RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2014) 17:165-174
doi:10.2436/20.1501.01.219. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*

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Received 23 May 2014 · Accepted 17 September 2014

Summary. In this work we analyzed the composition and structure of cultivable bacterial communities isolated from the stem/leaf and root compartments of two medicinal plants, *Echinacea purpurea* (L.) Moench and *Echinacea angustifolia* (DC.) Hell, grown in the same soil, as well as the bacterial community from their rhizospheric soils. Molecular PCR-based techniques were applied to cultivable bacteria isolated from the three compartments of the two plants. The results showed that the two plants and their respective compartments were characterized by different communities, indicating a low degree of strain sharing and a strong selective pressure within plant tissues. *Pseudomonas* was the most highly represented genus, together with *Actinobacteria* and *Bacillus* spp. The presence of distinct bacterial communities in different plant species and among compartments of the same plant species could account for the differences in the medicinal properties of the two plants. [**Int Microbiol** 2014; 17(3):165-174]

Keywords: *Echinacea purpurea* · *Echinacea angustifolia* · rhizosphere · medicinal plants · endophytes

Introduction

Herbal medicine has become a popular approach to the prevention and treatment of several diseases. Species of the genus *Echinacea* are among the most commonly used medicinal plants. *Echinacea purpurea* (L.) Moench, and *Echinacea angustifolia* (DC.) Hell, are currently used in Europe and the USA to treat the common cold and respiratory infections [1–3, 69]. The alkylamide, alkaloid, and polyacetylene fractions are considered to have immune-modulatory and anti-inflammatory effects [13,43]. The various bioactivities have been traced to the multiple components rather than to the multiple effects of individual chemical compounds present in *Echinacea* extracts [23]. The chemical diversity of such compounds has made it difficult to determine whether *Echinacea* extracts are genuinely medicinally effective and the benefits of these products are controversial [35,39]. However, several studies indicate that *Echinacea* indeed has antiviral, antioxidant, and anti-inflammatory properties, making it a very promising medicinal botanical species [9,29,61].

The chemical composition of the compounds from medicinal plants varies widely depending on the geographic

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provenance of the plant, the botanical species, its genetics and its biotic factors, as well as the specific anatomic part of the plant (e.g., seed, flower, root, leaf), the extraction techniques, etc. [18]. In addition, it is not known whether the compounds of interest in the *Echinacea* extracts are synthesized solely by the plant or by the microbial endophytes inhabiting the plant and producing bioactive compounds [7]. Advances in studies of the curative properties of medicinal plants have sparked interest in discovering the characteristics of their bacterial communities, in order to clarify whether the curative properties of a medicinal plant are directly or indirectly related to the presence of certain endophytic bacteria [45]. Endophytes are defined as "microorganisms that live for at least part of their life inside the internal tissues of a plant without causing any disease in the host" [68]. They are largely studied for their ability to produce a wide range of natural products, with pharmaceutical, agrochemical, and biotechnological properties of interest [17,27,59,70]. Endophytes are present in most plant species [42,59], both terrestrial and aquatic [6,63,64]. Their beneficial effects on their hosts include growth promotion through interactions in nitrogen metabolism [48] or phosphorus solubilization [57] and siderophore production [16]. The growth-promoting effects of endophytes include the induction of phytohormone biosynthesis, in particular that of indole-3-acetic acid [62], and of 1-aminocyclopropane-1-carboxylate deaminase activity [22]. Endophytes control plant pathogens through the production of antimicrobial compounds [49,65] and act as elicitors of systemic resistance in plants [36].

The colonization mechanisms, ecology, function, and plant interactions of endophytes have been widely investigated [12,27,44,46]. These studies have shown that the bacterial endophytes of plants derive from different sources, such as the spermosphere, anthosphere, caulosphere, and phyllosphere [25,27,58,59]. However, most endophytic bacteria are soil-derived [12], as hypothesized by Galippe already in 1887 [20,21]. Endophytes enter plant tissues through cracks in the roots or aerial parts [37] or through rhizosphere soil colonization and become distributed within the whole plant tissues through its vascular system [5]. Thus, endophytes can be detected in roots, stems, and leaves, inside plant reproductive organs and the apoplastic space and, in some cases, also in intracellular spaces (as is the case in symbiotic rhizobial species) [12,56,58].

The root compartment of *Echinacea* was shown to contain the highest levels of bioactive molecules [67]. The aim of this study was to determine the endophytic and rhizospheric bacterial biodiversity of two medicinal plants, *E. purpurea* and *E. angustifolia*, in order to evaluate their core and accessory cultivable microbiomes in relation to the roots and aerial parts of these plants. To the best of our knowledge, this work is the first to describe the diversity of the culturable endophytic and rhizospheric bacterial community of medicinal plants.

Materials and methods

Rhizospheric soil chemical variables. The main chemical-physical variables of the soil were: pH (H_2O) 8.4; total organic carbon, (TOC) 1.6%; N (g/kg); 1.50; C/N, 10.97; $CaCO_3$ total, 18.70%; cation exchange capacity (CEC, cmol[+]/kg), 9.74.

Plant material and soil properties. Five *E. purpurea* and five *E. angustifolia* plants were cultivated in the same square basin (160 cm length × 75 cm height), located in an open field of the botanical garden "Giardino delle Erbe", Casola Valsenio, Ravenna, Italy, and collected in October 2012. The basin was filled with the same soil as that sampled. The soil was air-dried at room temperature (21ºC), sieved through a 2-mm mesh, and then analyzed for pH, cation-exchange capacity, total $CaCO_3$, TOC, and total N.

Isolation of bacterial strains and preparation of cell lysates for DNA amplification. Collected plants were immediately taken to the laboratory. The anatomical part of the plants, i.e., the roots and stems/leaves, were separated and considered as independent samples throughout the experiment. Roots from the five individual plants of each *Echinacea* species were grouped and pooled, as were the stems/leaves. Two grams of fresh tissue from each pool was surface-sterilized with 1% HClO solution in sterile 50-ml Falcon tubes at room temperature and then washed three times with sterile water to remove the epiphytic bacteria. Aliquots (100 µl) of the last wash were plated in triplicate as sterility controls, which by the end of the experiment had not become contaminated (data not shown). Subsequently, the samples were homogeneously pottered in a sterile mortar with the addition of 2 ml of 0.9% NaCl (Sigma Aldrich, USA). One hundred µl samples of tissue extracts and their different dilutions were plated in triplicate.

Rhizospheric soil (RS) from five plants of each plant species was also analyzed and treated separately at room temperature for 1 h with 20 ml of 10 mM Mg_2SO_4 in 50-ml sterile Falcon tubes, which allowed detachment of the bacteria from soil particles. After sedimentation, 100-µl samples of the supernatant and different dilutions thereof were plated in triplicate.

Endophytic and rhizospheric bacteria were grown on solid tryptone soya broth (TSB) medium (Biorad, CA, USA) at 30°C for 48 h. The total number of aerobic heterotrophic fast-growing bacteria was expressed as colony-forming units (CFU), which were determined for each sample in triplicate based on an average value of the bacterial titer. From each sample, about 100 colonies were randomly selected and individually plated onto solid TSB Petri dishes.

A collection of 514 bacterial isolates was prepared for both *Echinacea* sp. plant species by dissolving the freshly isolated and plated colonies in 2-ml deep-well plates with 500 µl of TSB and 500 µl of 50% glycerol (25% final concentration). The plates were stored at –80°C.

Random amplified polymorphic DNA (RAPD) analysis. Cell lysates of endophytic and rhizospheric bacterial isolates were prepared by processing 100 µl of each glycerol suspension with thermal lysis (95°C for 10 min), followed by cooling on ice for 5 min.

Random amplification of DNA fragments [65] was carried out in a 25-µl total volume composed of 1× reaction buffer, 300 µM $MgCl_2$, deoxynucleoside triphosphate (200 µM each), 0.5 U of PolyTaq DNA polymerase (all re-

agents were from Polymed, Florence, Italy), 500 ng of primer 1253 [5′-GTT TCCGCCC-3′] [46], and 2 µl of cell lysate prepared as described above. The reaction mixtures were incubated in a PTC-100 Peltier thermal cycler (MJ Research, Quebec, Canada) at 90°C for 1 min, and 95°C for 90 s followed by 45 cycles at 95°C for 30 s, 36°C for 1 min, and 75°C for 2 min. Finally, the reaction mixtures were incubated at 75°C for 10 min, 60°C for 10 min, and 5°C for 10 min. Reaction products were analyzed by agarose (2% w/v) gel electrophoresis in Tris-acetate EDTA buffer (TAE) containing 0.5 µg ethidium bromide/ml.

Analysis of RAPD profiles. The genetic similarities in the different samples belonging to the same haplotype group were determined based on the fingerprinting pattern of each RAPD product and pairwise comparisons of the presence/absence of bands using the GelCompar II software (Applied Maths). For each recognized RAPD haplotype, a 16S rRNA gene sequence was obtained via PCR amplification (as described below) for taxonomic attribution of the bacterial isolates. For haplotypes represented by more than one strain, a single bacterial strain was randomly chosen for gene amplification.

PCR amplification and sequencing of 16S rRNA coding genes. PCR amplification of 16S rRNA genes was carried out in 20-µl reactions containing 1× reaction buffer, 150 µM $MgCl_2$, deoxynucleoside triphosphate (250 µM each), 2 U of PolyTaq DNA polymerase (all reagents were from Polymedaly), 0.6 µM of each primer [P0 5′-GAGAGTTTGATCCTGGCTCAG and P6 5′-CTACGGCTACCTTGTTACGA] [22], and 2 µl of each cell lysate. The samples were incubated in a PTC-100 Peltier thermal cycler (MJ Research) under the following conditions: primary 90-s denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. Amplicons were then excised from 0.6% agarose gels and purified using the MinElute gel extraction kit (Qiagen) according to the manufacturer's instructions. Direct sequencing of the amplified 16S rRNA genes was performed with primer P0 [5′-GAGAGTTTGATCCTGGCTCAG] using an ABI PRISM 310 genetic analyzer (Applied Biosystems) and the chemical dye terminator [59]. Each 16S rRNA gene sequence was submitted to GenBank and assigned an accession number from AJ642225 to AJ642604.

Analysis of 16S rRNA gene sequences. The 16S rRNA gene sequences were analyzed using the "classifier" tool of the Ribosomal Database Project –RDP [10] for taxonomic assignment. All sequences showed a degree of similarity ≥80% with the sequences available in the RDP database (at genus level) and were therefore considered of good quality. Diversity indices were calculated for both plant species and for each compartment using data on the genus-level taxonomic attribution of the RDP database. Abundance data were first normalized by calculating the percentage of each bacterial genus present in the three compartments of the two plant species. Diversity indices were calculated using PAST software version 3.0 [25]. The number of taxa for each plant compartment (S) was calculated together with the Simpson's diversity index (D), Shannon's diversity index (H), and evenness (e^H/S).

Results

Bacterial counts. Bacteria were extracted from the rhizospheric soil (RS), roots (R), and stems/leaves (S/L), diluted in saline (0.9% NaCl), and plated as described in Materials and methods. The lowest CFU/g values were detected in the stem/leaves compartments of both plant species ($4.80 \times 10^3 \pm 2.5 \times 10^3$ CFU/g in *E. purpurea* and $3.43 \times 10^3 \pm 2.1 \times 10^3$ CFU/g in *E. angustifolia*); the highest bacterial titers were in the root compartment of *E. angustifolia* ($1.34 \times 10^6 \pm 9.7 \times 10^5$ CFU/g) and the RS compartment of *E. purpurea* ($9.6 \times 10^5 \pm 6.4 \times 10^5$). Overall, the numbers of CFU/g isolated from the same compartment of the different plants were very similar.

RAPD fingerprinting. To determine the degree of genetic variability at the strain level and to analyze the structure of the isolated bacterial communities, 262 isolates were collected from *E. angustifolia* (83 from S/L, 88 from R, and 91 from RS) and 252 from *E. purpurea* (81, 89, and 82, respectively). Each of the 514 bacterial isolates was subjected to RAPD fingerprinting as follows: DNA from the lysed cell suspensions was amplified with the 10-mer oligonucleotide 1253 as described in Materials and methods. RAPD amplicons were then analyzed by agarose gel electrophoresis. Each RAPD profile (hereinafter referred to as haplotype) was then compared with all of the others as described in Materials and methods. Thus, from the 514 bacterial isolates 380 different RAPD haplotypes were identified, corresponding to at least 380 bacterial strains. Specifically, the 252 and 262 bacterial isolates from *E. purpurea* and *E. angustifolia* yielded 201 and 203 haplotypes, respectively. Among the 514 bacterial strains, 316 (61.5%) had a unique haplotype. Thirty-three haplotypes were composed of two strains each (8.7% of all haplotypes); 16 haplotypes (4.2%) were composed of 3 strains; 12 haplotypes (3.1%) were composed of 4–6 strains. Haplotype 1 comprised 7 different bacterial isolates; haplotype 66, 8 different bacterial isolates; and haplotype 2, 12 different bacterial isolates from both *Echinacea* species.

In *E. purpurea*, the RS compartment had the highest number of RAPD haplotypes (80 haplotypes out of 82 bacterial isolates), whereas the root compartment harbored 59 total different haplotypes, and the S/L compartment, 70 haplotypes. Similarly, the highest number of different haplotypes (84) isolated from *E. angustifolia* was detected in the RS compartment, whereas the S/L and R compartments yielded 62 and 67 different RAPD haplotypes, respectively.

The distribution of the RAPD haplotypes within the different compartments of the same plant species is shown in Fig. 1. Notably, no strain was shared among the three compartments in either *E. purpurea* or *E. angustifolia*, and a very low number of strains was shared between two different compartments. In *E. purpurea*, three haplotypes were shared between R and RS, four between S/L and RS, and two between S/L and R. In *E. angustifolia* eight, two and zero haplotypes

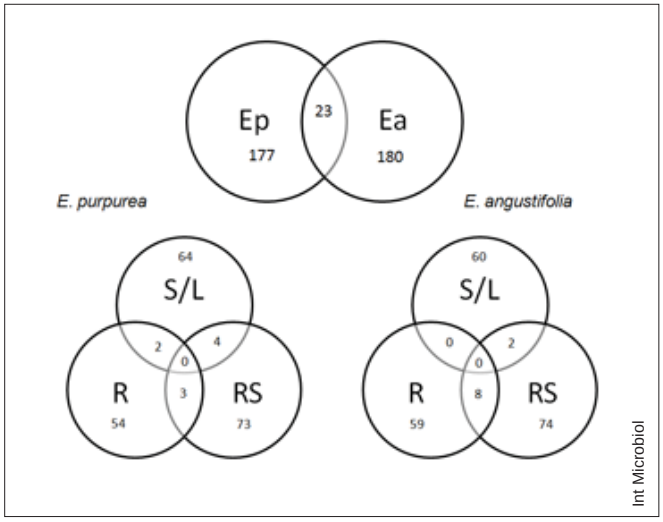

Fig. 1. Schematic representation of the core, accessory, and unique RAPD haplotypes detected in *Echinacea purpurea* and *E. angustifolia* rhizospheric soil (RS), roots (R) and stem/leaves (S/L).

were shared between R and RS, S/L and RS, and S/L and R, respectively. An analysis of the distribution of RAPD in the two *Echinacea* plant species showed that they shared only 23 haplotypes (about 6%; data not shown). The number of haplotypes common to the two plant species and the different compartments ranged from one (a single haplotype shared by *E. purpurea* RS and *E. angustifolia* RS, and by *E. purpurea* R and *E. angustifolia* RS) to six (shared by *E. purpurea* RS and *E. angustifolia* R compartments).

Analysis of 16S rRNA gene sequences. By assuming that bacterial isolates sharing the same RAPD profile corresponded to the same (or a closely related) strain, the composition of the six bacterial communities was assessed by analyzing the 16S rRNA gene sequence from a representative of each RAPD group. Thus, 16S rRNA genes were amplified from the 380 strains. An amplicon of the expected size was obtained from each one (not shown). The nucleotide sequences of these 380 amplicons were determined and compared with those available in databases. The entire dataset is summarized in Fig. 2. The analysis revealed that: (i) the 380 strains were affiliated with 29 different bacterial genera; (ii) the majority (47.4% of RAPD haplotypes) of the 16S rRNA gene sequences were affiliated with the genus *Pseudomonas*; (iii) *Staphylococcus* was the second most highly represented genus (9.5% of RAPD haplotypes); (iv) *Microbacterium* spp. and *Curtobacterium* spp. accounted for 6.1% and 6.3%, respectively, and *Arthrobacter* sp. for 4.2% of the strains; (v) 11 bacterial genera accounted for 1.3–2.6% of the strains; and (vi) the remaining 13 genera represented <1%.

Comparison of the bacterial cultivable communities inhabiting *Echinacea purpurea* and *E. angustifolia*. The bacterial communities inhabiting the two plant species were then compared to determine the degree of sharing at the genus level (Fig. 3). The analysis revealed that the two plants shared 14 genera (including all the most represented ones). Again, *Pseudomonas* was the most highly represented genus in both plant species, with no considerable

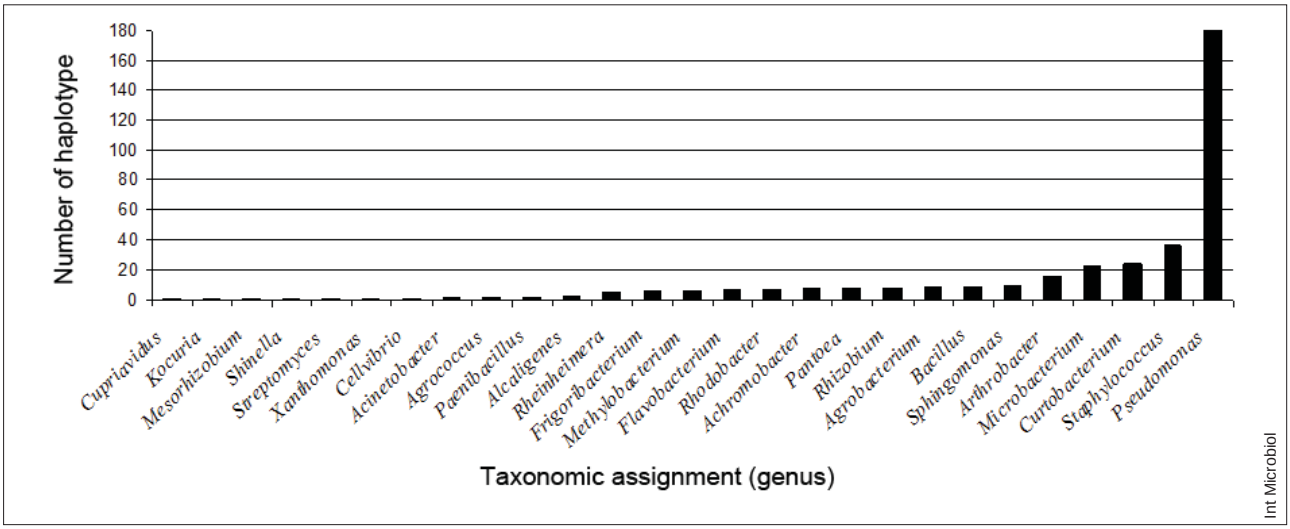

Fig. 2. Abundance of the different bacterial genera in all haplotype detected with RAPD analysis of both *Echinacea purpurea* and *E. angustifolia*.

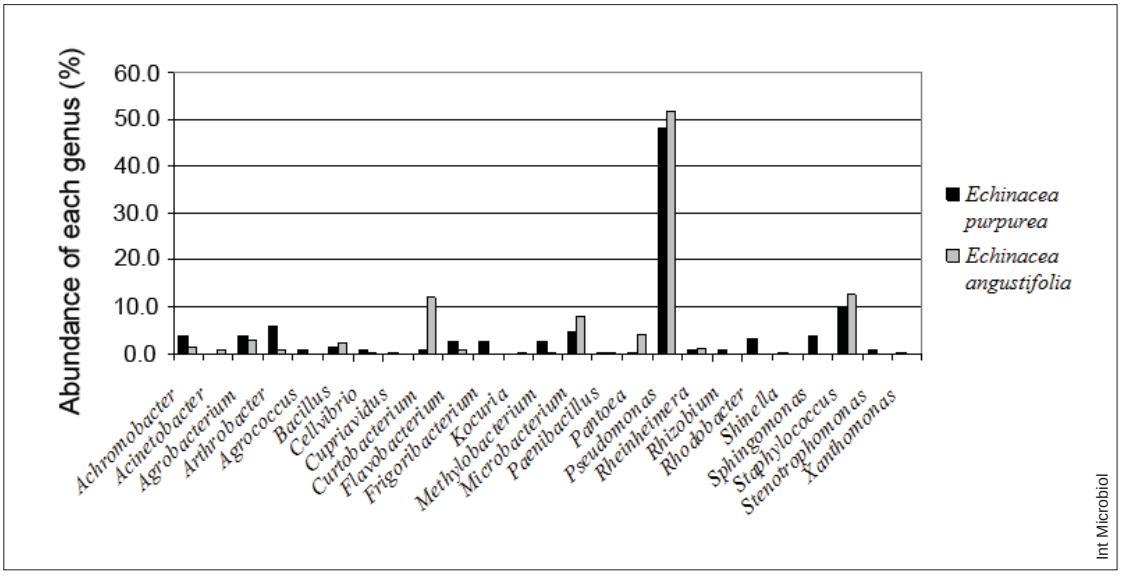

Fig. 3. Distribution of the different bacterial genera in the two medicinal plants analyzed in this study.

differences in their abundances (51.5% in *E. angustifolia* and 48% in *E. purpurea*). There was a large difference in abundances of *Curtobacterium* spp.: 0.8% in *E. purpurea* and 11.8% in *E. angustifolia*. Similarly, *Pantoea* spp. accounted for 0.4% of the genera in *E. purpurea* and 4.2% of those in *E. angustifolia*. By contrast, *Arthrobacter* spp. was much more abundant in *E. purpurea* than in *E. angustifolia* (6% vs. 0.8%), as were *Flavobacterium* spp. (2.8% vs. 0.8%) and *Methylobacterium* spp. (2.8% vs. 0.4%). *E. purpurea* hosted a larger number of bacterial genera than *E. angustifolia* (23 vs. 16), such that some bacterial genera were found only in *E. purpurea*, albeit at low percentages: *Agrococcus* spp., *Rhizobium* spp. and *Stenotrophomonas* spp. (0.8%), *Cupriavidus* spp., *Shinella* spp., and *Xanthomonas* spp. (0.4%), *Frigoribacterium* spp. (2.8%), *Rhodobacter* spp. (3.2%), and *Sphingomonas* spp. (4.0%). *Kocuria* spp. (0.4%) and *Acinetobacter* spp. (0.8%) were found only in *E. angustifolia*.

Comparison of the bacterial communities inhabiting different plant compartments. Figure 4 shows the composition of the bacterial communities isolated from the different compartments of the two *Echinacea* plant species. The diversity indices are shown in Table 1.

The composition of the cultivable bacterial community inhabiting the S/L compartment of both plants was highly different from that in the other two compartments (RS and R), which were much more similar to each other. The genus *Pseudomonas* was less represented in the S/L compartment than in the R and RS compartments, whereas a high percentage of bacteria associated with the genus *Staphylococcus* were detected in the S/L compartment of both plant species (25.9% in *E. purpurea* and 38.6% in *E. angustifolia*), thus underlining the differences in bacterial composition of this compartment compared with the other two. Differences were also evident concerning the genera *Bacillus* and *Curtobacterium*, which

Table 1. Diversity indices calculated from the percentages of the presence of the different bacterial genera in each plant compartment for both plant species

	Ep S/L	Ep R	Ep RS	Ea S/L	Ea R	Ea RS
Taxa_S	12	6	16	8	7	9
Simpson_D	0.8584	0.4574	0.7	0.6972	0.473	0.4448
Shannon_H	2.174	0.9625	1.785	1.405	1	1.063
Evenness_e^H/S	0.7329	0.4364	0.3724	0.5096	0.3885	0.3216

Ep: *Echinacea purpurea*; Ea: *Echinacea angustifolia*; S/L: stem/leaf compartment; R: root compartment; RS: rhizospheric soil compartment.

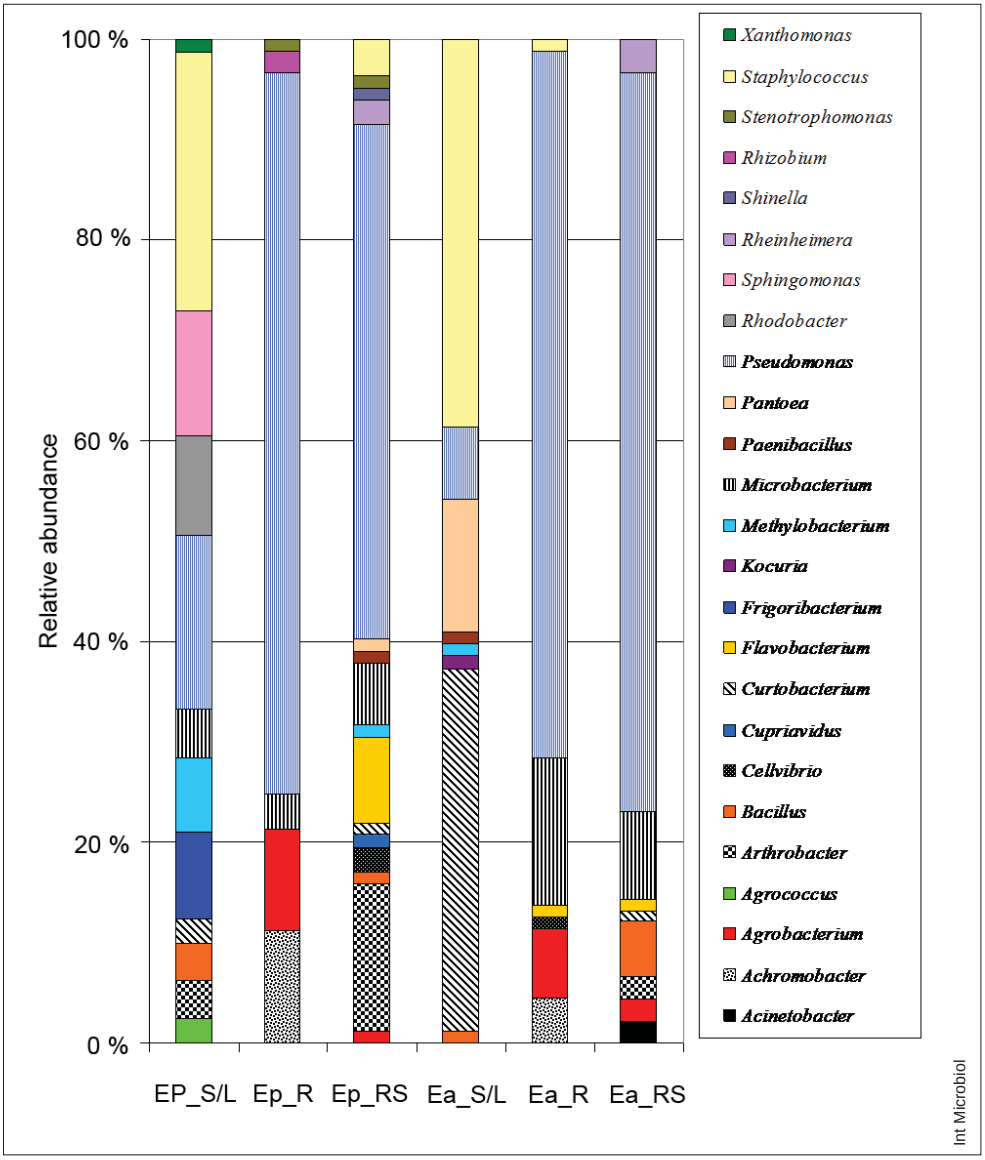

Fig. 4. Relative abundances (expressed as percentages) of cultivable bacterial taxa isolated from rhizospheric soil (RS), roots (R), and stem/leaves (S/L) of *Echinacea purpurea* and *E. angustifolia*.

were not detected in the root compartment of either of the two plant species; *Agrobacterium*, which was not identified in the S/L compartment of the two *Echinacea* species; *Rheinheimera*, which was present only in the rhizosphere of both plants; and *Achromobacter*, which was present only in the roots of the two plants.

The differences among the culturable bacterial communities were also highlighted by the diversity indices (Table 1). Overall, *E. purpurea* compartments were characterized by a higher number of bacterial taxa (genera) than *E. angustifolia*. The highest number of taxa were found in the rhizosphere of *E. purpurea* (16), followed by the stems/leaves of this species. The lowest number of taxa occupied the roots of both plant species, with just seven different bacterial genera in *E. angustifolia* and six in *E. purpurea*.

In *E. purpurea*, the Simpson diversity index was the highest (0.85) in the S/L compartment, and the lowest in the R compartment (0.46). Analogously, in *E. angustifolia*, the Simpson index was the highest (0.69) in the S/L, and the lowest (0.47) in the R compartment. The same was true for the Shannon diversity and evenness indices in both plant species.

Considering the same compartment in the two species, the Simpson index values in the root compartments of *E. purpurea* and *E. angustifolia* were similar (0.46 and 0.47, respectively), as

was the Shannon index (0.96 and 1, respectively). In the RS compartment of *E. purpurea*, Simpson's and Shannon's diversity indices were higher than in the RS compartment of *E. angustifolia*, but the evenness values were very similar (0.37 and 0.32, respectively).

Discussion

To the best of our knowledge, this is the first report in which the endophytic and rhizospheric cultivable bacterial communities were enumerated and described in the medicinal plant *Echinacea* spp. in an open field trial. In the only other available report in which *Echinacea* sp. endophytes were characterized, the plants were propagated in vitro for 9 months [41]. In a recent study, Pugh et al. [51] enumerated the total bacterial endophytes in *E. purpurea* roots and aerial samples in a molecular approach that used a PCR-based quantification method. Their study was aimed at determining whether differences in bacterial load correlated with the in vitro macrophage activity of the plant material.

In this work, bacterial endophytes were isolated from the S/L and R compartments of *E. purpurea* and *E. angustifolia*, as well as from their RS. We are aware that the approach used in this work has several limitations related to the experimental conditions, which selected for bacteria able to grow on TSB/TSA within 48 h of incubation at 30°C; however, this bias was negligible considering the aim of this preliminary study exploring the differences within and between the culturable microbial communities inhabiting two plants and their compartments. The effect of different growth conditions should be evaluated in additional experiments aimed at obtaining slow-growing bacteria and/or bacteria able to grow in different media or at different temperatures.

The yield of culturable heterotrophic fast-growing bacteria in both plant species ranged from 10^3 CFU/g in the S/L compartments to 10^5–10^6 CFU/g in the R and RS compartments; these values are in agreement with those in previously published studies showing greater abundances of plant-associated bacterial populations in R than in S/L [37,40]. The very low CFU/g values detected in the S/L compartment in both plant species is also in agreement with the findings of previous studies [51]. The RAPD data suggested a non-clonal structure and a very high degree of genetic variability at the strain level of the endophytic and rhizospheric bacterial communities from both *Echinacea* spp. There was also a low level of strain sharing in the different compartments of the same plant and between the same compartment of the two different plants. For example, just five strains were shared by the S/L compartments of the two plants and only four haplotypes were shared between their R compartments.

This finding raises the intriguing question of the nature of the (internal?) plant forces driving the selection of different bacterial endophytic strains in the diverse compartments of the same plant, both for *E. purpurea* and for *E. angustifolia*. One explanation may be related to the finding that plants have an innate immune system, with receptors that detect the presence of molecules both inside and on the surface of host cells [34]. Indeed, the selection of bacteria at the rhizospheric level (the rhizosphere effect) is well documented and was shown to be related to plant exudates released in the soil surrounding the roots, which results in the selection of a bacterial biota that is different from the one recovered in bulk soil (for a review see [8]).

Most of the bacterial genera identified through 16S rRNA gene amplification in *Echinacea* sp. have been detected in other plant species. Ikeda et al. [33] characterized 217 endophytic isolates from the roots of maize; the most highly represented bacterial genera were *Pantoea* and *Bacillus*. In the study of Gagne-Bourgue et al. [19], the endophytic components of the aerial parts of the switchgrass (*Panicum virgatum* L.) were analyzed; the most highly represented bacterial genera were *Microbacterium*, *Curtobacterium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Sphingomonas*, and *Serratia*. As seen in the diversity indices shown in Table 1, the higher numbers of bacterial genera in the S/L and RS compartments of *E. purpurea* than *E. angustifolia* suggests a higher microbial diversity level in the former. By contrast, there were no significant differences in the root compartments.

As expected, some genera present in the RS compartment were also detected within plant tissues, since most bacterial endophytes are presumably derived from soil. Note that the genera *Arthrobacter*, *Staphylococcus*, and *Methylobacterium* were detected in the RS and in the S/L compartments of *E. purpurea* but not in its R compartments, whereas the genus *Stenotrophomonas* was detected in the RS and R compartments. Analogously, in the two plant species the genera *Curtobacterium* and *Bacillus* were detected in the RS and S/L compartments, and the genus *Agrobacterium* in the RS and R compartments. The genus *Microbacterium* was present in all *E. purpurea* compartments and in the RS and R compartments of *E. angustifolia*. The distributions of these genera suggest that different endophytic bacterial species are selected not only at the RS level but also within plant tissues, thus indicating selection at the rhizospheric level and, once the bacteria reach plant tissues from the RS, their stabilization as endophytes.

In the two plant species, the highest values of D, H, and evenness were those of the S/L compartments. This finding, together with the observation that these same compartments had the lowest bacterial titers, led us to hypothesize that, although there were fewer endophytes in the S/L compartments than in the other two plant compartments, they were much more diversified and each taxon (in this case, genus) was represented more or less by an equal number of individuals, as indicated by the evenness values. By contrast, in the roots of both plants, the low evenness values together with the bacterial counts revealed higher bacterial abundances in the roots ($4.6 \times 10^5 \pm 2.8 \times 10^4$ in *E. purpurea* and $1.3 \times 10^6 \pm 9.6 \times 10^5$ in *E. angustifolia*) than in the stem/leaves ($4.5 \times 10^3 \pm 2.5 \times 10^3$ in *E. purpurea* and $3.4 \times 10^3 \pm 2.1 \times 10^3$ in *E. angustifolia*), consistent with the presence of only a few dominant bacterial taxa.

These differences in bacterial numbers and distribution between roots and stem/leaves of *Echinacea* spp. could be related to the different environmental and nutritional conditions to which the roots and the aerial parts are exposed and/or to anatomical and phytochemical features that, in turn, create specific ecological niches for endophytes. The aerial parts of plants are exposed to fluctuations in temperature, humidity, and UV irradiation and to a different trophism/physiology than roots [29]. Endophytic bacteria are likely selected on the basis of their adaptation strategies and tolerance of the different conditions in the various plant compartments, as reported for several plant species (e.g., [4,13,32,50]). Our analysis of the bacterial genera in the stems/leaves of *Echinacea* sp., showed *Sphingomonas* sp. in *E. purpurea* (12.3%) and *Methylobacterium* in *E. purpurea* and *E. angustifolia* (7.4% and 1.2%, respectively). Delmotte et al. [15] identified microbial proteins that appear to reflect differential adaptation strategies to the leaf environment of two abundant colonizers belonging to these two genera. This finding is in agreement with the data obtained in our study and could explain the presence of *Sphingomonas* and *Methylobacterium* in *Echinacea* sp., as endophytes adapted to this environment.

Among the compartements of *Echinacea,* the root compartment is richest in bioactive molecules responsible for the medicinal properties of these plants [67]. The potential of endophytes from medicinal plants to produce anticancer, antibacterial, and antifungal compounds was recently demonstrated [45]. Similarly, a few dominant bacterial genera inhabiting *Echinacea* sp. roots might be candidates for the synthesis of bioactive compounds and/or used to drive plant metabolism to synthesize these compounds. The dataset obtained with RAPD fingerprinting highlights a very low degree of sharing between the two plant species and, especially, between the two rhizospheric soils. Although, as discussed above, different plants select for different rhizospheric microbial communities [38], this result was unexpected given that the two plant species were grown in the same soil, that is, having the same chemical-physical characteristics, and within a few centimeters of each other. This suggests a high specific interaction between the plant roots and the microbial communities residing in soil.

Many rhizospheric bacteria switch from root-surface to endophytic lifestyles [12,58], including species of *Bacillus* and *Pseudomonas*. The structures and functions of their lipopeptides of these two genera were recently reviewed [54,55]. Lipopeptides are used by rhizosphere bacteria in antibiotic production and the induction of plant defense mechanisms. Thus, the dominance of *Pseudomonas* in the rhizospheres and roots of the two medicinal plants considered in this study could be related to the production by the plants of metabolites having medicinal properties, either directly, as already described for other species [10], or indirectly, through potential plant-growth-promoting properties [30].

In the present study, bacteria belonging to the phylum *Actinobacteria* (genera *Microbacterium*, *Frigoribacterium*, *Curtobacterium*, *Arthrobacter*, *Agrococcus* and *Kocuria*) accounted for 18.4% of the total endophytes in *E. purpurea* and 24.1% of those in *E. angustifolia*, which in both cases is a significant portion of the whole bacterial component. Endophytes belonging to *Actinobacteria* have been widely studied for their production of secondary metabolites [51,52], which were shown to include those with diverse biological activities, such as antibiotics, antitumor and anti-infection compounds, plant growth promoters, and enzymes [28,52]. This suggests that *Actinobacteria* together with *Pseudomonas* and *Bacillus* would be in large part responsible for the production of the compounds that account for the characteristics of the medicinal plant *Echinacea* spp. However, additional work should be carried out in order to specifically confirm such a hypothesis.

In summary, in this work, we analyzed the structure and composition of cultivable bacterial communities isolated from the stem/leaves, roots, and rhizospheric soils of two species of medicinal plants, *E. purpurea* and *E. angustifolia*, using a combination of PCR-based techniques. The results revealed differences in the microbial communities inhabiting the two plants, despite their growth in the same soil. In addition, different bacterial communities inhabited the different plant compartments. These findings together with the very low degree of strain sharing, raise intriguing question regarding the existence of a (strong) selective pressure that deter-

mines bacterial composition at the strain level, rather than at a higher taxonomic rank (genus). Although yet to be confirmed experimentally, bacterial endophytes would seem to elicit the synthesis of some if not all of the bioactive molecules produced by the plant, perhaps synthesizing some of these compounds themselves.

Acknowledgements. This work was supported financially by Ente Cassa di Risparmio di Firenze (Project 2013.0657). We are also very grateful to three anonymous referees for their helpful comments that improved the manuscript.

Competing interests. None declared.

References

1. Barnes J, Anderson LA, Gibbons S, Phillipson JD (2005) *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol 57:929-954
2. Barrett B (2003) Medicinal properties of *Echinacea*: a critical review. Phytomedicine 10:66-86
3. Barrett B, Brown R, Rakel D, Mundt M, Bone K, Barlow S, Ewers T (2010) *Echinacea* for treating the common cold: a randomized trial. Ann Intern Med 153:769-777
4. Barzanti R, Ozino F, Bazzicalupo M, Gabbrielli R, Galardi F, Gonnelli C, Mengoni A (2007) Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant *Alyssum bertolonii*. Microb Ecol 53:306-316
5. Bell CR, Dickie GA, Harvey WL, Chan JW (1995) Endophytic bacteria in grapevine. Can J Microbiol 41:46-53
6. Bhore SJ, Sathisha G (2010) Screening of endophytic colonizing bacteria for cytokinin-like compounds: Crude cell-free broth of endophytic colonizing bacteria is unsuitable in cucumber cotyledon bioassay. World J Agric Sci 6:345-352
7. Brader G, Compant S, Mitter B, Trognitz F, Sessitsch A (2014) Metabolic potential of endophytic bacteria. Curr Opin Biotechnol 27:30-37
8. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807-838
9. Chen Y, Fu T, Tao T, Yang J, Chang Y, Wang M, Kim L, Qu L, Cassady J, Scalzo R, Wang X (2005) Macrophage activating effects of new alkamides from the roots of *Echinacea* species. J Nat Prod 68:773-776
10. Christina A, Christapher V, Bhore SJ (2013) Endophytic bacteria as a source of novel antibiotics: An overview. Pharmacogn Review 7:11-16
11. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucl Acids Res 41(Database issue):D633-D642
12. Compant S, Clément C, Sessitsch A (2010) Plant growth promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42:669-678
13. Compant S, Mitter B, Colli-Mull JG, Gangl H, Sessitsch A (2011) Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microbial Ecol 62:188-197
14. Dalby-Brown L, Barsett H, Landbo A, Meyer A, Molgaard P (2005) Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on in vitro oxidation of human low-density lipoproteins. J Agric Food Chem 53:9413-9423
15. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von Mering C, Vorholt JA (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci USA 106:16428-16433
16. Duijff BJ, Recorbet G, Bakker PAHM, Loper JE, Lemanceau P (1999) Microbial antagonism at the root level is involved in the suppression of fusarium wilt by the combination of nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358. Phytopathology 89:1073-1079
17. Findlay JA, Bethelezi S, Li G, Sevek M (1997) Insect toxins from an endophytic fungus from wintergreen. J Nat Prod 60:1214-1215
18. Firenzuoli F, Gori L (2007) Herbal medicine today: clinical and research issues. Evid Based Complement Alternat Med 4:37-40
19. Gagne-Bourgue F, Aliferis KA, Seguin P, Rani M, Samson R, Jabaji S (2013) Isolation and characterization of indigenous endophytic bacteria associated with leaves of switchgrass (*Panicum virgatum* L.) cultivars. J Appl Microbiol 114:836-853
20. Galippe V (1887) Note sur la présence de micro-organismes dans les tissus végétaux. C R Hebd Sci Mem Soc Biol 39:410-416
21. Galippe V (1887) Note sur la présence de micro-organismes dans les tissus végétaux (2nd note). C R Hebd Sci Mem Soc Biol 39:557-560
22. Glick BR, Todorovic B, Czarny J, Cheng ZY, Duan J, McConkey B (2007) Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26:227-242
23. Grifoni A, Bazzicalupo M, Di Serio C, Fancelli S, Fani R (1995) Identification of *Azospirillum* strains by restriction fragment length polymorphism of the 16S rDNA and of the histidine operon. FEMS Microbiol Lett 127:85-91
24. Groom SN, Johns T, Oldfield PR (2007) The potency of immunomodulatory herbs may be primarily dependent upon macrophage activation. J Med Food 10:73-79
25. Hallmann J, Berg B (2007) Spectrum and population dynamics of bacterial root endophytes. In: Schulz BJE, Boyle CJC, Sieber TN (eds) Microbial root endophytes. Springer, Berlin, pp 15-31
26. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron 4:9
27. Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463-471
28. Hasegawa S, Meguro A, Shimizu M, Nishimura T, Kunoh H (2006) Endophytic actinomycetes and their interactions with host plants. Actinomycetologica 20:72-81
29. Hirano SS, Upper CD (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. Microbiol Mol Biol Rev 64:624-653
30. Hol WG, Bezemer TM, Biere A (2013) Getting the ecology into interactions between plants and the plant growth-promoting bacterium *Pseudomonas fluorescens*. Front Plant Sci 4:81
31. Hudson JB (2012) Applications of the phytomedicine *Echinacea purpurea* (purple coneflower) in infectious diseases. J Biomed Biotechnol:769896
32. Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A (2004) Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. Appl Environ Microbiol 70:2667-2677
33. Ikeda AC, Bassani LL, Adamoski D, Stringari D, Cordeiro VK, Glienke C, Steffens MB, Hungria M, Galli-Terasawa LV (2013) Morphological and genetic characterization of endophytic bacteria isolated from roots of different maize genotypes. Microb Ecol 65:154-160

34. Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323-329
35. Karsch-Völk M, Barrett B, Kiefer D, Bauer R, Ardjomand-Woelkart K, Linde K (2014) Echinacea for preventing and treating the common cold. Cochrane Db Syst Rev 20;2:CD000530. doi:10.1002/14651858.CD 000530.pub3
36. Kloepper J W, Ryu C M (2006) Bacterial endophytes as elicitors of induced systemic resistance. In: Schulz BJE, Boyle CJC, Sieber TN (eds) Microbial root endophytes. Springer, Berlin, pp 33-52
37. Kobayashi DY, Palumbo JD (2000) Bacterial endophytes and their effects on plants and uses in agriculture. In: Bacon CW, White JF (eds) Microbial endophytes. Marcel Dekker, New York, pp 199-233
38. Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PGL, van Veen JA (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Van Leeuwenhoek 81:509-520
39. LaLone CA, Hammer KD, Wu L, Bae J, Leyva N, Liu Y, Solco AK, Kraus GA, Murphy PA, Wurtele ES, Kim OK, Seo KI, Widrlechner MP, Birt DF (2007) *Echinacea* species and alkamides inhibit prostaglandin E2 production in RAW264.7 mouse macrophage cells. J Agric Food Chem 55:7314-7322
40. Lamb TG, Tonkyn DW, Kluepfel DA (1996) Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. Can J Microbiol 42:1112-1120
41. Lata H, Li XC, Silva B, Moraes RM, Halda-Alija L (2006) Identification of IAA producing endophytic bacteria fro34m micropropagated *Echinacea* plants using 16S rRNA sequencing. Plant Cell Tissue Organ Cult 85: 353-359
42. Malfanova N, Lugtenberg BJJ. Berg, G. (2013) Bacterial Endophytes: Who and Where, and What are they doing there? In: de Bruijn J (ed) Molecular microbial ecology of the rhizosphere. John Wiley, Hoboken, NJ, USA, (vol 1) pp 393-403. doi:10.1002/9781118297674.ch36
43. Melchart D, Clemm C, Weber B, Draczynski T, Worku F, Linde K, Weidenhammer W, Wagner H, Saller R (2002) Polysaccharides isolated from *Echinacea purpurea* herba cell cultures to counteract undesired effects of chemotherapy--a pilot study. Phytother Res 16:138-142
44. Mengoni A, Mocali S, Surico G, Tegli S, Fani R (2003) Fluctuation of endophytic bacteria and phytoplasmosis in elm trees. Microbiol Res 158:363-369
45. Miller KI, Qing C, Sze DM, Roufogalis BD, Neilan BA (2012) Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microb Ecol 64:431-449
46. Mocali S, Bertelli E, Di Cello F, Mengoni A, Sfalanga A, Tegli S, Viliani F, Surico G, Caciotti A, Fani R (2003) Fluctuations of endophytic bacterial communities isolated from tissues of elm plants. Res Microbiol 154:105-114
47. Mori E, Liò P, Daly S, Damiani G, Perito B, Fani R (1999) Molecular nature of RAPD markers amplified from *Haemophilus influenzae* Rd genome. Res Microbiol 150:83-93
48. Molina-Favero C, Creus CM, Simontacchi M, Puntarulo S, Lamattina L (2008) Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato. Mol Plant Microbe Interact 21:1001-1009
49. Pérez-García A, Romero D, de Vicente A (2011) Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Curr Opin Biotechnol 22:187-193
50. Pini F, Frascella A, Santopolo L, Bazzicalupo M, Biondi EG, Scotti C, Mengoni A (2012) Exploring the plant-associated bacterial communities in *Medicago sativa* L. BMC Microbiol 12:78. doi:10.1186/1471-2180-12-78
51. Pugh ND, Jackson CR, Pasco DS (2013) Total bacterial load within *Echinacea purpurea*, determined using a new PCR-based quantification method, is correlated with LPS levels and in vitro macrophage activity. Planta Med 79:9-14
52. Qin S, Xing K, Jiang J-H, Xu L-H, Li WJ (2011) Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. Appl Microbiol Biotechnol 89:457-473
53. Qin S, Chen HH, Zhao GZ, Li J, Zhu WY, Xu LH, Jiang JH, Li WJ (2012) Abundant and diverse endophytic actinobacteria associated with medicinal plant *Maytenus austroyunnanensis* in Xishuangbanna tropical rainforest revealed by culture-dependent and culture-independent methods. Environ Microbiol Rep 4:522-531
54. Raaijmakers JM, Mazzola M (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annu Rev Phytopathol 50:403-424
55. Raaijmakers JM, De Bruijn I, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev 34:1037-1062
56. Reinhold-Hurek B, Hurek T (2011) Living inside plants: bacterial endophytes. Curr Opin Plant Biol 14:435-443
57. Rodriguez H, Fraga R, Gonzalez T, BashanY (2006) Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant Soil 287:15-21
58. Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interaction with hosts. Mol Plant Microbe Interact 19:827-837
59. Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278:1-9
60. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467
61. Schoop R, Klein P, Suter A, Johnston S (2006) *Echinacea* in the prevention of induced rhinovirus colds: a meta-analysis. Clin Ther 28:174-183
62. Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial andmicroorganism-plant signaling. FEMS Microbiol Rev 31:425-448
63. Sturz AV, Christie BR (1996) Endophytic bacteria of red clover as agents of allelopathic clover-maize syndromes. Soil Biol Biochem 28:583-588
64. Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: Potential role in developing sustainable systems of crop production. CRC Crit Rev Plant Sci 19:1-30
65. Weller DM (2007) Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97:250-256
66. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531-6535
67. Wills RBH, Stuart DL (1999) Alkylamide and cichoric acid levels in *Echinacea purpurea* grown in Australia. Food Chem 67:385-388
68. Wilson D (1995) Endophyte: The evolution of a term, and clarification of its use and definition. Oikos 73:274-276
69. Wu CH, Wang CC, Kennedy J (2013) The prevalence of herb and dietary supplement use among children and adolescents in the United States: Results from the 2007 National Health Interview Survey. Complement Ther Med 21:358-363. doi:10.1016/j.ctim.2013.05.001
70. Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Sunand P, Qin L (2010) Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol Res 165:437-449