteria from 10^4 to 10^7 g^{-1} of plant tissue were obtained from the inoculated plants grown in cotton-sealed tubes, while no bacteria were isolated from the non-inoculated plants. Although bacteria could be isolated from the non-inoculated seedlings grown in flasks opened to air, the number of endophytic bacteria was much lower than those of the inoculated seedlings (Table 3). In the inoculation tests, the isolates Co9935 (*Klebsiella*), Co9936 (*Salmonella*) and Co9902 (*Enterobacter*) could significantly improve the growth of seedlings by enhancing the height of plants (Table 3) compared with the non-inoculated plants. But there were no significant differences in the weight of seedlings among the treatments.

Discussion

Based upon the observation in the field and the inoculation tests in the laboratory, it can be concluded that *Conzattia multiflora* does not form root nodules with rhizobia. However, many nodules are found on the trunk and main branches of healthy *Conzattia* trees. According to our results in this study, no rhizobia were isolated from the nodules, but a large number of facultative anaerobic bacteria were isolated from the nodule tissues.

Previously, three kinds of nodules or galls caused by bacteria have been recorded in different plants. They are the nitrogen-fixing root nodules induced by rhizobia or by *Frankia*; the crown galls caused by *Agrobacterium*; the leafy galls caused by *Phyllobacterium myrsinacearum* (Mergaert et al. 2002), *Rhodococcus fascians* (Goethals et al. 2001), and *Burkholderia* spp. (Van Oevelen et al. 2002, 2004). The trunk nodules of *Conzattia multiflora* are different from these reported nodules or gall structures in morphology (Fig. 1). The bacteria in the *Conzattia* nodules are found inside the plant cells, which is similar to the *Rhizobium*, but different from the leafy gall bacteria *Burkholderia* spp., which are found in intercellular spaces (Van Oevelen et al. 2002). The regular form and the woody core in the trunk nodules of *Conzattia* tree also differentiate them from the crown galls and the root-nodules, and the absence of nematodes or *Agrobacterium* in these nodules discards their participation in their morphogenesis, even in the very young ones.

A culture-independent study, such as by PCR, cloning and sequencing of nodulation or tumor-inducing genes, may help to find the nodule bacteria inside the *Conzattia* tree. Another possibility is that the nodules are induced by some uncultivable bacteria because uncultivable *Burkholderia* spp. have been found in the leafy galls of *Psychotria* (Van Oevelen et al. 2002, 2004).

The direct observation revealed that there were a lot of endophytic bacteria inside the cells of the *C. multiflora* nodules (Fig. 2). The isolation and counting results also evidenced the existence of bacteria inside the plant tissues (Tables 1, 2). We suppose that these bacteria have adapted to the microaerobic environment inside the plant cells because they could not grow when the nodule extracts were inoculated directly on PY or YMA plates, and they could be isolated in semisolid or liquid media. Although some bacteria were isolated by using the nitrogen-free medium, the poor growth and the absence of *nifH* gene made it uncertain if the isolates from *Conzattia* trunk nodules can fix nitrogen. The little amount of ethylene detected in the culture of some isolates may be synthesized by the bacteria, as reported in other phytopathogenic bacteria (Weingart and Volksch, 1997).

Table 2 Bacterial number estimated in PY broth by MPN (per g of fresh tissue) method

Sample	MPN (3 duplicate)	Mean	Sampling time
Nodule 1	2.5×10^6	4.5×10^6	October
Nodule 2	4.5×10^6		October
Nodule 3	7.5×10^6		October
Nodule 4	1.5×10^4		October
Nodule 5	2.5×10^5		October
Nodule 6	2.5×10^2	6.7×10^4	November
Nodule 7	8.3×10^4		November
Nodule 8	2.5×10^2		November
Nodule 9	27		November
Nodule 10	30		November

The nodules were collected in 1999 from fields

Table 3 Height and number of endophytic bacteria of 1 month-old plants inoculated with different strain

	Height of 6 seedlings (mm) inoculated with isolates									
	CK	Co9929	Co9935	Co9936	Co9937	Co9901	Co9902	Co9941	Co9945	Co9926
Average	154	166	176*	173*	164	146	171*	169*	167	152
STDEV	15.8	8.6	19.2	9.2	12.8	15.0	13.2	26.4	9.8	11.7
Average of bacteria number in 6 inoculated plants estimated by the MPN method (per g of fresh plant)										
MPN	5.2×10^4	1.1×10^6	3.9×10^6	2.3×10^6	2.4×10^7	4.6×10^5	1.1×10^6	2.4×10^6	2.4×10^5	9.3×10^6

The shoots of the seedlings were exposed to the air and the roots were maintained inside the flasks under aseptic condition

* Significantly different from the non-inoculated (CK) plants in the *t* test, $P = 0.01$

It seems clear that the isolation methods affect the evaluation of bacterial diversity inside a plant, because the bacteria isolated from the nodule extracts with and without dilution, and isolated with different media were identified as distinct 16S rDNA RFLP groups (Table 1). The different methods we used allowed us to obtain the bacteria that grow faster (with the nodule extracts without dilution); that are most abundant inside the nodules (serial dilution); and that require lower concentration of nitrogen nutrient (N-free medium).

The recovery of these isolates from the inoculated plants (Table 3) demonstrated that these isolates are endophytic bacteria according to the definition of Hallmann et al. (1997). The bacterial density inside the nodules is within the range of endophytic bacteria, that is around 10^3–10^6 cells per gram fresh weight (Hallman et al. 1997; McInroy and Kloepper, 1995). Some of these bacteria may be plant growth promoting microbes that produce phytohormones, because they can significantly enhance the seedling length, but not the weight. Since similar bacteria are also found inside all the seeds we tested, the endophytic bacteria in *Conzattia* trees might be inherited, as revealed on the *Burkholderia* spp. in leafy galls (Van Oevelen et al. 2002).

In this study, we found that the number of endophytic bacteria reduced dramatically (100 times) from the growth season (in the start of October) to the dormant season (at the end of November). These data indicate that (a) the number of these endophytic bacteria may be related to carbon supply from the host plant; and (b) the endophytic bacteria can serve as a nutrient pool for the plant, even if they do not excrete nitrogen compounds to the plant when they grow, but the dead cells should supply the plant their nitrogen and other contents in the dormant season. The absence of nitrogen-deficient symptom in the bacterialized seedlings may be the evidence that some of the endophytic bacteria can improve the nutrient condition of the plant.

In the inoculation tests, no nodules were induced by the bacteria, but the bacteria were recovered from the seedlings at a high number similar to that inside the nodules (Table 3). These results indicated that these bacteria also could be endophytes in other plant tissues. Microscopic analysis showed that the bacteria existed only in roots and in stems, but not in leaves of the inoculated plants (not shown).

Research of endophytic bacteria has been performed on the bacteria associated with economically important plants, such as potato (Garbeva et al. 2001), citrus plants (Araujo et al. 2002), cotton and sweet corn (McInroy and Kloepper, 1995), maize (Seghers et al. 2004), and pea (Elvira–Recuenco and van Vuurde, 2000). The endophytic bacteria in these plants are very diverse and the most abundant groups are *Entrobacter* spp., *Pantoea* spp. and *Pseudomonas* spp. In soybean plants, most of the endophytic bacteria were members of *Pseudomonadaceae, Burkholderiacea* and *Enterobacteriaceae* (Kuklinsky-Sobral et al. 2004). In the pea plants, the most frequently recovered bacterial types were *Pantoea agglomerans* and *Pseudomonas fluorescens* (Elvira-Recuenco and van Vuurde 2000). Compared with soybean and pea plants, all the endophytic bacteria isolated from *Conzattia* trees were enterobacteria (Fig. 4). Thus it seems that the enterobacteria are common endophytic bacteria of both the root nodule-forming legumes (soybean and pea) and the *Conzattia* trees. The differences in the community of endophytic bacteria may reflect the specificity between the bacteria and their host plants, because the colonization of bacteria in plants is regulated by the genotype of plants (Iniguez et al. 2005).

The *Pantoea* strains have been isolated from various plants and some isolates of *Pantoea agglomerans, Enterobacter kobei, Enterobacter cloacae, Leclercia adecarboxylata, Escherichia vulneris*, and *Pseudomonas* sp. have been reported as nitrogen-fixing nodule bacteria (Benhizia et al. 2004). In our work, *Salmonella, Pantoea* and *Entrobacter* were identified among the

endophytic bacteria isolated from the trunk nodules of *Conzattia*. However, the endophytic bacteria in *Conzattia* may not nitrogen fixers, because no *nifH* gene was amplified from the isolates or from the metagenomic DNA extracted from the nodule tissues (data not shown).

Salmonella is a bacterial genus comprising important pathogens. Based upon the results of this study, we report the existence of plant-borne lineages within the genus *Salmonella* (Table 1; Fig. 3). Therefore, the genus *Salmonella* seems to include pathogens, soil-borne lineage (Shelobolina et al. 2004) and plant-borne lineage. The genetic differences among the *Salmonella* from different sources could be an interesting topic for further study.

This is the first report on the association of bacteria and *Conzattia multiflora*, a leguminous tree native to Mexico. No rhizobia were obtained but very diverse enterobacteria were isolated from the epidermis of the trunk nodules of *Conzattia* trees. These bacteria have been confirmed as endophytes, but the nitrogen-fixing and nodule-inducing abilities of these bacteria is doubtful.

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