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Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion

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Summary

Endophytic and epiphytic bacteria were isolated from two soybean cultivars (Foscarin and Cristalina). Significant differences were observed in bacterial population densities in relation to season of isolation, soybean growth phase and the tissues from which the isolates were obtained. The isolates were identified by partial 16S rDNA sequence analysis, with most of the isolates belonging to the *Pseudomonaceae, Burkholderiacea* and *Enterobacteriaceae* groups. The potential of the isolates for plant growth promotion was evaluated by screening for indoleacetic acid (IAA) production and mineral phosphate solubilization; 34% of endophytic bacteria produced IAA and 49% were able to solubilize mineral phosphate whereas only 21% of epiphytic bacteria produced IAA although 52% were able to solubilize mineral phosphate. A high frequency of IAA producing isolates occurred in the early ripening Foscarin cultivar whereas a high percentage of phosphate solubilizing isolates were obtained from plants in the initial development stage (V6). We also found that 60% of endophytic and 69% of epiphytic isolates that produced IAA and solubilized mineral phosphate were also able to fix nitrogen *in vitro*. The soybean-associated bacteria showing characteristics related to plant growth promotion were identified as belonging to the genera *Pseudomonas, Ralstonia, Enterobacter, Pantoea* and *Acinetobacter*.

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Introduction

Bacteria are common inhabitants of both the surfaces and the internal tissues of most plants and may have diverse effects on host plant development. Plant-associated bacteria isolated from rhizoplane and phylloplane surfaces are known as epiphytes (Andrews and Harris, 2000) whereas those isolated from the interior of tissues, which they inhabit without causing harm to the host, are called endophytes (Petrini *et al*., 1989; Azevedo *et al*., 2000), with some bacterial populations fluctuating between endophytic and epiphytic colonization (Hallmann *et al*., 1997).

Endophytic and epiphytic bacteria can contribute to the health, growth and development of plants. Plant growth promotion by endophytic and epiphytic bacteria may result either from indirect effects such as the biocontrol of soilborne diseases through competition for nutrients, siderophore-mediated competition for iron, antibiosis or the induction of systemic resistance in the plant host, or from direct effects such as the production of phytohormones or by providing the host plant with fixed nitrogen or the solubilization of soil phosphorus and iron (Glick, 1995; Shishido *et al*., 1999; Kinkel *et al*., 2000; Sturz *et al*., 2000).

The utilization of endophytic and epiphytic bacteria in agricultural production depends on our knowledge of the bacteria–plant interaction and our ability to maintain, manipulate and modify beneficial bacterial populations under field conditions (Hallmann *et al*., 1997). The study of plant-associated bacteria is important not only for understanding the ecological role of such bacteria in their interaction with plants but also for the biotechnological application of these bacteria to areas such as the plant growth promotion.

The objective of this study was to identify epiphytic and endophytic bacteria from soybean and evaluate the genetic and physiologic diversity and population distribution of these bacteria under different factors. As additional aim epiphytic and endophytic isolates was evaluated in relation to their potential for plant growth promotion by screening of indoleacetic acid (IAA) production, mineral phosphate solubilization and/or nitrogen fixation.

Table 1. Taxonomic distribution of bacterial isolates obtained from soybean plants. Classification based on amplified rDNA restriction analysis (ARDRA).

Group	N°[a]	Taxonomic affiliation	Species
Group 1	04	β-*Proteobacteria*	*Ralstonia picketti*
Group 2	41	γ-*Proteobacteria*	*Pseudomonas citronellolis*, and *P. oryzihabitans*
Group 3	23	γ-*Proteobacteria*[b]	*Enterobacter agglomerans, Pantoea* sp., and *Enterobacteriaceae*
Group 4	08	β-*Proteobacteria*	*Burkholderia* sp.
Group 5	07	γ-*Proteobacteria*	*Pseudomonas* sp., *P. straminea*, and *P. fulva*
Group 6	01	β-*Proteobacteria*	*Ralstonia pickettii*
Group 7	71	γ-*Proteobacteria*[b]	*Erwinia* sp., *Klebsiella pneumoniae, K. oxytoca, Enterobacteriaceae*
Group 8	03	γ-*Proteobacteria*	*Acinetobacter* sp. and *A. calcoaceticus*
Group 9	03	γ-*Proteobacteria*[b]	*Enterobacter sakazakii*
Group 10	02	γ-*Proteobacteria*	*Pseudomonas* sp.
Group 11	06	α-*Proteobacteria*	*Agrobacterium* sp. and *Caulobacter/Asticcacaulis*
Total	169		

a. Number of analysed isolates.
b. *Enterobacteriaceae*.

Results

Determination of cultivable epiphytic and endophytic bacteria counts

The soybean [*Glycine max* (L.) Merrill] cultivars Foscarin (early ripening) and Cristalina (late-ripening) were cultivated in clay soil during September to January 2000/2001 (year 1) and 2001/2002 (year 2). Four plants of each cultivar were collected during three growth phases: vegetative (V6), flowering (R2) and senescent (R6) (Neumaier *et al*., 2000). The number of cultivable epiphytic bacteria recovered from plants during the senescent phase was higher during year 1 [$\log_{10}$ 6.69 CFU g^{-1} fresh-weight (fw) of tissue] than year 2 ($\log_{10}$ 4.90 CFU g^{-1} fw of tissue). The density of cultivable endophytic bacteria decreased in agreement with soybean growth phase, in the order senescent larger than florescent and this larger than vegetative. In the vegetative phase the bacterial density was not significantly different as regards cultivar or year of planting, although in the flowering (R2) and senescent (R6) phases there were significantly more bacteria in year 1 ($\log_{10}$ 3.95 CFU g^{-1} fw of tissue) than in year 2 ($\log_{10}$ 2.94 CFU g^{-1} fw of tissue).

Although the total population of epiphytic and endophytic bacteria was not significantly different between the early ripening Foscarin cultivar and the late-ripening Cristalina cultivar, the cultivable epiphytic and endophytic bacteria decreased in agreement with soybean tissues, in the order roots larger than stems and this larger than leaves. For epiphytes, we recovered $\log_{10}$ 6.21 CFU g^{-1} fw from roots, $\log_{10}$ 5.59 from stems and $\log_{10}$ 4.86 CFU g^{-1} fw from leaves whereas for endophytes we recovered $\log_{10}$ 4.55 CFU g^{-1} fw from roots, $\log_{10}$ 3.03 from stems and $\log_{10}$ 2.19 CFU g^{-1} fw from leaves.

Isolation, identification and phylogenetic analysis of epiphytic and endophytic bacteria

A total of 361 epiphytic and 373 endophytic bacteria isolated from leaves, stems and roots of two soybean cultivars were randomly picked up, and this population was partially characterized by ARDRA technique with *Alu*I enzyme, resulting in 11 profiles (haplotypes). At least 20% of the isolates within each haplotype was identified by 16S rDNA sequencing. The results (Table 1) showed that the cultivable epiphytic and endophytic bacteria associated with soybean cultivars belong mainly to *Proteobacteria* and that the γ-proteobacteria subgroup was the one dominant, within which the *Enterobacteriaceae* (groups 3, 7 and 9) most frequently showed the highest diversity (Tables 1 and 2). Some correlation between bacterial groups and plant tissues was observed, for example, some groups, such as groups 1 and 4 (*Burkholderiaceae*), colonized only roots whereas group 8 (*Moraxellaceae*) was isolated only from stem tissues (Table 2). The highest diversity was observed inside the roots, as shown from the fact that 10 haplotypes were isolated from this niche.

Partial sequences of 16S rDNA were aligned and the relationships between epiphytic (EP) and endophytic (EN) isolates were evaluated by neighbor-joining algorithm (Figs 1 and 2). Some epiphytic and endophytic isolates presented the same restriction profile and had closely related sequences (Fig. 2). For example, isolates EN141 and EP108, close to *Enterobacter sakazakii,* had 100% of similarity. Similar result was observed for isolates EN315

Table 2. Endophytic and epiphytic bacteria isolated from soybean. Grouped by amplified rDNA restriction analysis (ARDRA) haplotypes and percentage occurrence in the leaves, stems and roots of soybean plants.

	Endophytes (%)[a]			Epiphytes (%)[b]		
Group	Leaf	Stem	Root	Leaf	Stem	Root
Group 1	0	0	8.3	0	0	5.0
Group 2	37.5	27.3	11.1	26.2	17.6	20.0
Group 3	25.0	12.1	2.8	13.1	29.4	0
Group 4	0	0	16.7	0	0	10.0
Group 5	2.5	9.1	5.5	4.3	0	0
Group 6	0	0	2.8	0	0	0
Group 7	30.0	45.5	41.7	47.8	35.3	60.0
Group 8	0	3.0	0	0	11.8	0
Group 9	0	3.0	2.8	4.3	0	0
Group 10	0	0	2.8	4.3	0	0
Group 11	5.0	0	5.5	0	5.9	5.0

a. Identification based on 40 leaf, 33 stem and 36 root isolates.
b. Identification based on 23 leaf, 17 stem and 20 roots isolates.

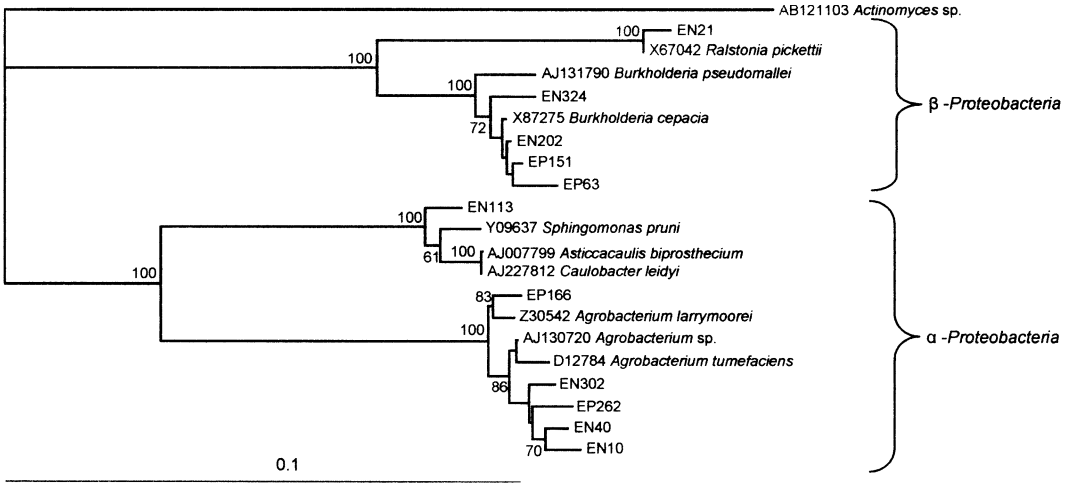

Fig. 1. Phylogenetic relationships of α and β-*Proteobacteria* based on partial 16S rDNA sequences obtained from the soybean-associated bacteria and closely related sequences, based on a distance analysis (neighbour-joining algorithm with Jukes-Cantor model; 1000 bootstrap replicates performed). EP = epiphytic bacteria; EN = endophytic bacteria.

and EP122, close to *Pseudomonas straminae*, and EN251 and EP175, close to *Acinetobacter calcoaceticus.* No relation was observed between plant tissues, growth stage, cultivars and sequencing analysis.

Screening for potential plant growth promoting endophytic and epiphytic bacteria

A total of 361 epiphytic and 373 endophytic isolates were analysed for their ability to produce the auxin indoleacetic acid (IAA) and solubilize phosphate *in vitro*. The overall percentage of IAA producing isolates was higher for the endophytic (34%) than the epiphytic (21%) isolates, although the percentage of IAA producing epiphytic bacteria was significantly higher (26%) for the early ripening Foscarin cultivar than for the late-ripening Cristalina cultivar (16%) ($\chi^2 = 5.23$; $\alpha = 0.05$). There was no significant difference in the frequency of IAA producing bacteria recovered during different seasons of from the different growth phases or tissues.

The percentage of mineral phosphate solubilizing epiphytic (52%) and endophytic (49%) bacteria was similar. However, the frequency of endophytic bacteria able to solubilize phosphate recovered from the different plant growth phases decreased in the order vegetative > flowering > senescent (Fig. 3A). In the other hand, it was observed that the frequency of phosphate solubilizing epiphytic bacteria decreasing significantly from leaf > root > stem (Fig. 3B). Endophytic (20%) and epiphytic (13%) bacteria showing the ability to both produce auxin and to solubilize mineral phosphate were selected for further analysis to evaluate if they were able to fix nitrogen. For both endophytes (EN) and epiphytes (EP), the most important bacterial groups containing these two characteristics were groups 2 (17% EN and 22% EP), 3 (17% EN and 16% EP) and 7 (43% EN and 44% EP).

Screening for nitrogen fixing endophytic and epiphytic bacteria

Of the isolates that produced IAA and were able to solubilize mineral phosphate 75 endophytic and 45 epiphytic isolates were evaluated for their possible ability to fix atmospheric nitrogen. Two methodologies were used: bacterial growth in nitrogen free medium (NFb medium) and PCR specific for the *nif*H gene (encode nitrogenase protein Componet II). The NFb medium methodology revealed that 60% of analysed endophytic and 69% of epiphytic isolates were able to grow in nitrogen free medium. These isolates belonged to α and β *Proteobacteria*, although the predominant groups were *Enterobacteriaceae (*group 7) and *Pseudomonadaceae* (group 2). The PCR method revealed the presence of *nifH* in 21% of the endophytic isolates, which were identified as *Acinetobacter calcoaceticus, Burkholderia* sp., *Pseudomonas* spp., *Ralstonia* sp. and species belonging to the *Enterobacteriaceae* group. However, only 9% of epiphytic isolates displayed the *nif*H gene, these isolates belonging to the *Pseudomonadaceae* and *Enterobacteriaceae*.

Discussion

The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue (Mocali *et al*., 2003), plant species

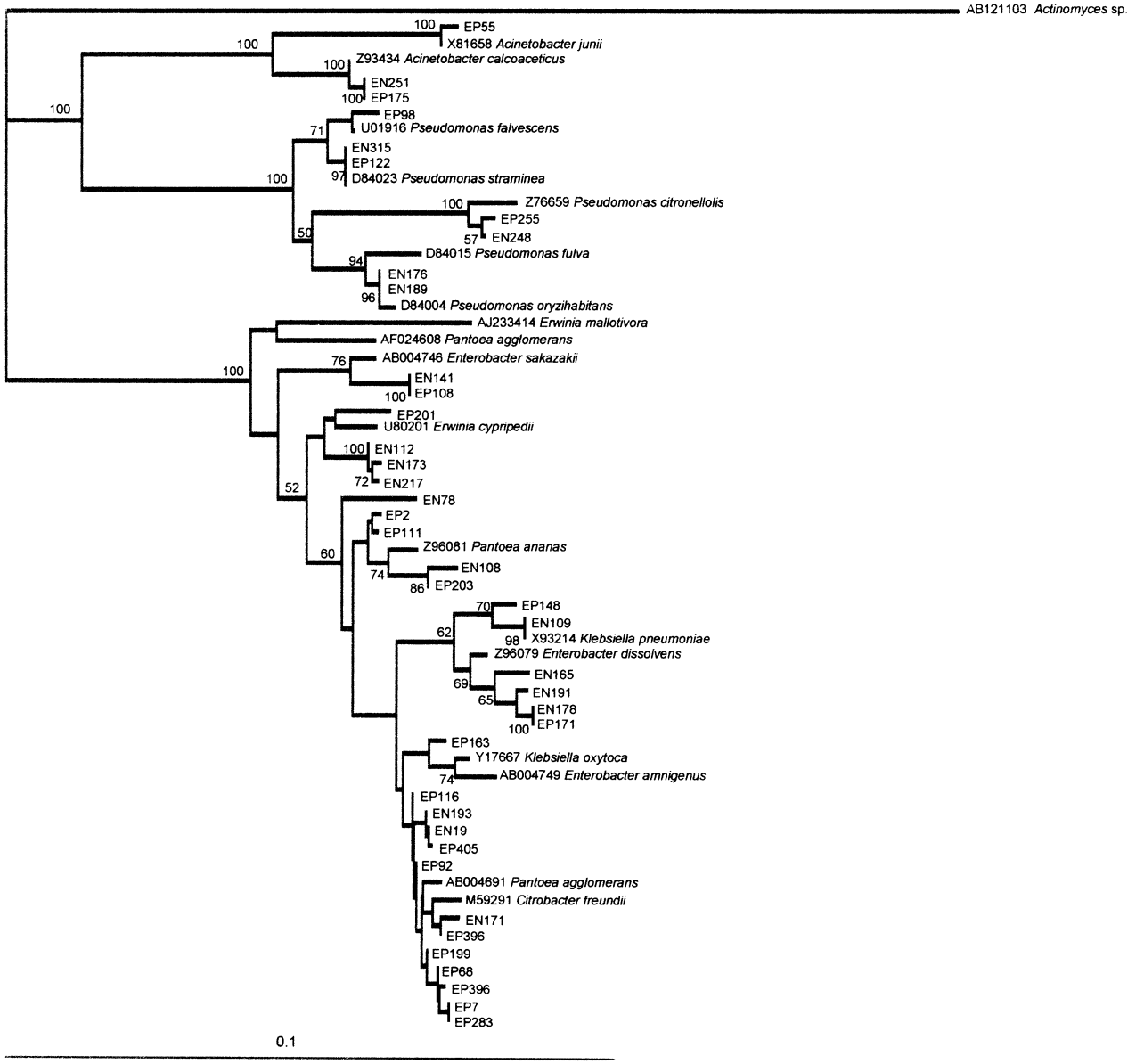

Fig. 2. Phylogenetic relationships of γ-*Proteobacteria* based on partial 16S rDNA sequences obtained from the soybean-associated bacteria and closely related sequences, based on a distance analysis (neighbour-joining algorithm with Jukes-Cantor model; 1000 bootstrap replicates performed). EP = epiphytic bacteria; EN = endophytic bacteria.

and cultivar, soil type (Dalmastri *et al*., 1999; Kinkel *et al*., 2000; Fromin *et al*., 2001) and interaction with other beneficial or pathogenic microorganisms (Araújo *et al*., 2001; 2002). An understanding of the structure and species composition of plant-associated bacterial populations is fundamental to understanding how plant-associated biological processes are influenced by environmental factors and, consequently, has important biotechnological implications.

Most studies about soybean-associated bacteria have been related to nodulation and the interaction between leguminous plants and the nitrogen-fixing bacterium *Bradyrhizobium* (Polenko *et al*., 1987; Oehrle *et al*., 2000; Bai *et al*., 2002) or to plant/pathogen relationships (Kloepper *et al*., 1991; Volksch *et al*., 1992; May *et al*., 1997). On the other hand, the present study is one of the first to explore environmental and physiological factors interacting with soybean-associated bacteria.

Our results show that the type of plant tissue influenced the population density and the taxonomic diversity (Table 2) of epiphytic and endophytic bacterial populations as well as seasonal changes, growth phase or genotype of the host. Therefore, the bacteria–soybean interaction is influenced by several factors that may be used for a better

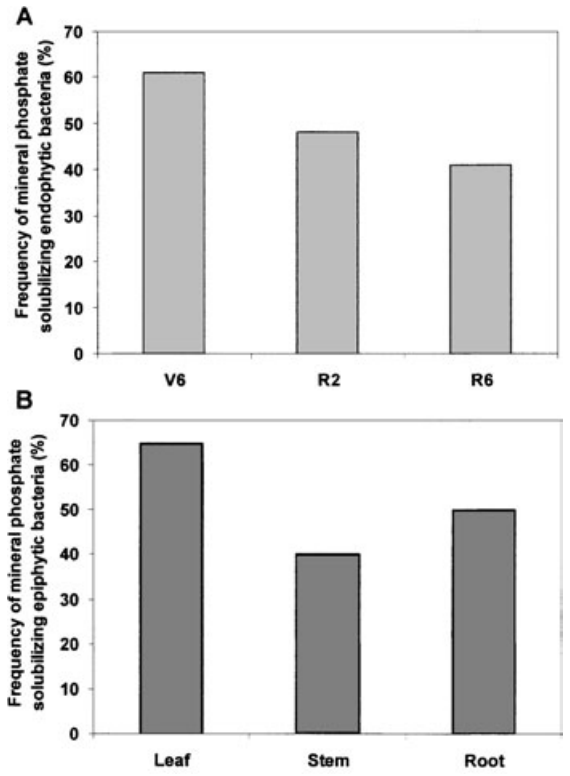

Fig. 3. Isolation frequency of mineral phosphate solubilizing bacteria: (A) endophytes during different growth stages of soybean plants (V6 = vegetative phase; R2 = flowering phase; R6 = senescent phase) and (B) epiphytes from different tissue (leaf, stem and root) of soybean plants. The results were significantly different as calculated by χ^2 ($P < 0.05$).

crop management as beneficial bacterial populations are involved in this interaction. As described previously (McInroy and Kloepper, 1995; Lamb *et al*., 1996; Elvira-Recuenco and van Vuurde, 2000) the roots seems to be the preferential site for epiphytic and endophytic bacteria, suggesting that endophytic bacteria may travel upward from the roots into the stem during plant development. This preference for root tissue also reflects the presence of high levels of nutrients in the rhizosphere, such nutrients being able to support higher bacterial growth and metabolism in roots compared with other tissues (Glick, 1995).

The knowledge about genetic and physiological relationships between epiphytic and endophytic communities may be useful to determine if epiphytic and endophytic isolates are related. The phylogenetic trees revealed a close relationship between the epiphytic and endophytic isolates, supporting the hypothesis that one possible origin of endophytic bacteria is epiphytic and rhizosphere bacterial population (Hallmann *et al*., 1997; Sturz *et al*., 2000). The capacity of bacteria to colonize plant tissues both externally and internally is a desirable characteristic for seeds inoculation because such bacteria would have a greater chance of influencing host development.

Our results indicate that the number of bacteria present was independent of the soybean cultivar, although the physiological diversity of the isolates seems to be related to the cultivars from which the isolate was obtained. For example, a higher percentage of IAA producing epiphytic bacteria was isolated from the early ripening Foscarin cultivar than from the late-ripening Cristalina cultivar. This is interesting because IAA is a plant hormone with no apparent function in bacterial cells, and it could be speculated that IAA production may improve the fitness of the plant–bacterium interaction. Production of IAA is widespread among bacteria–plant associated, but studies showed that in phytopathogenic bacteria IAA is produced from tryptophan via the intermediate indoleacetamide and in beneficial bacteria IAA is synthesized predominantly via indolepyruvic acid (Manulis *et al*., 1998; Patten and Glick, 2002). Furthermore, Patten and Glick (2002) have shown that bacterial IAA stimulates the development of the root system of the host plant and Brandl and Lindow (1998) have studied the contribution of IAA for bacterial epiphytic fitness, observations supported by the investigations of other workers (Glick, 1995; Patten and Glick, 1996; Bastián *et al*., 1998; Dobbelaere *et al*., 1999; Verma *et al*., 2001).

Another interesting interaction observed was the higher percentage of phosphate solubilizing endophytic bacteria occurring in the vegetative (V6) stage (i.e. the initial development stage) of soybean growth. Phosphorus is one of the most important plant nutrients and a large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and becomes unavailable to plants (Nautiyal, 1999; Rodriguez and Fraga, 1999). Previous experiments have shown that endophytic bacteria possess the capacity to solubilize immobilized mineral phosphates (Rodriguez and Fraga, 1999; Verma *et al*., 2001), suggesting that during initial colonization, endophytic bacteria could enhance phosphate availability to the host soybean plant.

Biofertilizers increase crop growth by combinations of mechanisms, which include biological nitrogen fixation (BNF), phytohormone production, increasing the availability of soil nutrients and disease control (Cocking, 2003). Keeping this in mind, we evaluated the potential to fix nitrogen in those isolates (mainly members of the *Enterobacteriaceae* and species of *Pseudomonas* and *Ralstonia*) able to both IAA production and phosphate solubilization. A higher percentage of these types of isolates with this potential were found. However, it was observed a discrepancy of positive isolates between the applied two methodologies to study nitrogen fixation. Endophytic and epiphytic bacteria revealed a discharge discrepancy between grown isolates in nitrogen free medium and positive isolates in PCR to *nif*H. This may be explained by the variability of *nif*H gene (Zehr *et al*., 2003) or by residual bacterial growth.

In the present study was found that some species might develop epiphytic and endophytic colonization, suggesting that these bacteria could fluctuate between endophytic and epiphytic niche and isolates able to produce IAA, solubilize phosphate and fix nitrogen could be used for soybean growth promotion. Also, this study highlighted the effect of seasonal shifts, plant growth stage, cultivar and plant tissue in endophytic and epiphytic bacterial community and provides more subside to understand the mechanisms involved in plant–bacteria interaction. However, practical application of these results should be further evaluated in field experiments.

Experimental procedures

Plant and experimental field design

The soybean [*Glycine max* (L.) Merrill] cultivars Foscarin (early ripening) and Cristalina (late ripening) were cultivated in clay soil (pH 5.4 (water), 2.7 mmol kg^{-1} potassium, and 21.1 mg kg^{-1} total phosphorus) during September to January 2000/2001 (year 1) and 2001/2002 (year 2) in two 2×6 m plots using a completely randomized block design of 10 lines of plants with 15 plants per line. The plots were located at the Escola Superior de Agricultura 'Luiz de Queiroz', São Paulo University, Piracicaba, SP, Brazil (22°42′S and 47°38′W). Four plants of each cultivar were collected during three growth phases: vegetative (V6), flowering (R2) and senescent (R6) (Neumaier *et al*., 2000).

Sample processing and isolation of cultivable epiphytic and endophytic bacteria

Soybean plants were removed from the soil with a trowel, placed in a plastic bag and immediately transported to the laboratory where they were washed in running tap water to remove soil and the leaves, stems and roots were separated.

Epiphytic bacteria were isolated by placing three grams of appropriate tissue in a 500 ml Erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 ml of phosphate-buffered saline (PBS, containing (g l^{-1}) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl 8.00; pH 7.4) and agitating the flasks at 150 r.p.m., 28°C for 1 h. After agitation, appropriate dilutions of the contents of the flasks were plated onto 10% trypicase soy agar (TSA) supplemented with 50 μg ml^{-1} of the fungicide Imazalil (Magnate 500 CE, Agricur) and the plates incubated at 28°C for 2–15 days, after which colonies were picked off the plates, inoculated on 10% TSA agar slants, incubated at 28°C for 2 days and stored at 4°C. These colonies also were cultivated in 10% TSA, incubated at 28°C for 18 h and following each culture was suspended in 20% glycerol solution and stored at −70°C.

Endophytic bacteria were isolated after removing epiphytes by surface disinfection using serial washing in 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl^-) for 3 min, 70% ethanol for 30 s and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto 10% TSA supplemented with 50 μg ml^{-1} of the fungicide Imazalil and incubating the plates at 28°C for 2–15 days. After surface disinfection, the leaf, stem or root tissue was cut and triturated in 10 ml of sterile PBS contained in a 50 ml flask maintained at 28°C and agitated at 150 r.p.m. for 1 h, after which appropriate dilutions were plated onto 10% TSA supplemented with 50 μg ml^{-1} of Imazalil and incubated at 28°C for 2–14 days. After incubation, colonies were picked off the plates, inoculated on 10% TSA agar slants, incubated at 28°C for 2 days and stored at 4°C. These colonies also were cultivated in 10% TSA, incubated at 28°C for 18 h and following each culture was suspended in 20% glycerol solution and stored at −70°C.

Polymerase chain reaction (PCR) amplification, restriction, sequencing and analysis of bacterial 16S rDNA

Bacterial DNA was extracted according to the method given by Araújo *et al*. (2002), amplification of 16S rDNA being performed in a 50 μl final volume containing 1 μl (0.5–10.0 ng) of total DNA, 0.2 μM of P027F primer (5′-GAGAGTTTGATC CTGGCTCAG-3′), 0.2 μM of 1378R primer (5′-CGGTGTG TACAAGGCCCGGGAACG-3′), 200 μM of each dNTP, 3.75 mM $MgCl_2$ and 0.05 U of Taq DNA polymerase (Invitrogen) in 20 mM of pH 8.4 Tris-HCl containing 50 mM KCl. A negative control (PCR mixture without DNA) was included in all PCR experiments. The reaction conditions were as follows: 94°C for 4 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 1 min and primer extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min. The reaction products were separated by running 5 μl of the PCR reaction mixture in 1.2% (w/v) agarose gel and staining the bands with ethidium bromide (Sambrook *et al*., 1989). For amplified rDNA restriction analysis (ARDRA) analysis, 1 μg of amplified 16S rDNA fragment (1350 bp) was digested with *Alu*I restriction enzyme (Invitrogen) according to manufacture recommendations and the products run in 2.5% (w/v) agarose gel and stained with ethidium bromide. For identification, the PCR products of at least 20% of isolates of each haplotype were purified using a GFX PCR DNA and gel band purification kit (Amersham Biosciences) and sequenced using the 1378R primer. Analyses of sequences were performed with the basic sequence alignment BLAST program run against the database [National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST)] and the. The determined sequence were aligned using CLUSTALW and the distance matrices and phylogenetic trees were calculated by Jukes and Cantor, 1969) and neighbour-joining (Saitou and Nei, 1987) algorithms, respectively, using PAUP software (Swofford, 2002). The nucleotide sequences obtained in this study have been submitted to the GenBank and assigned accession numbers AY487809 to AY487818 and AY489067 to AY489107.

Screening for IAA producing epiphytic and endophytic bacteria

Indoleacetic acid (IAA) production was analysed using a modification of the qualitative method developed by Bric *et al.* (1991). Sixteen strains per plate were plated onto 10% TSA

amended with 5 mM of L-tryptophan, overlaid with a nitrocellulose membrane (Amersham Pharmacia) and incubated at 28°C for 24 h. After bacterial growth had occurred the membrane was removed from the plate and treated with Salkowski reagent (2% (w/v) 0.5 M $FeCl_3$ in 35% perchloric acid) for 15 min at room temperature, bacteria producing IAA being identified by the presence of a red halo on the membrane corresponding to the position of the IAA-producing colony.

Screening for mineral phosphate solubilizing epiphytic and endophytic bacteria

Mineral phosphate solubilization activity was assayed according to Verma *et al*. (2001). Briefly, isolates were plated onto agar medium containing inorganic phosphate (constituents ($g\ l^{-1}$) agar, 15; glucose, 10; NH_4Cl, 5; NaCl, 1; $MgSO_4$•7H2O, 1; $Ca_3(HPO_4)_2$, 0.8; pH 7.2) and incubated at 28°C for up to 48 h, solubilization of mineral phosphate being characterized by a clear halo around bacterial colonies with phosphate solubilization capacity.

Screening for nitrogen fixing epiphytic and endophytic bacteria

The ability to fix nitrogen was evaluated by: (i) PCR specific for the *nif*H gene (Ueda *et al*., 1995) using the 16F (5′-GCIWTYTAYGGIAARGGIGG-3′) and 407R (5′-AAICCRC CRCAIACIACRTC-3′) primers; and (ii) the ability to grow in semisolid nitrogen-free NFb medium (Dobereiner *et al*., 1995), a halo of bacterial growth within the medium indicating nitrogen fixation.

Statistical analysis

Analysis of the data was carried out using the SAS software package [Copyright (c) 1989–96 by SAS Institute, Cary, NC USA] with a completely randomized analysis of variances ($P < 0.05$). Bacterial counts were transformed using $\log_{10}$ of X + 1 before analysis of variance. The Tukey-test was used for comparison of means and χ^2 analysis ($P = 0.05$) was performed on the screening data for IAA production and mineral phosphate solubilization (Steel and Torrie, 1980).

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