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REGULAR ARTICLE

The seed endosphere of *Anadenanthera colubrina* is inhabited by a complex microbiota, including *Methylobacterium* spp. and *Staphylococcus* spp. with potential plant-growth promoting activities

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Abstract
Background and aims Plant seeds are emerging micro–habitats, whose importance as reservoir and vector of beneficial microbes just begins to be recognized. Here we aimed to characterize the bacterial microbiota of the *Anadenanthera colubrina* seed endosphere, with special focus to beneficial traits and to the colonization pattern.
Methods Cultivation–dependent (isolation from surface–sterilized seeds) and cultivation–independent (pyrosequencing of 16S rRNA gene from metagenomic seed DNA) analyses, functional tests and microscopical

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investigations (fluorescence *in situ* hybridization coupled with confocal laser scanning microscopy (FISH-CLSM) were performed.
Results We isolated several *Methylobacterium* and *Staphylococcus* spp., exhibiting both plant growth promotion and antimicrobial activities. The two taxonomic groups showed complementary traits, which supports a functional selection. Both genera were detected also by pyrosequencing, together with further taxa. The genera *Friedmaniella*, *Bifidobacterium, Delftia*, *Anaerococcus* and *Actinomyces* appeared here for the first time as seed endophytes. We detected bacterial cells and micro–colonies in seed cryosections by FISH-CLSM. Alphaproteobacteria, Firmicutes and other bacteria colonized intercellular spaces of the parenchyma and associated to transport vessels.
Conclusions This work sheds light onto the diversity, functions and colonization pattern of the *Anadenanthera colubrina* seed endophytes, and strongly suggest a role as beneficial partners for seed-associated microbiota.

Keywords Seed endophytes · Beneficial plant–microbe interactions · Plant growth promoting activities · Plant microbiota · Pyrosequencing · Fluorescence in situ hybridization–confocal laser scanning microscopy (FISH–CLSM)

Introduction

The role of the plant microbiota in supporting host's fitness is being recognized more and more (Bulgarelli et al. 2013), and plants are now regarded as single units of evolution called "holobionts", which include the plant and the associated microbiota genomes (Zilber-Rosenberg and Rosenberg 2008; Vandenkoornhuyse et al. 2015). Due to its morphological and physiological heterogeneity, the plant can be considered, from the microbial point of view, as a "mosaic of microhabitats", each one hosting a community of microbes adapted to specific micro–environmental conditions. Some of these microhabitats were well investigated, including rhizosphere (Gaskins et al. 1985; Barea et al. 2005; Berendsen et al. 2012; Doornbos et al. 2012; Pii et al. 2015), phyllosphere (Lindow and Leveau 2002; Lindow and Brandl 2003; Whipps et al. 2008; Vorholt 2012; Müller and Ruppel 2014) as well as shoot– and root–endosphere (Azevedo et al. 2000; Hardoim et al. 2008; Compant et al. 2010; van Overbeek and Saikkonen 2016). Other plant habitats remained relatively neglected and were much less investigated, generally due to the low microbial abundance compared to leaves and root systems (Hallmann 2001). Seeds are among the less–known habitats, and for long time it was supposed that only the seed surface was colonized by microbes. However, a consistent recent literature reported about occurrence of microbes in surface–sterilized seeds in various plant species, including important commercial crops such as coffee, grapevine, bean, tobacco, rice, maize, wheat, tomato, peanut, pumpkin, soybean and sugar beet (reviewed by Truyens et al. 2015).

Some studies considered only the cultivable strains, while others investigated the total bacterial population present in seeds. To identify the cultivable strains, the recent studies used 16S rRNA gene sequencing, while earlier studies used fatty acid methyl ester profile analysis or biochemical methods. To identify bacterial communities, in which there are non–cultivable bacteria, currently is used the 16S rRNA gene, using DGGE technology (denaturing gradient gel electrophoresis, e.g. Hardoim et al. 2012) and, more recently, through Next Generation Sequencing (NGS).

Seed endophytes include members of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, whose roles still remain quite elusive; however, bacteria belonging to several genera showed plant growth promotion (PGP) and/or biocontrol activities (Xu et al. 2014; Gagne-Bourgue et al. 2013; Truyens et al. 2015), which support the fascinating hypothesis of a bacterial–plant co–evolution and functional selection.

Indeed, it can be hypothesized that seed microbiota is actively used by the mother plant as both reservoir and vector of beneficial microbes specifically selected to support the fitness of its progeny, as it can play a role in seed preservation, preparation of the environment for germination, seedling development and plant growth (Holland and Polacco 1994; Chee-Sanford et al. 2006; Johnston-Monje and Raizada 2011; Truyens et al. 2015).

In this work, we investigated the seed endophytic microbiota of *Anadenanthera colubrina* (common name: Curupaú), a bee–pollinated legume tree growing in South America, and native of Brazil, Paraguay, Bolivia, Peru, and northern Argentina (Justiniano and Fredericksen 1998; Cialdella 2000; Barrandeguy et al. 2014; de Viana et al. 2014). This legume species has several synonyms, including *Piptadenia colubrina* (Vell. Conc.), *Piptadenia macrocarpa* Benth., *Niopa macrocarpa* (Benth.) Britt and Rose, and *Anadenanthera macrocarpa* (Benth.) Brenan (Justiniano and Fredericksen 1998), and it is characterized by a high genetic diversity due to ancient fragmentation (Barrandeguy

et al. 2014). It is the dominant species of Seasonally Dry Tropical Forests (SDTFs; Barrandeguy et al. 2014), can reach 25–35 m height (Justiniano and Fredericksen 1998; Barrandeguy et al. 2014) and it is important for its cultural, economic, and medicinal uses (Justiniano and Fredericksen 1998; Torres and Repke 2006); moreover, it was considered as sacred by local cultures since shamans have used its seeds in rituals for over 3000 years (Torres and Repke 2006). Our aim was to determine the diversity and the potential functions of the bacterial seed endophytes of *A. colubrina*. Isolation and high–throughput sequencing were used to assess the composition of cultivable– and total bacterial microbiota, respectively, while fluorescence in situ hybridization coupled with confocal laser scanning microscopy (FISH–CLSM) was used to specifically localize the niches of colonization inside the seed. Finally, PGP– and antimicrobial tests were performed to shed light onto possible beneficial functions of the *A. colubrina* seed microbiota.

Materials and methods

Plant material and surface sterilization of seeds

The plant species investigated in this work was *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb.) Altschul, a legume arboreal species belonging to the Mimosoideae subfamily of the Fabaceae, native of South America (Brenan 1955; Cialdella 2000; Barrandeguy et al. 2014). The fruits were harvested in the year 2010 from two different populations in Argentina (San Bernardo and El Gallinato, Provincia de Salta; de Viana et al. 2014), and kept into plastic bags for transport to the laboratory. Four seeds coming from San Bernardo (sample B2) and four from El Gallinato (samples G6) were removed from the fruits (typical weight of the seeds varies from 0.17 to 0.19 g, the width from 13 to 13.5 mm, the length from 14.4 to 16 mm and thee thickness from to 1.2 to 1.3 mm; de Viana et al. 2014; Online resource 1) and then surface–sterilized by stepwise immersion in 70% ethanol for 1 min, then sodium hypochlorite solution 2,5% for 2 min and finally 70% ethanol for 1 min, followed by two rinses in sterile distilled water. To confirm that the sterilization process was successful, washing water (1 ml) and imprinting of sterilized seed surface were incubated on different culture agar–media (LB, MS, R2YE) and examined for growth after incubation at 30 °C for 4 days.

Isolation of endophytic microrganisms from *Anadenanthera colubrina* seeds

The surface–sterilized seeds were individually immersed in falcon tubes with sterile distilled water for 1 h, ground with a Potter–Elvehjem Tissue Grinder (Sigma–Aldrich, St Louis, USA), resuspended in 50 ml phosphate buffer saline (PBS: 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) per 7.5 g of seeds, and finally shaken at 150 rpm for 1 h. One–hundred μl of phosphate buffer were plated on Luria Bertami (LB), Mannitol Soya flour (MS) and R2YE agar–media (Kieser et al. 2000). The plates were incubated at 30 °C until appearance of microbial colonies.

The colonies, grown on the culture media and obtained from each homogenized sample, were selected on the basis of pigmentation and morphology, and repeatedly incubated on agar–media to obtain pure cultures. The isolates were named according to the seed population (B2 or G6) followed by a progressive number indicating the isolate (for example, G2_10 is the tenth isolate obtained from the seeds of El Gallinato).

Colony PCR of 16S rRNA gene, BLAST alignment and phylogenetic analysis

The microbial isolates were taxonomically characterized on the base of their 16S rRNA gene sequence using the universal bacterial primers 27F and 1492R (Frank et al. 2008) for 16S rRNA gene amplification by colony PCR as previously described (Gallo et al. 2012; Milanesi et al. 2015). The PCR products were purified by using NucleoSpin Gel and PCR Clean-up (MACHERY-NAGEL, Germany). Sequencing was performed by Macrogen Inc. (The Netherlands, http://www.macrogen.com); paired raw forward and reverse sequences were checked for quality with the Geospiza's FinchTV software (PerkinElmer Inc., Waltham, USA; www.geospiza.com/Products/finchtv.shtml) and used to reconstruct the 16S rRNA sequences. Unambiguous regions of the reconstructed 16S rRNA sequences were aligned with the reference GenBank sequences using the BLASTN tool of the NCBI website (McGinnis and Madden 2004). Phylogenetic relationships to known species were inferred by both the neighbour–joining and the maximul likelihood methods using the software Mega6 (Tamura

et al. 2013), with the aim to determine the most closely related species. The tree topology obtained by neighbour–joining was shown, while the percentage of both bootstrap values (obtained with 1000 re–samplings) was indicated. All *Staphylococcus* reference sequences were directly retrieved from the National Centre for Biotechnology Information (NCBI) by BLAST, using the BLAST search tool implemented in Mega6. The reference sequences of *Methylobacterium* spp. were obtained from Chaudhry et al. (2015), after removing a few sequences showing ambiguous (n) bases. Two different phylogenetic trees were created for the isolates belonging to the genera *Staphylococcus* and *Methylobacterium*, respectively, in order to maximize the resolution level of the trees.

The 16S rRNA gene sequences produced in this work and used for the phylogenetic analysis were submitted to Genbank under the accession numbers KX608926 to KX608937.

Characterization of pink pigmented facultative methylothrophs (PPFMs) isolated from seeds

Isolates displaying a pink pigmentation (potential PPFMs) and identified as *Methylobacterium* sp. by 16S rRNA sequence analysis were streaked on solid Ammonium mineral salt (AMS) culture medium (per liter of distilled water: 1.0 g $MgSO_4x7H_2O$, 0.7 g K_2HPO_4, 0.54 g $K_2H_2P0_4$, 0.5 g NH_4Cl, 0.2 g $CaCl_2x2H_2O$, 4.0 mg $FeSo_4x7H_2O$, 0.3 mg H_3BO_4, 0.2 mg $CoCl_2x6H_2O$, 0.1 mg $ZnSO_4x7H_2O$, 0.06 mg $NaMoO_4x2H_2O$, 0.03 mg $MnCl_2$, 0.02 mg $NiCl_2$, 0.01 mg $CuCl_2x2H_2O$, 15 g Agar, and sterile methanol at a concentration of 0.5% added after cooling down of the medium; pH 6.8). The growth of the pink colonies was observed after 5 days of incubation at 30 °C.

To provide further evidence, a fragment of the *mxaF* gene (methanol dehydrogenase) was amplified with the primers mxa1003f/mxa1561r (McDonald and Murrell 1997), using the following thermal protocol: initial denaturation 94 °C for 5 min, followed by 30 cycles of denaturation at 92 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension for 5 min at 72 °C. PCR products were checked for size and purity on 1% (wt/vol) agarose gel. PCR products were sequenced as described above for the 16S rRNA gene. The sequences were submitted to Genbank under the accession numbers KY091854 to KY091857.

Screening for production of antimicrobial compounds

In order to assess antibacterial activity, microbiological assays were performed by using *Kokuria rizophila* ATCC 10240 and *Escherichia coli* DH5α™ (Invitrogen) as Gram-positive and Gram-negative tester strains, respectively, in agar–diffusion test (Baldi et al. 2016; Scaffaro et al. 2015). In particular, for each tester strain, 5 ml of a bacterial suspension, containing 10^8–10^9 colony-forming units (CFUs) *per* mL, was prepared in warm LB soft–agar. In order to obtain a bacterial tester strain overlay, each suspension was then poured over R2YE plates containing on the surface patches of bacterial isolates. After LB soft–agar solidification, the plates were incubated at 37 °C to allow tester strain growth. Then the presence of growth inhibition halos in the surrounding zone of the bacterial isolate patches was checked.

Screening for plant growth promoting (PGP) activities

All bacterial isolates were evaluated for the following growth promoting traits: nitrogen fixation; inorganic and organic phosphate solubilisation; siderophore; 1–aminocyclopropane–1–carboxylate (ACC) deaminase; Indol Acetic Acid (IAA) production. Each test was performed in duplicate and further repeated in case of unclear results. The strains *Microbacterium natoriense* E38 and *Pseudomonas brassicacearum* E8 isolated from plant rhizosphere in a natural salt meadow near Münzenberg, Hessen, Germany (Cardinale et al. 2015), were used as positive controls.

Inorganic– and organic phosphate solubilization

Since the soil pH of the isolation sites is variable from slightly acidic to slightly basic (Larenas Parada et al. 2004; Chavez et al. 2014), all isolates were plated on basal medium agar (BM) containing different sources of P, as suggested by Bashan et al. (2013): $AlPO_4$ (AP), $Ca_3O_8P_2$ (CP) and $FePO_4$ (FP), (according to Gadagi and Sa 2002), for characterizing their ability to solubilize inorganic phosphates, and on inositol hexaphosphate (IHP) agar medium (Richardson and Hadobas 1997) for testing the organic phosphate solubilization activity. After 3, 7 and 14 days of incubation at 28 °C, the plates were checked for growth, development of a solubilisation halo around the colonies and colour change of the culture medium.

Nitrogen fixation

All the isolates were inoculated in semi–solid NFB medium (Kirchhof et al. 1997) prepared with washed agar and supplemented with 1% saccharose, to check their ability of growing on nitrogen free medium. After 3, 7 and 14 days of incubation at 28 °C, the plates were checked for growth.

IAA production

IAA production was detected with the method of Bric et al. (1991). All strains were inoculated in LB–agar amended with L–tryptophan (LBT); each inoculated plate was overlaid with sterilized cellulose nitrate filter membrane (Sartorius AG, Goettngen, Germany). After colony appearance, the membrane was removed from the plate, immersed in Salkowski reagent (Glickmann and Dessaux 1995) and incubated at 28 °C for 1 h. Bacteria producing IAA were identified by the formation of a characteristic red halo on the membrane corresponding to the position of the colonies.

ACC-deaminase production

DF salt minimal medium amended with ACC (Penrose and Glick 2003) was used to test the ACC–deaminase activity. After 3, 7 and 14 days of incubation at 28 °C, the plates were checked for growth.

Siderophore production

Bacterial isolates were assayed for siderophores production in liquid King's medium B (Schaad et al. 1980). The inoculated broth was incubated at 28 °C in a rotary shaker for 7 days. From the culture, 1 ml was centrifuged for 5 min at 1100 g at room temperature with a Microstar 17R centrifuge (VWR, Darmstadt, Germany). From the supernatant, 100 μl were placed in a microtiter plate and 100 μl of 2 mM chrome azurol S (CAS) solution (Schwyn and Neilands 1987; Alexander and Zuberer 1991) were added. After 30 min incubation at room temperature, a shift from blue to yellow-orange or purple colour indicated the production of siderophores of the type hydroxamate or catechol, respectively (Neilands 1995; Milagres et al. 1999).

Salt– and drought tolerance assays

Bacteria isolates were tested for salt and drought resistance using two different media: LB agar for the isolates with phenotypic colour white/beige (later identified as *Staphylococcus* spp.) and AMS agar with methanol for the isolates with phenotypic colour pink (later identified as *Methylobacterium* spp.). The media were adjusted by 2.5%, 5% and 7.5% (*w*/*v*) NaCl (salt stress), and 15% of polietilenglicol (PEG; drought stress). Each test was performed in duplicate and further repeated in case of unclear results. After 2, 7 and 15 days of incubation at 28 °C, the growth was observed and compared with that of the same isolates grown in the same media without NaCl or PEG.

Extraction of total DNA from seeds

Total DNA was extracted from ground surface–sterilized seeds using a modified Phenol/Chloroform method. To each 70 mg of homogenized seed, 0.4 ml of extraction buffer (2% CTAB, 100 mM Tris-HCl, 3.5 M NaCl, 20 mM EDTA, 0.2 M mercaptoethanol, 2% Polyvinyl pyrrolydine PVP, pH 8,0) was added, the samples were incubated for 90 min at 60 °C with inversion mixing during incubation and subsequently put for 2 min into ice. To remove the proteins, 10 μl of Proteinase K (10 μg/μl) were added to the mixture; after an incubation at 65 °C for 40 min, an equal volume of chloroform:isoamyl alcohol (24:1, *v*/v) was added, and the samples were mixed thoroughly by inversion. The tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was transferred to new tubes. DNA was precipitated by adding one volume of 0.3 M sodium acetate, pH 5.2, and 2.5 volumes of ice–cold absolute ethanol. The tubes were incubated over-night at −20 °C or for 1 h at −80 °C, and then centrifuged at 10,000 rpm for 10 min; the supernatant was discarded and the pellet was washed with 80% (*v*/v) ethanol, air-dried for 1 h at room temperature (or 20 min at 45 °C) and finally resuspended in 100 μl of double distilled water (ddH_2O). Twenty μl of RNAse (10 μg/μl) were added and the tubes were incubated for 1 h at 37 °C. For further purification and concentration, an additional extraction with an equal volume of chloroform:isoamyl alcohol (24:1, *v*/v) was performed; DNA was precipitated by adding one volume of 0.3 M sodium acetate, pH 5.2, and 2.5 volumes of ice-cold absolute ethanol; the tubes were inverted gently, maintained over night at −20 °C and then centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 80% (*v*/v) ethanol, dried out and finally resuspended in 50 μl ddH_2O.

DNA concentration and quality was measured by reading absorbance at 260 nm with NanoDrop® ND-/

100 (NanoDrop Technologies, Wilmington, DE) and by running aliquots on 0.8% agarose gel. The DNA samples were stored at −20 °C until further use.

Pyrosequencing of the V3-V5 region of bacterial 16S rRNA gene

For each DNA sample (two pooled B and two pooled G seeds), 16S rRNA gene was amplified using fusion primer set specific for V3–V5 hypervariable regions (F357: 5′- TCCTACGGGAGGCAGCAG -3′ and R937: 5′- TGTGCGGGCCCCCGTCAATT -3′ (Muyzer et al. 1993) containing adaptors, key sequence and MID (Multiplex IDentifier) barcode sequences as described by the 454 Sequencing System Guidelines for Amplicon Experimental Design (Roche, Basel, Switzerland).

PCR were performed using the FastStart High Fidelity PCR system (Roche, Basel, Switzerland) according to the following protocol: 5 min at 95 °C, 28 cycles of 30 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C, followed by a final extension of 8 min at 72 °C. The PCR mix contained 1X FastStart High Fidelity PCR buffer; 1.8 mM $MgCl_2$, 200 μM of dNTPs, 0.4 μM of each primer (synthesized by Eurofins, Milano, Italy), 2.5 U of FastStart High Fidelity Polymerase Blend and 5 μl of a 1:10 dilution of each DNA as template. All PCRs were carried out in triplicate using a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and pooled before sequencing.

The PCR products obtained were analyzed by gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions, quantified via quantitative PCR using the Library quantification kit – Roche 454 titanium (KAPA Biosystems, Boston, MA) and pooled in equimolar mixture in the final amplicon library. The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+ chemistry following the manufacture's recommendations (Roche, Basel, Switzerland).

Analysis of pyrosequencing data

Raw 454 files were demultiplexed using the Roche's sff file software. Reads were preprocessed using the MICCA pipeline (version 0.1) (Albanese et al. 2015). Operational taxonomic units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity and their representative sequences were classified using the RDP classifier version 2.7 (Wang et al. 2007). Template–guided multiple sequence alignment (MSA) was performed using PyNAST version 0.1 (Caporaso et al. 2010) against the multiple alignment of the Greengenes database (release 13_05) (DeSantis et al. 2006) filtered at 97% similarity. The phylogenetic tree was inferred using micca-phylogeny (Price et al. 2010). Sampling heterogeneity was reduced by rarefying samples at 90% of the less abundant sample (43,866 sequences per sample). Alpha–(within–sample) diversity estimates were computed using the phyloseq R package (McMurdie and Holmes 2013).

The sequences were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) under the accession number PRJEB14944.

Fluorescence in situ hybridization (FISH) of seed sections

Surface–sterilized seeds were embedded in Tissue freezing medium Jung (Leica Instruments GmbH, Nussloch, Germany), and longitudinal cryosections of 35–50 μm were obtained using the low–temperature constant–cooling cryostat HM 500 OM (MICROM, Walldorf, Germany) at −20 °C; the cryosections, including the testa (seed coat), the cotyledons and the embryo, were gently washed twice in phosphate–buffered saline 1× (PBS) to remove the embedding medium and fixed in 3:1(v:v) 4% paraformaldehyde:PBS for 12 h at 4 °C, then washed three times in ice–cold PBS (for 10/20/30 min stepwise, at 4 °C), and finally stored at −20 °C in 1:1 (v:v) ice–cold PBS:ice-cold 96% ethanol until FISH staining. Additionally, seed coats were removed under sterile conditions and fixed without sectioning.

Fixed cryosections or seed coats were stained by in–tube FISH according to Cardinale et al. (2008). Specifically, sections were put into 1.5 ml Eppendorf tubes, rinsed with PBS, and incubated with 100–150 μl of 1 μg/μl Lysozyme (Sigma–Aldrich, Steinheim, Germany) for 10 min at room temperature, to increase the bacterial cell wall permeability to the FISH probes. The samples were rinsed twice with PBS, dehydrated by immersion in ethanol (50, 70, and 96%, for 3 min each), washed in ice–cold PBS (3 min at room temperature) and then incubated in hybridization buffer (0.9 M NaCl, 0.02 M Tris-HCl, pH 8, 0.01% vol sodium dodecyl sulphate (SDS), 30% deionized formamide and 1.5–2.5 ng/μl of each FISH probe) at 42 °C in the dark for 120 min. For the detection of all bacteria, an equimolar mixture of Rhodamine Red-labelled EUB338, EUB338II and EUB338III probes (EUB338MIX; Amann et al. 1990; Daims et al. 1999) was used. Either Cy5-labelled

ALF968 probe (Neef 1997) or an equimolar mixture of Cy5-labelled LGC354A, LGC354B and LGC354C probes (LGC354MIX; Meier et al. 1999) were used for the specific detection of *Alphaproteobacteria* and *Firmicutes*, respectively. The seed coats were stained with FITC–labelled ALF968 probe and Rhodamine red–labelled EUB338MIX. A mixture of FITC–, Rhodamine Red– and Cy5– labelled NONEUB probes (Wallner et al. 1993) were used as negative control to check for aspecific attachment of probes and fluorochromes to the seed tissues. Following hybridization, the samples were rinsed with pre–warmed washing buffer solution (0.02 M Tris-HCl, pH 8, 0.102 M NaCl, and 5 mM EDTA) and incubated with 1 ml of the same pre–warmed washing buffer in a water bath for 15 min at 43 °C. The washing buffer was removed and the samples were rinsed with ice–cold double-distilled water, air dried on glass slides, immediately mounted with the antifadent reagent Citifluor AF 2 (Citifluor Ltd., London, UK) and finally stored at 4 °C in the dark until microscopic observation, which was performed within 3 days after FISH staining.

Confocal laser scanning microscopy (CLSM), epifluorescence microscopy and image analysis

FISH–stained cryo–sections were observed by confocal laser scanning microscopy (CLSM), using a Leica TCS SP8 (Leica Microsystems, Heidelberg, Germany) equipped with argon and helium/neon lasers. We observed about 30 seed sections, in at least 15 independent FISH experiments, of the seeds belonging to the same populations used for metagenomics and cultivation analysis.

Three confocal light channels were observed simultaneously along with an additional (non–confocal) bright–field channel. The signals of the two FISH probes and the seed autofluorescence signal were acquired sequentially (excitation/emission–range in nm: Rhodamine red 561/570–610; Cy5 633/650–720; seed autofluorescence 405/415–500). Confocal stacks (15–30 μm–thick) were acquired with a Leica 63X 1.0 NA water–immersion objective, by applying a Z–step of 0.6–0.8 μm, and visualized by maximum projections

Table 1 BLAST analysis results of isolated seed endophytes of *Anadenanthera colubrina*

Isolate name	16S rRNA gene sequence lenght (nts)	Best BLAST hit(s)	Accession number	Sequence similarity (%)
G2_2	1114	*Methylobacterium indicum* SE2. 11	NR_135210	98.8
		Methylobacterium variabile GR3	NR_042348	98.7
G2_6	1335	*Methylobacterium extorquens* IAM 12631	NR_112230	100
G2_7	1332	*Methylobacterium hispanicum* DSM 16372	NR_112613	99.7
B6_7	1341	*Methylobacterium rhodesianum* DSM 5687	NR_041028	100
G2_3	1388	*Staphylococcus epidermidis* Fussel	NR_036904	99.9
G2_9	1370	*Staphylococcus epidermidis* Fussel	NR_036904	99.9
G2_1	1373	*Staphylococcus epidermidis* NBRC 100911	NR_113957	99.9
G2_5	1381	*Staphylococcus epidermidis* NBRC 100911	NR_113957	99.9
G2_10	1070	*Staphylococcus aureus* NBRC 100910	NR_113956	100
		Staphylococcus aureus subsp. anaerobius MVF–7	NR_036828	100
		Staphylococcus aureus ATCC 12600	NR_115606	100
		Staphylococcus argenteus MSHR1132	FR821777	100
		Staphylococcus schweitzeri FSA084	CCEL01000025	100
B6_5	1103	*Staphylococcus aureus* NBRC 100910	NR_113956	100
		Staphylococcus aureus subsp. *anaerobius* MVF–7	NR_036828	100
		Staphylococcus aureus ATCC 12600	NR_115606	100
		Staphylococcus argenteus MSHR1132	FR821777	100
		Staphylococcus schweitzeri FSA084	CCEL01000025	100
B6_1	1170	*Staphylococcus pasteuri* ATCC 51129	NR_114435	99.9
G2_4	1008	*Staphylococcus haemolyticus* JCM 2416	NR_113345	100

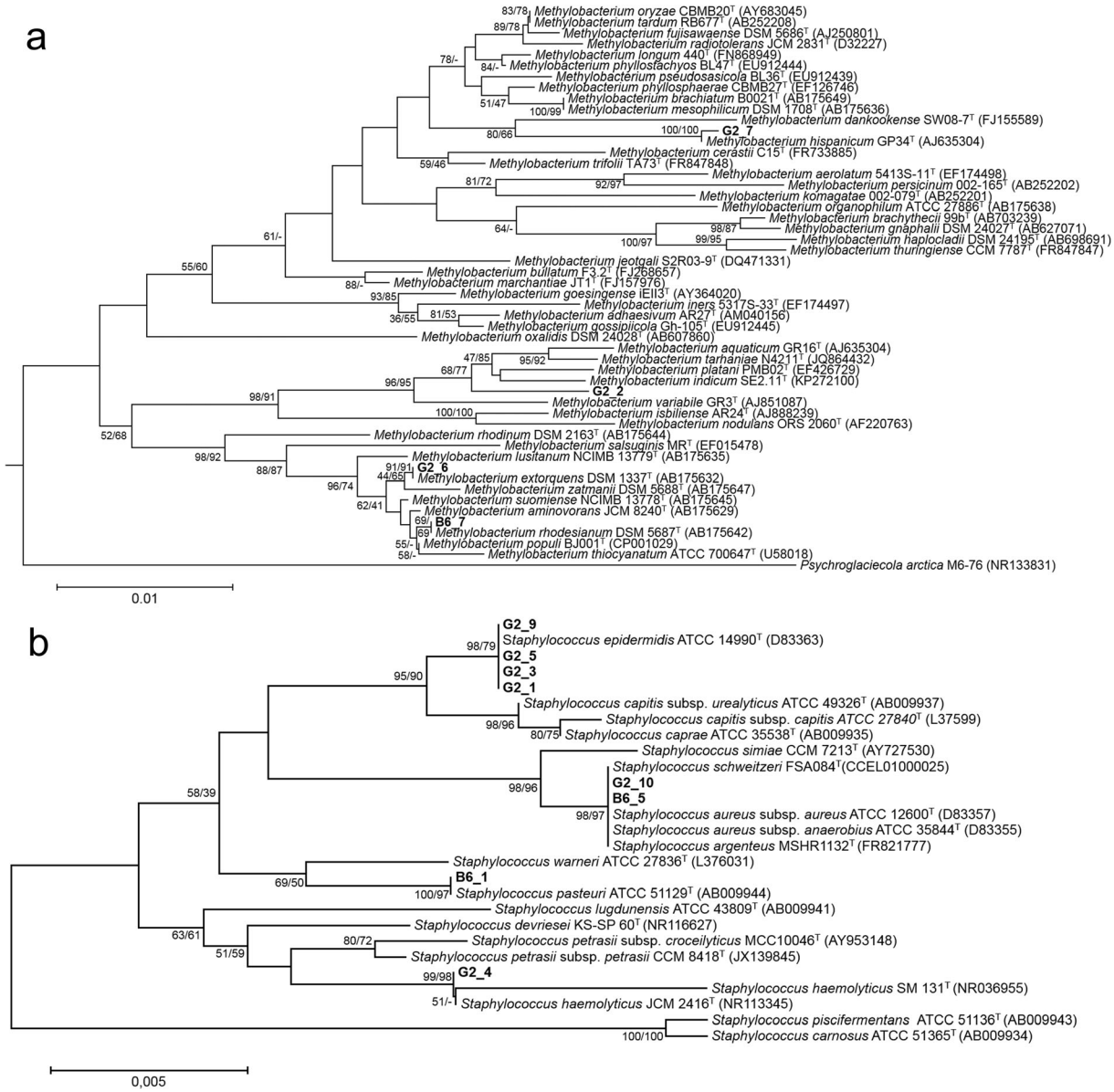

Fig. 1 Phylogenetic analysis of *A. colubrina* seed endopytes. **a** Neighbour–joining 16S rRNA gene phylogenetic tree obtained with a 1115 nts–alignment, including the *A. colubrina* isolates belonging to the genus *Methylobacterium* (bold) and almost all 16S reference sequences of *Methylobacterium* type strains. **b** Neighbour–joining 16S rRNA gene phylogenetic tree obtained with a 970 nts–alignment, including the *A. colubrina* isolates belonging to the genus *Staphylococcus* (bold) and several 16S reference sequences of closely related *Staphylococcus* type strains. Numbers at the branch nodes indicate the bootstrap percentage values obtained from 1000 resamplings with both the neighbour–joining and the method maximum likelihood methods (NJ/ML; only values ≥50% for at least one method are shown; - means that this cluster did not occur in the tree obtained with the respective method). Scale bars indicate substitutions per site

and volume–renderings with the software Imaris version 8.2 (Bitplane, Zurich, Switzerland). The internal side of the unsectioned seed coats were observed with an epifluorescence microscope Zeiss Axioplan 2 (Carl Zeiss Jena GmbH, Jena, Germany), using the Zeiss filter F36–720 HC-mFISH Sp. Green with Brightline HC 515/LP for FITC, and the Zeiss filter set 15 (BP 546, FT 580, LP 590) for Rhodamine.

Final figures were assembled with Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, USA).

Table 2 Plant growth promoting activity tests

Isolate	PGP Test[#]							
	Solub. inorganic phosphate[##]			Solub. organic phophate	Growth on N–free medium	Synthesis of ACC–deaminase	Production of siderophores[###]	Synthesis of auxin
	Ca–P	Al–P	Fe–P					
G2_2	+	−	+	+/−	+/−	+/−	−	−
G2_6	−	−	+	+	+	+/−	−	−
G2_7	+	−	+	+/−	+/−	+/−	−	−
B6_7	+	−	+	+	+/−	+/−	−	−
G2_3	+ (y)	−	−	−	−	−	+ (p)	+
G2_9	+ (y)	−	+	−	−	−	+ (p)	+
G2_1	+ (y)	−	+/− (y)	−	−	−	+ (p)	+
G2_5	+ (y)	−	−	−	−	−	+ (p)	+
G2_10	+	+/− (y)	+/− (y)	−	−	−	+ (p)	+
B6_5	+ (y)	−	+	−	−	−	+ (p)	+
B6_1	+ (y)	−	+	−	−	−	+ (p)	+
G2_4	+ (y)	−	−	−	−	−	+ (p)	+

[#] + growth – No growth; +/− Reduced growth

[##] (y) Colour change of the medium to yellow

[###] (p) Colour change of the medium to purple, after addition of CAS reagent

Results

Taxonomic diversity of cultivated *A. colubrina* seed endophytes

After 5–7 days of incubation, bacterial colonies appeared on the surface of agar–medium plates inoculated with the surface–sterilized seed suspensions. The colonies showed three different phenotypes: most had a white, translucent pigmentation, others were pink, while few colonies, that were observed only from B seeds, were yellow. A total of 12 isolates were selected and obtain as pure cultivations (Table 1). Inoculation of washing step water and surface imprinting of surface–sterilized seeds did not result in bacterial growth on agar–medium plates, thus confirming the successful external sterilization of the seeds.

From all 12 isolates, a 16S rRNA gene sequence was obtained. All the sequences showed high similarities (≥ 98.7%) with sequences obtained from NCBI database (Table 1). The isolates belonged basically to two bacterial genera, *Staphylococcus* and *Methylobacterium*, the most related species being *S. epidermidis*, *S. haemolyticus*, *S. aureus/S. argenteus/S. schweitzeri*, *S. pasteuri*, *M. variable*, *M. extorques*, *M. indicum*, *M. hispanicum* and *M. rhodesianum* (Table 1). Phylogenetic analysis confirmed the taxonomic affiliations retrieved by BLAST alignment (Fig. 1). The isolate G2_2 was the only ones that did not uncontrovertibly cluster with a known species, resulting placed instead between the species *M. variable* and *M. indicum* (Fig. 1a), while G2_10 and B6_5 clustered with the unresolved *Staphylococcus aureus/argenteus/schweitzeri* group (these three species have the same 16S rRNA gene sequence).

The isolates of the genus *Methylobacterium* were able to grow on AMS–methanol, so confirming their ability to use methanol as only carbon source. Additionally, a fragment of the *mxaF* gene was successfully amplified from the four isolates, and the electrophoretic analysis of the PCR product showed the expected size of about 550 bp (Online resource 2). Sequencing and BLAST alignment showed that all PCR products were actually *mxaF* gene of *Methylobacterium* spp.; moreover, for all of them, the closest species according to the 16S phylogeny appeared within the first three best BLAST hits of the *mxaF* gene sequences: *M. platani* for G2_2, *M. rhodesianum* for B6_7, *M. extorquens* for G2_6, *M. hispanicum* for G2_7.

Screening of bacterial isolates for plant growth promoting traits antimicrobial activity, and salt/drought tolerance

The isolates showed variable plant growth promoting activities (Table 2). All strains except G2_6 solubilized calcium phosphate. All *Methylobacterium* isolates and some *Staphylococcus* isolates were able to solubilize also iron phosphate, while only G2_10 showed (reduced) growth on aluminium phosphate (Table 2). The isolates belonging to *Methylobacterium* genus were also able to solubilize organic phosphate and grow, without inducing a colour change, thus suggesting an enzymatic mechanism of phosphate solubilisation (Table 2). However, no clear solubilisation halo was observed around the bacterial colonies. All *Staphylococcus* isolates also produced auxin and siderophores of type cathecol (colour change from blue to purple after CAS reagent addition, Table 2). All *Methylobacterium* strains were positive for ACC–Deaminase production test and could grow in the nitrogen–free medium (Table 2).

All strains were able to grow in presence of 2.5, 5 and 7.5% NaCl and 15% PEG, except isolate G2_5 that did not grow on PEG (Table 3). Finally, three out of four *Staphylococcus epidermidis* isolates showed antimicrobial activity against both *K. rizophila* and *E. coli*, as demonstrated by the inhibition zones of bacterial tester growth (Table 3). Furthermore, none of these strains inhibited the growth of the *Methylobacterium* isolates.

High-throughput sequencing analysis of *Anadenanthera* seed metagenome

Pyrosequencing of the amplicon libraries of the 16S rRNA gene V3–V5 hypervariable regions resulted in a total of 105,271 reads, with a mean of 52,635.5 sequences per sample (one pooled B and one pooled G sample). More than 99% of reads were of plant origin (mainly chloroplastidial and mitochondrial sequences), confirming the low abundance of seed endophytes found by plate cultivation. Rarefaction analysis resulted in a sequencing depth adequate to capture the ecological diversity of the samples up to saturation (online resource 3). The analysis of alpha–diversity (i.e. the ecological richness within samples; online resource 4) identified 12 OTUs in the G sample and 7 OTUs in the B samples for a total of 11 bacterial taxa classified (Fig. 2), including *Staphylococcus* and *Methylobacterium* that were found also by culture–dependant methods. Moreover, sequences belonging to the genus *Methylobacterium* were the most abundant retrieved by pyrosequencing, thus confirming its occurrence as main seed endophyte of *A. colubrina.* It

Table 3 Antimicrobial activity and stress–tolerance tests

Isolate	Stress test[#]				Antimicrobial activity[##]	
	NaCl (%)			PEG 15%	*Kokuria rhizophila*	*Escherichia coli*
	2.5	5	7.5			
G2_2	+/−	+/−	+/−	+/−	−	−
G2_6	+/−	+/−	+/−	+/−	−	−
G2_7	+/−	+/−	+/−	+/−	−	−
B6_7	+/−	+/−	+/−	+/−	−	−
G2_3	+/−	+/−	+/−	+/−	−	−
G2_9	+/−	+/−	+/−	+/−	+	+
G2–1	+	+	+	+/−	+	+
G2–5	+/−	+/−	+/−	−	+	+
G2–10	nt	nt	nt	nt	−	−
B6_5	+/−	+/−	+/−	+/−	−	−
B6_1	+/−	+/−	+/−	+/−	−	−
G2–4	nt	nt	nt	nt	−	−

[#] + growth (single colonies of the same size than on control plates without NaCl/PEG); − No growth; +/− Reduced growth (no single colonies or single colonies smaller than on control plates without NaCl/PEG); nt Not tested.

[##] + Presence of inhibition halo; − Absence of inhibition halo

is worth noting that Actinobacteria were identified almost exclusively in the G sample with the exception of the genus *Bifidobacterium* that was found in both samples (i.e. B and G samples).

FISH analysis of *A. colubrina* seed sections

Fluorescence in situ hybridization was used to confirm the results of cultivation–dependent and –independent methods, and to visualize the colonization niches of the seed endophytic bacteria. Since Alphaproteobacteria and Firmicutes bacteria were both isolated and detected by pyrosequencing, we applied Alphaproteobacteria– and Firmicutes–specific FISH probes, along with the universal bacterial probe EUB338MIX (see materials and methods for details). FISH signals were obtained simultaneously with the probes EUB338MIX and with the specific probes for Alphaproteobacteria or Firmicutes, thus confirming the results of both pyrosequencing and bacterial isolation. Both Alphaproteobacteria (Fig. 3) and Firmicutes (Figs. 4 and 5) cells were visualized inside seeds, at different sites. Alphaproteobacteria cells with methylobacteria–compatible morphology colonized the intercellular spaces of the parenchyma (Fig. 3a–e and k, arrows) and the internal side of seed coats (Online resource 5); besides the EUB338MIX and ALF968 FISH probe–conferred signals, these cells also appeared slightly autofluorescent in the same range of wavelenghts of the plant cells (Fig. 3b). Firmicutes cells with staphylococci-compatible morphology were observed in the internal parenchyma of the cell coat (Fig. 4a–e, arrows), together with additional cells stained by the EUB338MIX probe only (Fig. 4c, e, circles) in their vicinity. Interestingly, colonization by Firmicutes cells with staphylococci-compatible morphology was also detected close to the vascular tissues (Fig. 5a–e, arrow), together with additional cells (Fig. 5c, e, arrowhead). No signals were detected in the seed sections stained with the nonsense NONEUB probe (Figs. 3, 4 and 5, panels f–j).

In general, the bacterial density was scarce and in fact, a large screening was necessary to find bacterial cells. This low abundance is coherent with both the low isolate number and the few bacterial sequences obtained by pyrosequencing. A scheme of the seed structure showing the localization of bacteria according to our FISH microscopy observation is shown in the Online resource 6.

Discussion

In this work, a polyphasic approach was employed to elucidate identity, colonization pattern and potential beneficial functions of the bacteria inhabiting the *Anadenanthera colubrina* seed endosphere. Bacterial strains belonging to the genera *Staphylococcus* and *Methylobacterium* were isolated and both genera showed a surprising diversity at species level with four species of *Methylobacterium* and four species of *Staphylococcus* retrieved on the basis of 16S rRNA gene phylogenetic analysis (Table 1; Fig. 1). Methylobacteria have been isolated from the seed endosphere of bean (López-López et al. 2010), eucalyptus (Ferreira et al. 2008), rice (Mano et al. 2006) and maize (Johnston-Monje and Raizada 2011), and from ovuliferous buds of *Pinus sylvestris* (Pirttilä et al. 2000). Methylobacteria are typical plant–associated bacteria, ubiquitously occurring as phyllosphere colonizers of several species (Gourion et al. 2006; Vorholt 2012) but also as endophytes (Lacava et al. 2006; Ryan et al. 2008). Their beneficial effects for the plant are well recognized, including production of auxin and cytokinins (Tsavkelova et al. 2006; Kutschera 2007; Meena et al. 2012), ACC–deaminase activity (Fedorov et al. 2013), phosphate solubilization (Jayashree et al. 2011), induction of systemic resistance (Madhaiyan et al. 2004) and nitrogen fixation either as free–living (Raimam et al. 2007) or as specific symbionts in legume root nodules (Sy et al. 2001; Jourand et al. 2004). All the methylobacteria isolates were negative for the production of auxin, but they were able to grow on semisolid N–free medium and to solubilize both inorganic and organic phosphates (Table 2). The observation that phosphate solubilisation was not coupled with medium acidification as elsewhere reported (Jayashree et al. 2011) suggests the occurrence of solubilisation mechanisms other than the secretion of organic acids. Indeed, acidification of the medium does not always correlate with phosphate solubilization efficiency (Hamdali et al. 2010). Altogether, these results indicate a possible role of seed endophytic methylobacteria in supporting plant mineral nutrition more than in hormone–induced enhancement of plant growth, assuming that seed endophytes will grow in the rhizosphere of the seedling after germination. Furthermore, our *Methylobacterium* isolates did also grow on ACC–amended minimal medium and in presence of 7.5% NaCl and 15% PEG (Table 3), suggesting an additional potential role as abioic stress alleviators.

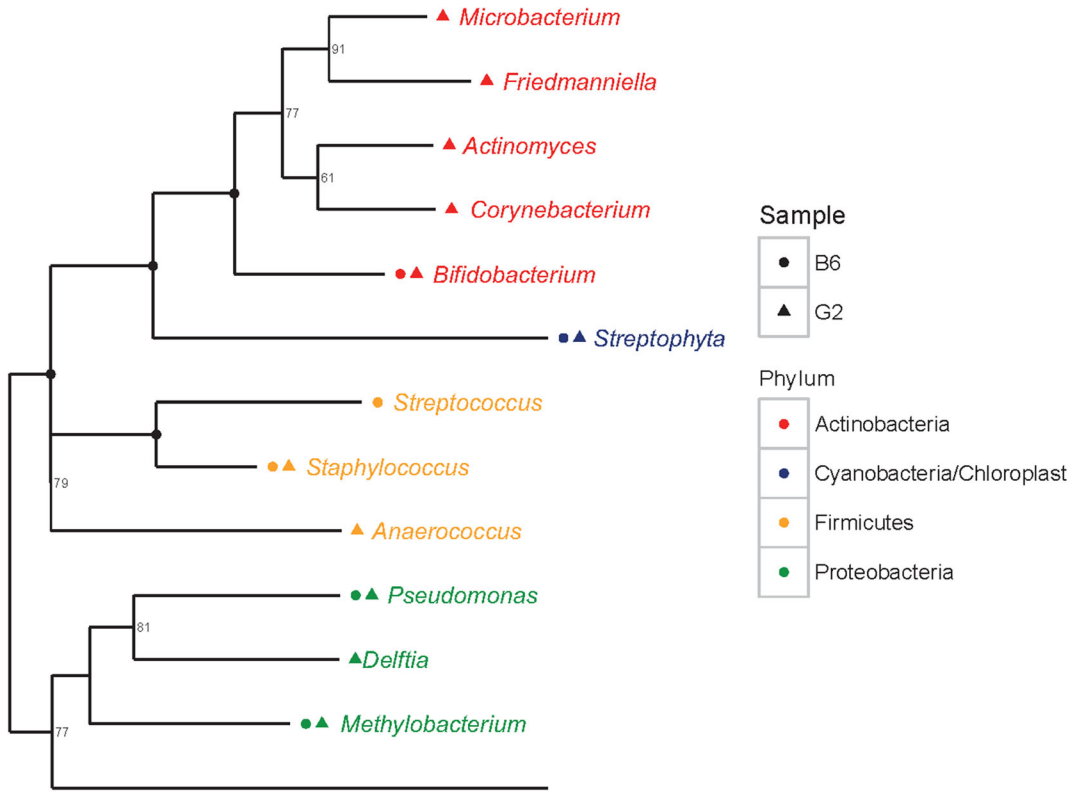

Fig. 2 Phylogenetic analysis of *A. colubrina* seed microbiota from bacterial V3–V5 16S rRNA gene sequencing. The different shapes indicate the samples in which each OTU was observed while colors indicate the Phylum of each OTU. When possible OTUs were classified at the genus level. G and B seed populations were collected from two different locations (see materials and methods for details)

Although staphylococci are regarded as typical human–associated bacteria with pathogenic potential, they were often found in association with plants. In fact, they were found as seed endophytes of maize (Liu et al. 2012, 2013) and were additionally isolated from other plant tissues of *Chlorophytum borivilianum* (Panchal and Ingle 2011), soybean (Hung and Annapurna 2004), and carrot (Surette et al. 2003). *Staphylococcus* spp. were detected in cotton roots but not in the leaves of the same plant (Mcinroy and Kloepper 1995), which indicates a substantial level of specificity in the interaction with the host. In our study, we retrieved four different *Staphylococcus* species, which were phylogenetically close to *S. epidermidis* (the most frequently isolated), *S. pasteuri, S. haemolyticus* and *S. aureus* group (including *S. aureus*, *S. argenteus* and *S. schweitzeri*). Interestingly, *S. epidermidis* and *S. pasteuri* were found as dominant members of the endophytic community of ginseng (Vendan et al. 2010). All our *Staphylococcus* isolates were able to synthetize auxin, to produce siderophores and to solubilize calcium phosphate, indicating a potential role as plant growth promoters. Although the phosphate solubilization test did not show a clear halo around our *Staphylococcus* (as well as *Methylobacterium*) colonies, we relied on consistent growth of single colonies and on colour change of the medium, when occurring, since it was already recognized that even efficient phosphate–solubilizing bacteria might not be able to produce a visible solubilization halo (Fankem et al. 2006; Baig et al. 2010; Jayashree et al. 2011; Lopez et al. 2011). *Staphylococcus sciuri* isolated from *Catharanthus roseus* was shown to have plant growth promoting effect on the host plant (Tiwari et al. 2013), and the ability to produce auxin was assessed in vitro for different *Staphylococcus* spp. (Ali et al. 2010; Vendan et al. 2010; Kumar et al. 2011). Environmental *Staphylococcus* isolates were able to solubilize phosphate (Kumar et al. 2011), including calcium phosphate (Acevedo et al. 2014). Siderophore production is a common feature of staphylococci (Lindsay and Riley 1994; Beasley and Heinrichs 2010), and Lisiecki et al. (1993) found that 14 out of 180 analysed *Staphylococcus*

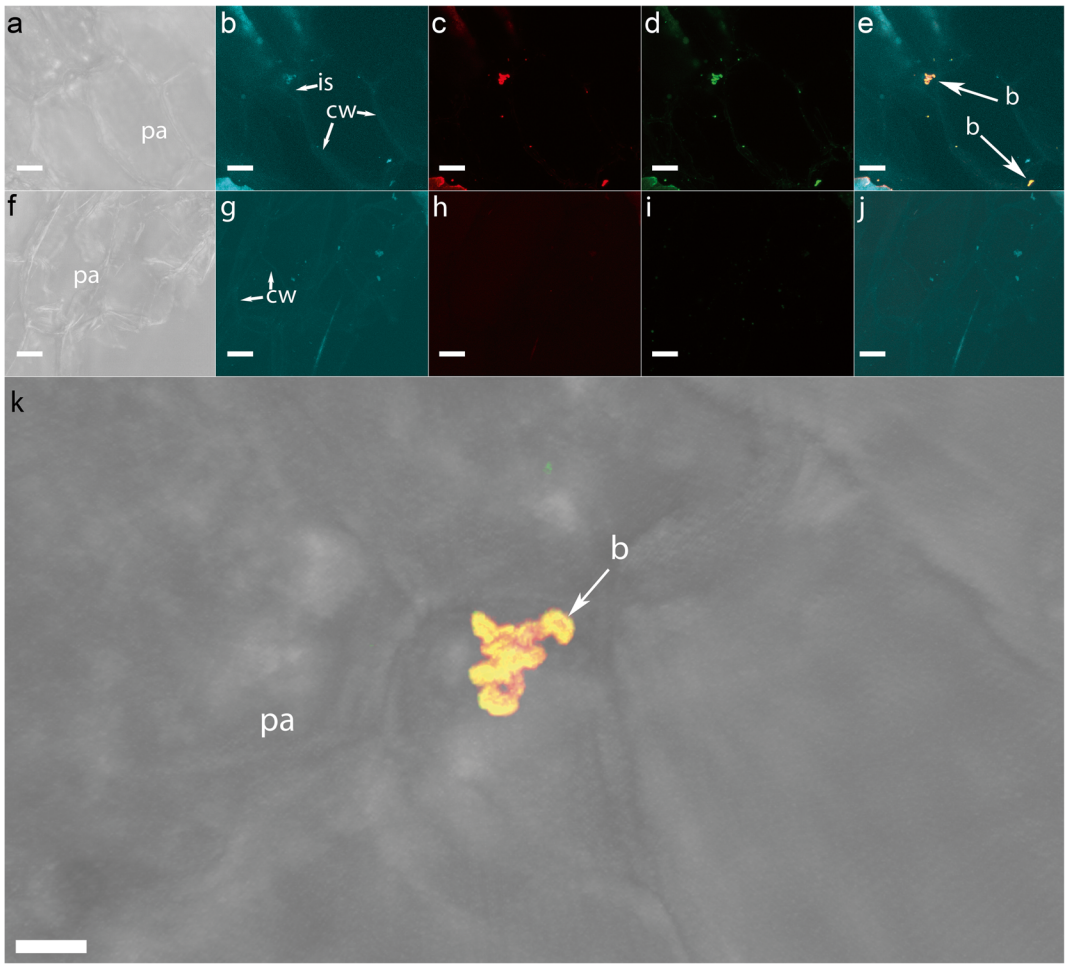

Fig. 3 Bacterial colonization of *A. colubrina* seeds. Confocal laser scanning microscopy images showing fluorescence in situ hybridization (FISH)–stained bacteria inside *Anadenanthera colubrina* seed cryosections. First raw shows the result after staining with the Rhodamine red–labelled universal FISH– probes EUB338MIX (shown as red in panel c) and the Cy5–labelled Alphaproteobacteria–specific probe ALF968 (shown as green in panel d); panels a and b show the bright–field image and the autofluorescence of the seed tissues, respectively; panel e is the overlap of the images b–c–d (arrows indicates alphaproteobacterial cells). The second raw (panels f to j) shows the result after staining of a separate subsample with a mixture of Rhodamine red– and Cy 5–labelled nonsense NONEUB FISH probes. K shows the zoomed, volume–rendered overlap of panels a, c and d; arrows indicate an alphaproteobacterial cell with a darker zone corresponding to the nucleoid. Parameters used for image acquisition were the same for both positive and negative subsamples. Scale bars: a–j 10 μm; k 5 μm. pa = parenchyma of the seed coat (mesotesta); is = intercellular space (apoplast); cw = cell wall (of the plant); b = bacteria

strains produced cathecol–like siderophores. Our results confirm the previous studies and suggest that, in general, plant–associated *Staphylococcus* spp. might exhibit plant growth promoting effects more frequently than supposed.

Interestingly, the results of the antimicrobial– and plant growth promotion tests showed complementary traits among methylobacteria and staphylococci (Tables 2 and 3); this suggests that the different members of the seed endosphere might be part of a unique beneficial consortium, which provides a broad spectrum of functions to the plant. This is coherent with the concept of "functional redundancy", which implies the selection of beneficial microbial partners in the host, based on their functional traits and irrespective of the taxonomic identity, thus obtaining co–evolutionary and mutualistic advantages (Wohl et al. 2004; Burke et al. 2011). Although functional redundancy was not shown in this work, our results suggest that pursuing a complementarity of functions might be a general mechanism underlying microbiome associations with eukaryotes.

In this work, we did not test the plant growth promoting effect of the isolated *Methylobacterium* and

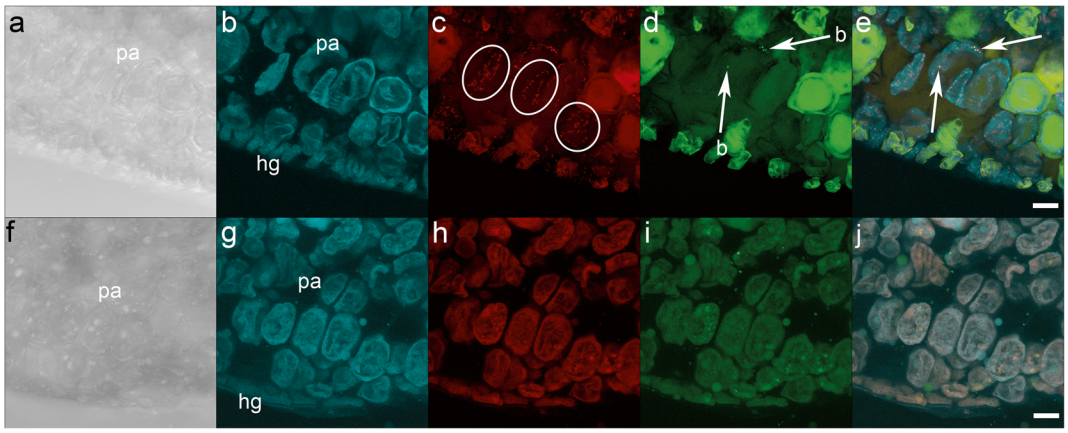

Fig. 4 Bacterial colonization of *A. colubrina* seeds. Confocal laser scanning microscopy images showing fluorescence in situ hybridization (FISH)–stained bacteria inside *Anadenanthera colubrina* seed cryosections. First raw shows the result after staining with the Rhodamine red–labelled universal FISH– probes EUB338MIX (shown as red in panel c, circles) and the Cy5–labelled Firmicutes–specific probe LGC354MIX (shown as green in panel d); panels a and b show the bright–field image and the autofluorescence of the seed tissues, respectively; panel e is the overlap of the images b–d (arrows indicate coccoid Firmicutes cells). The second raw (panels f to j) shows the result after staining of a separate subsample with a mixture of Rhodamine red– and Cy 5–labelled nonsense NONEUB FISH probes. Parameters used for image acquisition were the same for both positive and negative subsamples. Scale bars: 20 μm (applies to all panels). pa = parenchyma of the seed coat (mesotesta); hg = hourglass-cells; b = bacteria

Staphylococcus spp. *ad planta*, however it is striking to notice that most of these seed endophytes show on plate plant growth promoting activities that could be beneficial to the host although the demonstration remains to be done in planta.

Both groups of cultivated taxa from *Anadenanthera* seed endosphere (*Methylobacterium* spp. and *Staphylococcus* spp.) were detected by pyrosequencing of seed metagenomic DNA. This confirms that their isolation is not a cultivation bias, but instead they are actually relevant members of the *Anadenanthera* seed endophytic microbiota. Barret et al. (2015) also detected both taxa by high throughput sequencing in *Brassicaceae* seeds not subjected to surface sterilization.

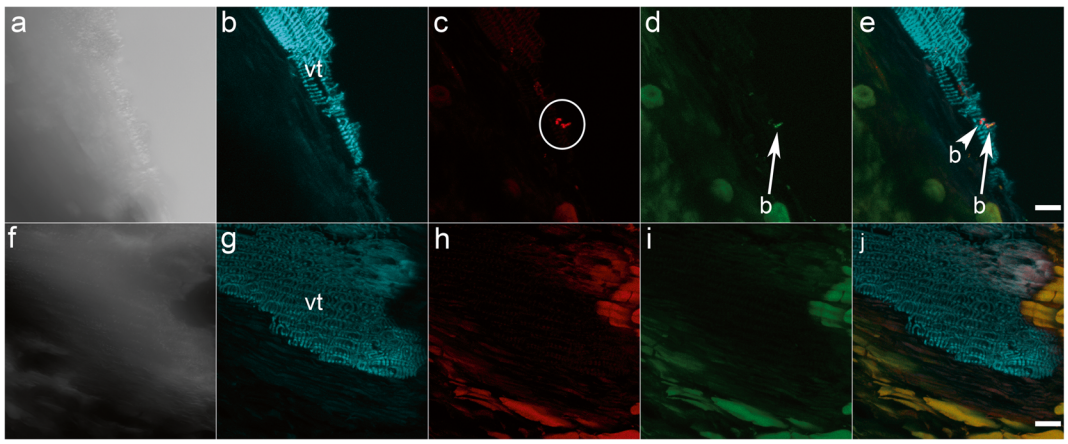

Fig. 5 Bacterial colonization of *A. colubrina* seeds. Confocal laser scanning microscopy images showing fluorescence in situ hybridization (FISH)–stained bacteria inside *Anadenanthera colubrina* seed cryosections. First raw shows the result after staining with the Rhodamine red–labelled universal FISH– probes EUB338MIX (shown as red in panel c, circles) and the Cy5–labelled Firmicutes–specific probe LGC354MIX (shown as green in panel d, arrow); panels a and b show the bright–field image and the autofluorescence of the seed tissues, respectively; panel e is the overlap of the images b–d (arrow indicates staphylococci–like Firmicutes cells, while arrowhead points to two additional cells stained by the universal probe EUB338MIX only). The second raw (panels f to j) shows the result after staining of a separate subsample with a mixture of Rhodamine red– and Cy 5–labelled nonsense NONEUB FISH probes. Parameters used for image acquisition were the same for both positive and negative subsamples. Scale bars: 10 μm (all panels). vt = vascular tissues of the tracheid bar; b = bacteria

Characterization of *Anadenanthera* seed microbiota by mean of metataxonomic analysis identified even more taxa, which is not surprising; all of them belong to the typical seed–associated bacterial phyla, although to the best of our knowledge new genera appear here for the first time as seed endophytes (Fig. 2): *Friedmaniella*, *Delftia*, *Anaerococcus*, *Bifidobacterium* and *Actinomyces*. Further taxa detected by pyrosequencing were *Pseudomonas*, *Corynebacterium* and *Microbacterium* (Fig. 2). It must be noticed that we could only retrieve few bacterial sequences, which was probably due to the low bacterial density inside the seeds more than to a PCR bias. This is supported by our microscopic observations and by the available data that indicate seed endophyte population sizes ranging from 55 colony-forming units (CFUs) per gram in bean (Rosenblueth et al. 2010) to as high as 10^7 CFUs g^{-1} in rapeseed (Granér et al. 2003). *A. colubrina* appears to be on the lower end of the wide spectrum of seed bacterial endophyte loads observed so far. We therefore suggest that retrieving a few bacterial sequences can be the expected result for low–abundance seed endosphere habitat, where it cannot be avoided plant DNA being drastically dominant.

Further cultivation–independent investigation is needed to assess the actual diversity of seed endophytes among a broader spectrum of plants, by applying a suitable sequencing efforts to unravel less abundant taxa. In fact, cultivation alone, although of paramount importance at this early stage of seed endophyte investigations (Donachie et al. 2007; Nichols 2007), does not deliver a satisfactory coverage of the total microbiota, as usual for almost all natural habitats where cultivable fraction rarely overcomes 1–3% of the total species (Staley and Konopka 1985; Hugenholtz et al. 1998; Hugenholtz 2002; Donachie et al. 2007).

Microscopic analysis of colonization pattern is a critical aspect when investigating natural host–associated microbial communities. Spatial organization at micro–scale, presence of favourite micro–niches of colonization, physical host–microbe and microbe–microbe interactions, and other similar features give additional useful information to complement (and confirm) cultivation–dependent as well as molecular analysis (Cardinale 2014; Cardinale and Berg 2015). In this work, we showed the bacterial niches of colonization in a seed endosphere after FISH staining, for the first time by confocal 3D–microscopy. We confirmed the presence of the taxa (Alphaproteobacteria and Firmicutes) detected by isolation, but also confirmed the occurrence of additional taxa coherently with the pyrosequencing outcome. The generally low cellular density confirms previous observations of seed–associated bacteria (Compant et al. 2011) and is coherent with our pyrosequencing results consisting of a quite low number of bacterial sequences. Therefore, it was not possible to perform a quantitative or semi–quantitative analysis of the microscopy images. Nevertheless, we detected mostly Alphaproteobacteria, which support the pyrosequencing results. Inside the seeds, most of the bacterial cells are expected to be in a semi–dormant state due to the long–term persistence and also because an intense metabolic activity could potentially affect the seed viability through, for example, consumption of the stored nutrients. However, we were able to localize cells and micro–colonies, especially in the intercellular spaces of the seed coat parenchyma, facing the cotyledons, and associated with the vascular tissues of the tracheid bar, and the intense FISH–conferred signals indicate that the ribosomal content is coherent with a metabolically active state. We hypothesize that the low bacterial abundance prevents the detrimental effects of the bacterial metabolic activity in the seed endosphere, while ensuring the sufficient inoculum to provide relevant ecological services to the plant after seed germination. This is coherent with the theory of "Dose–response relations in plant–endophyte interactions" (Partida-Martinez and Heil 2011). The detection of cell in the intercellular spaces is coherent with the typical colonization pattern of plant endophytes, which usually colonize apoplastic spaces (Cardinale 2014). Due to the scarce number of bacterial cells found, it remains to be confirmed whether the localization next to the vascular tissues have or not a biological meaning. However, the detection of bacterial cells associated with transport vessels supports the theory of internal translocation of endophytes (Maude 1996; Compant et al. 2005).

Conclusions and outlook

In this work we assessed, for the first time simultaneously, structure, potential functions and niches of colonization of a bacterial seed endophytic microorganisms, by coupling cultivation–dependent and–independent analyses corroborated by microscopical observations of *Anadenanthera colubrina* seed microbiota. We showed that, in this legume tree, diverse *Methylobacterium* spp. and *Staphylococcus* spp. are important and potentially active members of the seed microbiota, showing notable plant growth promotion traits. Our results add new and

robust evidences to the importance of the seed–endosphere as microbial habitat. Further investigations are needed to unravel the role(s) of seed endophytes for the different plants, their fate after seed germination and their effect(s) on the surrounding rhizosphere community during/after seed germination. Important ecological questions still need to be answered in the future, especially by cultivation–independent methods, including i) the level of specificity of the seed endophytic microbiota, ii) the assessment of the "core seed–microbiota" within and across plant species, iii) the environmental factors driving structure and composition of seed microbiota. Moreover, metagenomic approaches based on whole genome sequencing instead of metataxonomic analysis based on targeted 16S rRNA gene sequencing will be required to gain insight into the functional roles of seed endophytic microbiomes.

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