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Plant growth promoting potential of bacterial endophytes from three terrestrial mediterranean orchid species

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ABSTRACT

Orchids are highly dependent on symbiotic microorganisms during their entire life cycle. Whereas an important role in orchid seed germination and early plant development is well established for mycorrhizal fungi, the influence of endophytic bacteria on orchid growth has been less investigated. Here, we report the isolation of endophytic bacteria from different organs of three terrestrial Mediterranean orchid species (*Spiranthes spiralis, Serapias vomeracea* and *Neottia ovata*), the investigation of their potential Plant Growth-Promoting (PGP) traits and their interaction with the orchid mycorrhizal (OM) fungus *Tulasnella calospora in vitro*. Little overlap was found among endophytic bacteria isolated from the different organs of the three orchid species. Taxonomic identification, based on the 16S rRNA gene, of fifty dereplicated bacterial isolates revealed that they belong to the genera *Pseudomonas*, *Pantoea*, *Rahnella*, *Staphylococcus*, *Sphingomonas*, *Microbacterium*, *Streptomyces*, *Fictibacillus* and *Bacillus*. Most bacterial isolates exhibited some potential PGP traits, such as nutrient solubilization, ACC deaminase activities and/or IAA biosynthesis. Although some *Pseudomonas* reduced growth of the OM fungus *Tulasnella calospora*, most isolates did not affect fungal growth. These results increase our understanding of the diversity and potential PGP functions of bacterial endophytes in terrestrial orchids, and suggest a role as beneficial partners in the orchid microbiota.

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Terrestrial orchids; bacterial endophytes; plant growth promoting bacteria; mycorrhizal fungi; phytohormones

Introduction

Plant-bacteria interactions have been studied for several decades. Hiltner (1904) was the first to observe that microorganisms were more abundant in the soil surrounding the roots (the rhizosphere) than in the bulk soil far from the root. Subsequent investigations have clearly demonstrated that several plant-associated bacteria positively influence plant health and growth (Hardoim et al. 2008; Liu et al. 2017). Moreover, plants can select beneficial bacteria, including those living within their tissues as endophytes (Marasco et al. 2012; Rashid et al. 2012; Agnolucci et al. 2019).

Beneficial plant-associated bacteria, including endophytic ones, are also known as Plant Growth-Promoting Bacteria (PGPB). PGPB can affect plant growth through direct as well as indirect mechanisms (Gamalero and Glick 2011; Glick 2015). Direct mechanisms include mobilization of plant nutrients, such as iron and phosphorus, nitrogen fixation, and production of various phytohormones such as auxin, cytokines or ethylene (Patten and Glick 2002; Battini et al. 2017). Indirect mechanisms involve, for example, the ability of PGPB to reduce the deleterious effects of plant pathogens by producing antibiotics, lytic enzymes and siderophores, or by strengthening the plant defense responses by triggering induced systemic resistance (Thomashow et al. 1990; Arora et al. 2001; Whipps 2001).

In orchids, the largest family of monocotyledonous plants, the study of microbial endophytes has been mainly focused on mycorrhizal fungi because of their important role in orchid seed germination and early plant development (Rasmussen and Rasmussen 1991; Smith and Read 2008). Mycorrhizal fungi colonize the roots of both terrestrial and epiphytic orchids, where they form intracellular hyphal coils named "pelotons". Their taxonomic position largely reflects the habitat and photosynthetic abilities of the host. Basidiomycetes in the form-genus *Rhizoctonia* (a polyphyletic group comprising Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae) are commonly found in the roots of photosynthetic orchid species, whereas Asco- and Basidiomycetes able to form ectomycorrhizal symbioses with neighbouring plants usually colonize the roots of non-photosynthetic or partially photosynthetic orchid species (Selosse and Roy 2009). Various studies on mycorrhizal interactions in orchids have highlighted the role of the mycorrhizal fungal

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Figure 1. Photographs of the three Mediterranean orchids in their natural environment: a) *Spiranthes spiralis*. b) *Serapias vomeracea*. c) *Neottia ovata*.

symbionts as plant growth promoters (Yeh et al. 2019). By contrast, the diversity and role of endophytic bacteria have not been extensively explored in orchids, although bacterial endophytes have been identified in commercially valuable epiphytic orchids, such as *Vanilla* (White et al. 2014), *Dendrobium* (Yang et al. 2014; Pei et al. 2017) and *Cymbidium* (Gontijo et al. 2018) species or hybrids. A role in promoting orchid seed germination (Tsavkelova et al. 2016) and plant growth (Faria et al. 2013; Gontijo et al. 2018) was demonstrated in vitro for some of these bacterial endophytes, indicating their potential PGP activity. For terrestrial orchids, investigations have mostly focused on root-associated bacteria. Wilkinson et al. (1989, 1994) were the first to isolate endophytic bacteria from the roots of terrestrial Australian orchids, and Tsavkelova et al. (2007) more recently isolated bacterial endophytes from the roots of a Vietnamese orchid species. To our knowledge, no information is currently available on the bacteria inhabiting the tissues of Mediterranean orchids.

The aim of this work was to isolate and characterize endophytic bacteria colonizing different plant organs (roots, stems, leaves and capsules) of three Mediterranean orchid species. We hypothesized that each orchid species would harbor a fraction of potentially beneficial bacteria in some or all of their tissues, and that different tissues would be colonized by a different microbiota. Isolation and sequencing of the 16S rRNA was used to identify the bacterial isolates, while PGP-and antimicrobial tests were performed in vitro to identify possible beneficial functions of the orchid bacterial microbiota. We also investigated the influence of endophytic bacteria on the growth of the orchid mycorrhizal fungus *Tulasnella calospora* (Boud.) Juel, in order to understand possible interactions between bacterial and fungal orchid endophytes.

Materials and methods

Plant species

The plant species investigated in this work were *Spiranthes spiralis*, *Serapias vomeracea* and *Neottia ovata* (Figure 1). *Spiranthes spiralis* (L.) Chevall. is an herbaceous orchid that flowers in autumn with a particular spiral-shaped inflorescence. It is widely distributed in Southern Europe and in the Mediterranean region, where it grows in pine, oak, chestnut, hornbeam and birch forests, dry meadows as well as in flat grasslands and semi-rocky areas. The preferred substrate is both calcareous and siliceous, with neutral pH. The biological form of this orchid is rhizomatous geophyte, the rhizome is periodically generated every year with new roots and stems (Arditti 2002).

Serapias vomeracea (Burm. f.) Briq. is a bulbous herbaceous plant, with two underground globose rhizotubers and erect stems of purplish-vinous color and varying in height from 20 to 60 cm. The inflorescence, loose and elongated, is composed of a few spaced flowers. It can be found in sunny and wet meadows, on the edges of paths, in bushy environments from the plain up to 1200 m of altitude. It is the most widespread species in the genus *Serapias* and it is distributed in most Europe (Arditti 2002).

Neottia ovata (L.) Bluff & Fingerh. (homotypic synonym: *Listera ovata* (L.) R.Br) is a perennial rhizomatous orchid regularly found in a wide range of habitats including woods, shrubs, hedges, calcareous pastures, dunes and marshes and, to a lesser extent, meadows. It grows on acid and calcareous substrates in damp and cold woods, mainly conifers, on sphagnum moss and carpets, often together with blueberry (*Vaccinium myrtillus* L.) from 900 to 2100 m a.s.l. The lower capsules in the inflorescence can mature and disperse the seeds even before the flowers placed higher in the

inflorescence are pollinated. It is common throughout Europe (Arditti 2002).

Sampling and isolation of endophytic bacteria

The roots, stems, leaves and capsules of the three different orchids were harvested in autumn 2016 and spring 2017 in three areas in the Imperia (*Serapias vomeracea*), Savona (*Spiranthes spiralis*) and Genova (*Neottia ovata*) provinces (Liguria, Italy), stored on ice in sterile plastic bags or falcon tubes, transported to the laboratory and kept at 4 °C before processing, no later than two days after sampling. The plant samples from aerial organs (stems, leaves and capsules) were surface–sterilized by stepwise immersion in 70% ethanol for 1 min, then in 2.5% sodium hypochlorite for 2 min and finally in 70% ethanol for 1 min (Alibrandi et al. 2018), followed by five rinses in sterile distilled water. The root samples were thoroughly rinsed with sterile water, sonicated and surface-sterilized with 95% ethanol for 20 s followed by a treatment in 5% sodium hypochlorite for 3 min and washed seven times with sterile distilled water. In order to confirm that the sterilization process was successful, the last washing water (1 ml) and an imprinting of sterilized surfaces were incubated on different culture agar media (Alibrandi et al. 2018) for bacteria (Luria Bertani - LB, King'B) and examined for growth after 4–7 days at 28 °C. Five different individuals for each plant species were used for isolation.

To isolate endophytic bacteria, the surface–sterilized samples were individually immersed in falcon tubes with sterile distilled water for 1 h, grounded with a homogenizer, resuspended in 50 ml phosphate buffer saline (PBS: 140 mM NaCl, 3 mM KCl, 10 mM Na2 HPO4, 2 mM KH2PO4, pH 7.4) per 7.5 g of tissues, and finally shaken for 1 h. Fifty μl of the undiluted homogenates, as well as of 10–1 and 10–3 dilutions, were plated in duplicate on LB and King's B agar and incubated at 28 °C for 4 days.

The colonies obtained from each homogenized sample were selected on the basis of morphology and pigmentation, and individually replicated on the same agar medium to obtain pure cultures. The isolates were dereplicated first according to colony phenotype, and subsequently by comparing the 16S rRNA sequences. They were named according to the orchid species they were isolated from (SP for *Spiranthes spiralis*, SV for *Serapias vomeracea* and NO for *Neottia ovata*), followed by a number indicating the bacterial isolate and by a letter indicating the plant organ (R for root, L for leaf, S for stem, C for capsule).

Colony-PCR of the 16S rRNA gene and identification of isolates

The bacterial isolates were identified taxonomically on the basis of their 16S rRNA gene sequence, obtained by colony PCR with the universal bacterial primers 27 F and 1492 R (Frank et al. 2008), as previously described (Gallo et al. 2012). The PCR products were purified using NucleoSpin Gel and PCR Clean-up (MACHERY-NAGEL, Germany) and sequenced (BMR Genomics Srl, Italy, www.bmr-genomics.it).

Sequence chromatograms were visually checked with Geospiza's FinchTV software (PerkinElmer Inc., USA; www.geospiza.com/Products/finchtv.shtml) and used to reconstruct the 16S rRNA gene sequences. Sequences are available in GenBank (Accession numbers from MN624212 to MN624261). To identify the isolates, the reconstructed 16S rRNA gene sequences were aligned by BLAST against the Genbank of the National Center for Biotechnology Information (NCBI) database. A phylogenetic analysis was performed by aligning the 16S rRNA gene sequences to the nearest BLAST matches as well as to other reference sequences of each identified genus, using the Muscle alignment implemented in the megaX software (Kumar et al. 2018). Phylogenetic trees were inferred using the maximum likelihood methods, using RaxML (maximum randomized axillary probability) implemented in the open source CIPRES Science Gateway (https://www.phylo.org/). Five-hundred bootstrap resamplings per tree were generated, and values above 50% are shown at the branches. The trees were visualized and edited using the open source iTOL (Interactive tree of life, https://itol.embl.de/)

Screening for potential plant growth promoting (PGP) activities

All the bacterial isolates were evaluated for the following PGP traits: inorganic and organic phosphate solubilization, potassium solubilization, potential nitrogen fixation (measured as growth on N-free medium), siderophore production, 1–aminocyclopropane–1–carboxylate (ACC)-deaminase activity, and Indol Acetic Acid (IAA) production. Each test was performed at least in triplicate.

Inorganic– and organic phosphate solubilisation

Phosphorus in an essential nutrient but its availability to plants is low due to slow diffusion and high fixation in soil (Shen et al. 2011). All isolates were cultivated on broth media (either LB or King's B) for 1 day. Ten μL of the bacterial cultures (containing approximately 106 CFUs ml-1) were spotted on National Botanical Research Institute's phosphate growth agar medium (NBRIP), as described by Nautiyal (1999), containing the following ingredients (l − 1): glucose, 10 g; MgCl2·6H2O, 5 g; MgSO4·7H2O, 0.25 g; KCl, 0.2 g and (NH4)2SO4, 0.1 g, with one source of insoluble inorganic (calcium, aluminum or iron) or organic (phytate) phosphate for each assay. After 4 days of incubation at 30 °C, the plates were checked for bacterial growth and for the development of a solubilisation halo around the colonies. As many bacterial isolates could solubilize different sources of phosphate without forming a clearly distinguishable halo around the colony; bromophenol blue (0.075 g l-1) was added to the culture medium in order to enhance its visibility.

Potassium solubilisation

Potassium represents one of the main and essential plant nutrients, playing important biological and physiological

functions (Read et al. 2006). Potassium solubilizing bacteria decompose silicate minerals such as K-feldspar and mica, increasing the availability of potassium in the soil and promoting its absorption by plants (Friedrich et al. 1991; Sheng 2005; Basak and Biswas 2010). The bacterial isolates were grown in liquid medium and plated (10 µl, containing approximately 106 CFUs ml-1) on Aleksandrov agar medium (Parmar and Sindhu 2013) containing insoluble potassium as mica powder (3.0 g/l). After 7 days of incubation at 30 °C, the plates were checked for bacterial growth and for the development of a solubilisation halo around the colonies.

Quantification of indole-3-acetic acid (IAA) production

Tryptophan is the main precursor of IAA biosynthesis and redundancy of IAA biosynthetic pathways starting from tryptophan has been described in bacteria, although a tryptophan-independent pathway has been also suggested (Spaepen and Vanderleyden 2011). IAA production was detected with the modified colorimetric method of Gordon and Weber (1951), using microplates. All isolates were inoculated in LB and in LB supplemented with 0.1% L–tryptophan. The inoculated broth was incubated at 30 °C in a rotary shaker for 4 days. From the culture, 1 ml was centrifuged at room temperature for 10 min at 14,000 rpm with a Microstar 17 R centrifuge (VWR, Germany). Two volumes of Salkowski's reagent (Glickmann and Dessaux 1995) were added to 100 µl supernatant in the microplate wells. The plates were incubated at room temperature for 30 min and were then analyzed for red colour development using a Microplate Reader SpectroStar Nano (BMG Labtech) at 530 nm (OD530). Uninoculated broth and inoculated broth without Salkowski's reagent were used as negative controls, whereas *Pseudomonas aeruginosa* was used as positive control. Standard curve for quantification was prepared with 5 to 100 µg ml-1 of IAA.

Growth in nitrogen-free medium

All bacterial isolates were tested for their ability to grow on a nitrogen-free medium, indicative of potential capabilities as diazotrophs. Bacteria were inoculated in N-free semi-solid medium (NFb medium) prepared with washed agar and supplemented with 1% sucrose, as modified by Alibrandi et al. (2018). The plates were incubated at 30 °C and checked for growth after 3, 7 and 14 days from inoculation.

Siderophore production

Siderophore-production was tested by the chrome azurol sulphonate (CAS) assay (Schwyn and Neilands 1987). This assay was performed according to the method described by Arora and Verma (2017). CAS agar plates were prepared by mixing 100 ml CAS reagent with 900 ml sterilized LB agar medium. Four different bacterial strains were spot-inoculated on each plate. An uninoculated plate was used as negative control. After inoculation, plates were incubated at 30 °C for 5–7 days and observed for the formation of a colored halo around the bacterial colonies (Louden et al. 2011).

Salt and drought tolerance assays

Bacteria isolates were tested for salt and drought tolerance on the isolation medium (either LB or King's B), adjusted to 5%, 7.5% and 10% (w/v) NaCl (salt stress), or to 10% polyethylene glycol (PEG 6000; drought stress). Bacterial growth was monitored after 3, 7 and 14 days of incubation at 30 °C, and compared with growth on the same media without NaCl or PEG.

ACC-deaminase activity

The role of ACC deaminase in reducing ethylene levels has been suggested as one of the main mechanisms of plant growth promotion by bacteria under abiotic stress (Kumar et al. 2019). DF salt minimal medium amended with ACC was used as described by Penrose and Glick (2003) to test ACC-deaminase activity. Plates were checked for growth after 3, 7 and 14 days of incubation at 28 °C.

Bacterial endophytes - mycorrhizal fungus interaction and antibacterial activity

The influence of all dereplicated bacterial endophytes on mycorrhizal fungal growth was assessed using a modified protocol from Sen et al. (2009). Each bacterial isolate was inoculated in the center of a PDA (Potato Dextrose Agar) and a MEA (Malt Extract Agar) plate. Simultaneously, two agar plugs from an actively growing culture of the mycorrhizal fungus *Tulasnella calospora* (Boud.) Juel AL13, deposited in the mycological collection of the University of Turin (accession number MUT4182) were inoculated on the opposite sides of the same plate (ca. 1.5 cm from the bacterial inoculum). After 15 days of incubation at 28° C, the bacterium-fungus interaction was evaluated by comparing the co-cultures with the control plates containing only the fungus.

Antibacterial activity was assessed using *Kokuria rizophila* ATCC 10240 and *Escherichia coli* DH5α^{TM} (Invitrogen) as Gram-positive and Gram-negative tester strains, respectively, in agar–diffusion test (Scaffaro et al. 2015; Baldi et al. 2016). In particular, 5 ml of a bacterial suspension containing 108–109 colony-forming units (CFUs) per mL was prepared for each tester strain in warm LB soft–agar. Each bacterial suspension was then poured over R2YE plates containing patches of bacterial isolates on the surface, in order to obtain a bacterial tester strain overlay. After solidification of the LB soft–agar, plates were incubated at 37 °C to allow growth of the tester strain. Plates were then monitored for the presence of growth inhibition halos in the zone surrounding the bacterial isolate patches.

Table 1. Bacterial isolates from different orchid species and organs after phenotypic and molecular dereplication.

	Spiranthes spiralis				*Serapias vomeracea*				*Neottia ovata*			
Bacterial genus	Root	Stem	Leaf	Capsule	Root	Stem	Leaf	Capsule	Root	Stem	Leaf	Capsule
Pseudomonas	11	0	5	0	0	3	2	0	0	2	0	0
Pantoea	0	0	5	0	0	5	0	0	0	0	0	0
Rahnella	0	0	0	0	0	2	0	0	0	1	0	0
Staphylococcus	2	0	0	0	1	0	0	1	0	1	0	0
Microbacterium	1	0	0	0	2	0	0	0	0	0	0	0
Fictibacillus	0	0	0	0	1	0	0	0	0	0	0	0
Streptomyces	1	0	0	0	0	0	0	0	0	0	0	0
Sphingomonas	0	0	0	1	0	0	0	0	0	0	0	0
Bacillus	0	0	0	0	1	0	0	0	0	0	0	2
Total N. of isolates	**15**	**0**	**10**	**1**	**5**	**10**	**2**	**1**	**0**	**4**	**0**	**2**

Results

Isolation and identification of bacterial endophytes

After 5 days of incubation at 30 °C, numerous bacterial colonies grew on the agar media inoculated with surface-sterilized tissue homogenates, showing different colony morphology. A total of 139 isolates were obtained in pure culture (Table S1). In particular, 60 colonies were isolated from *S. spiralis*, 62 from *S. vomeracea* and 14 from *N. ovata*. Bacterial endophytes were isolated from all organs (roots, stems, leaves and capsules) only for *S. vomeracea*. Plate inoculation with the final washing water and surface imprinting of surface–sterilized organs did not result in any bacterial growth, thus confirming the successful surface sterilization of the plant organs. After comparison of their 16S rRNA gene sequences, the number of the isolated bacteria was reduced to a total of 50 dereplicated strains. Most of the 16S rRNA gene sequences showed very high similarities (>99%) with sequences in the NCBI database, and only for two isolates (SV_72S and SV_66S) the sequence similarity was slightly lower (Table S2). The isolates belonged to nine genera: *Pseudomonas*, *Pantoea*, *Rahnella*, *Staphylococcus*, *Sphingomonas*, *Microbacterium*, *Streptomyces*, *Fictibacillus*, *Bacillus* (Table S2). Phylogenetic trees were built to support the results of BLAST searches, but only for some bacterial isolates the phylogenetic analysis confirmed the taxonomic affiliation at species level (e.g., some *Pseudomonas*), whereas most isolates were not unequivocally grouped to a species (Figures S1–S9). The number of dereplicated bacterial strains obtained from the different organs and orchid species is summarized in Table 1 at genus level. In Figure 2, the phylogenetic position of the endophytic bacteria is summarised, together with their species and organ of isolation. The figure clearly shows that *Pseudomonas* and *Staphylococcus* were isolated from all orchid species, from hypogeous (roots) and epigeous (stems and leaves) organs (Table 1; Figure 2), while other genera showed a less widespread occurrence. In particular, *Pantoea* and *Rahnella* were exclusively found in stems and leaves, although they were not isolated from all three species (Table 1; Figure 2). In particular, endophytes belonging to the genus *Pantoea* were isolated from leaves and stems of *S. spiralis* and *S. vomeracea*, while those belonging to the genus *Rahnella* came only from stems of *S. vomeracea* and *N. ovata*.

In vitro testing for potential plant growth promoting activities

Activities commonly found in plant growth promoting (PGP) bacteria were assessed for all the dereplicated isolates from orchid tissues (Table 2). In particular, the ability to increase nutrient availability by solubilizing insoluble forms, to produce siderophores or to produce/modify phytohormones (IAA production and ACC deaminase activity) were measured. Some of these activities (e.g., phosphate and potassium solubilization, IAA production) were very commonly observed, whereas others, such as ACC deaminase activity and siderophore production, were more infrequent. In particular, all isolates in the genus *Pseudomonas* solubilized potassium, most of them solubilized calcium phosphate (SP_1L and NO_103S being the best solubilizers), while only few were able to solubilize the other phosphate forms. Most *Pseudomonas* isolates grew in the nitrogen-free medium, except SP_26L, SP_29R, SP_38R, SP_49R and SV_87L, while only five isolates (SP_25L, SP_38R, SP_41R, SV_63_S and SV_103S) showed ACC deaminase activity (Table 2). About 50% of the *Pseudomonas* isolates produced IAA when grown on medium supplemented with tryptophan, the major IAA producer being isolates SP_38R, SV_73S and SV_75S. About 37% of the *Pseudomonas* isolates produced siderophores (Table 2).

Isolates belonging to the genus *Pantoea* were the best IAA producers, with some IAA being measured also in the medium without tryptophan, and about 36% displayed ACC deaminase activity (Table 2). With the exception of SP_9L and SP_16L, all other *Pantoea* isolates could solubilize some insoluble phosphate forms. Solubilization was higher for calcium phosphate than for aluminum phosphate, and only three *Pantoea* isolates were able to dissolve organic phosphate. The three isolates in the genus *Rahnella* all produced IAA, SV_78S being the best producer, and solubilized all phosphate forms. By contrast, none of them produced siderophores. Isolates in the genera *Staphylococcus*, *Bacillus*, *Fictibacillus*, *Streptomyces*, *Microbacterium* and *Sphingomonas* did not show particular PGP traits as they were in general poor phosphate and potassium solubilizers. Although some of them synthesized low amounts of IAA on tryptophan-containing medium, none of them produced siderophores (Table 2).

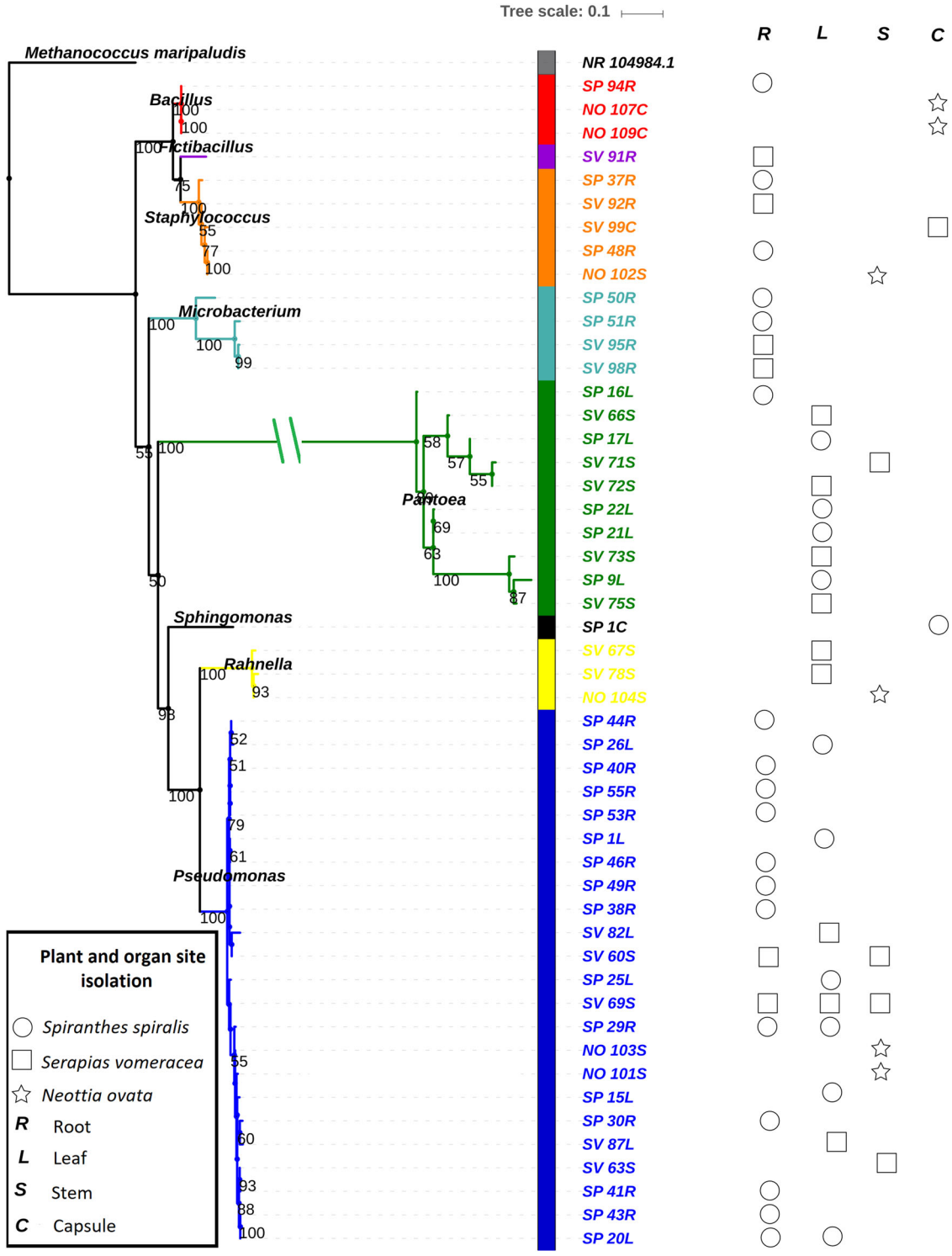

Figure 2. Phylogenetic tree of dereplicated endophytic bacteria and their plant-organ of isolation. The tree was generated using the RAxML method; the numbers at the branch nodes indicate the bootstrap percentage values obtained from 500 resampling (only bootstraps >50% are shown). Scale bar indicates substitutions per site. *Methanococcus maripaludis* (NR_104984.1) was used as outgroup.

Salt/drought tolerance

All isolates tested were able to grow in the presence of 10% PEG, a condition that mimicked drought stress (Table 3). The ability to grow in the presence of 5 and 7.5% NaCl was observed for all four *Staphylococcus* isolates, for 73% of the *Pantoea* isolates, and for about 35% of *Pseudomonas* isolates, irrespective of the organ of origin (Table 3).

Biotic interactions

None of the isolates tested, with the exception of the *Pseudomonas* isolate SP_26L, showed antimicrobial activity against the two bacteria tester strains *Kokuria rhizophila* and *Escherichia coli*, indicating poor antagonistic capabilities (Table 3). Similarly, most endophytic bacteria isolated from the different organs of the three orchid species did not

Table 2. Plant growth promoting activities of the bacteria isolated in this study, assessed by plate/liquid culture assays.

	PGP Tests[#]										
	Phosphate solubilization								IAA Production[###]		
Isolate	Ca-P[##]	Al-P	Fe-P	Ph-P	K Solubilization[##]	Putative diazotrophs	ACC deaminase activity	Siderofore production	LB	LB + Trp	Taxon Group
SP_1L	4.50	–	–	–	3.00	+/-	–	–	0.38 ± 0.01	0.86 ± 0.05	*Pseudomonas*
SP_15L	3.00	+/-	–	–	2.24	+/-	–	+	0.00	0.77 ± 0.03	*Pseudomonas*
SP_20L	–	–	–	–	2.00	+/-	–	–	4.00 ± 0.01	12.24 ± 0.04	
SP_25L	3.60	+/-	–	+	4.13	+/-	+	+	0.00	4.90 ± 0.04	
SP_26L	–	–	–	–	2.50	–	–	+	0.00	4.23 ± 0.02	
SP_29R	4.00	–	+	+	3.67	–	–	+	0.00	0.00	
SP_30R	–	–	–	–	2.31	+/-	–	+	0.00	0.00	
SP_38R	2.20	–	–	+	2.78	–	+/-	+	2.33 ± 0.04	43.93 ± 0.11	
SP_40R	–	–	–	–	2.45	+/-	–	++	1.65 ± 0.03	26.17 ± 0.34	
SP_41R	2.40	+/-	–	+	2.39	+/-	++	+	0.00	0.00	
SP_43R	2.40	+/-	–	+	2.25	+/-	–	–	0.00	0.00	
SP_44R	2.40	+/-	+	–	2.37	+/-	–	–	0.00	0.00	
SP_46R	2.40	–	–	–	2.67	+/-	–	++	0.00	0.00	
SP_49R	2.60	+/-	–	+	3.11	–	–	–	0.00	0.00	
SP_53R	2.80	–	+	+	2.58	+/-	–	–	3.15 ± 0.03	4.11 ± 0.00	
SP_55R	–	–	–	–	2.59	+/-	–	+	0.98 ± 0.00	6.90 ± 0.02	
SV_60S	2.80	–	+/-	+/-	3.06	+/-	–	–	0	17.40 ± 0.14	
SV_63S	3.00	+/-	+/-	+/-	2.17	+/-	++	+	0.00	5.28 ± 0.01	
SV_69S	3.50	+/-	–	+/-	2.33	+/-	–	–	0.00	8.97 ± 0.02	
SV_82L	2.43	–	+/-	+	2.53	+/-	–	–	0.00	28.47 ± 0.23	
SV_87L	3.29	+/-	+/-	+	2.23	–	–	–	0.00	5.32 ± 0.03	
NO_101S	3.20	+/-	+	+	4.38	+/–	–	–	0.00	2.593 ± 0.06	
NO_103S	4.20	+/-	+	+	3.67	+/-	+	–	0.00	0.00	
SP_9L	3.00	–	–	–	3.18	+	+	–	4.05 ± 0.02	24.79 ± 0.04	*Pantoea*
SP_16L	–	–	–	–	3.83	+	+/-	–	5.66 ± 0.02	19.236 ± 0.08	
SP_17L	3.00	+/-	+	–	3.69	+	+/-	+	6.06 ± 0.03	27.975 ± 0.03	
SP_21L	3.40	+/-	+	+	3.17	+	–	–	3.21 ± 0.02	28.25 ± 0.05	
SP_22L	3.60	+/-	+	+	3.28	+	–	–	7.26 ± 0.01	49.71 ± 0.35	
SV_66S	3.33	+/-	+	+	2.00	+/-	–	–	4.70 ± 0.05	21.91 ± 0.01	
SV_71S	4.17	+/-	+/-	+/-	0.00	+/-	–	+	0.00	77.97 ± 0.09	
SV_72S	2.17	+/-	+/-	+	2.50	+/-	–	+	1.98 ± 0.01	49.33 ± 0.23	
SV_73S	2.17	+/-	+/-	+	0.00	–	–	+	1.23 ± 0.01	108.47 ± 0.25	
SV_75S	2.33	+/-	+/-	+/-	0.00	–	–	–	0.12 ± 0.00	67.40 ± 0.26	
SV_67S	3.33	+/-	+	+	3.87	+/-	+/-	–	0.00	6.89 ± 0.02	*Rahnella*
SV_78S	2.50	+/-	+	+	1.58	–	+/-	–	0.00	97.43 ± 0.24	
NO_104S	4.40	+/-	+	+	1.63	+/-	–	–	0.00	35.32 ± 0.39	
SP_37R	–	–	–	–	0.00	–	–	–	0.00	0.00	*Staphylococcus*
SP_48R	–	–	–	–	0.00	–	–	–	0.00	0.00	
SV_92R	–	–	–	–	0.00	–	–	–	1.37 ± 0.01	8.292 ± 0.01	
SV_99C	–	–	–	–	3.43	–	–	–	0.00	1.052 ± 0.01	
NO_102S	–	–	–	–	0.00	–	–	–	0.00	0.00	
SV_94R	–	–	–	–	0.00				2.35 ± 0.50	3.23 ± 1.20	*Bacillus*
NO_107	–	–	–	–	0.00	–	–	–	1.94 ± 0.02	2.56 ± 0.27	
NO_109C	–	+/-	–	+/-	0.00	+/-	+/-	–	0.00	0.00	
SP_50R	2.60	–	+	+	2.88	+	–	–	0.00	0.00	*Streptomyces*
SP_51R	–	–	–	–	0.00	+/-	–	–	0.00	14.29 ± 0.04	*Microbacterium*
SV_R95	–	+/-	–	–	0.00	–	–	–	0.00	19.33 ± 0.25	
SV_98R	–	+/-	–	–	0.00	–	–	–	0.00	17.07 ± 0.07	
SV_91R	–	–	–	–	2.25	+	+	–	0.00	33.67 ± 0.09	*Fictibacillus*
SP_1C	-	–	–	–	0.00	+	–	-	0.00	0.00	*Sphingomonas*

[#] + growth – No growth; +/– Reduced growth.
[##] solubilization index (SI).
[###] IAA values ± SD (μg ml^{-1}).
Ca-P = Calcium phosphate; Al-P = Aluminum phosphate; Fe-P = Iron phosphate; Ph-P = Phytate phosphate.

inhibit growth of the orchid mycorrhizal fungus *Tulasnella calospora*, as fungal growth in co-culture was similar to the control plates (Table 3 and Figure 3). Only few isolates belonging to the genus *Pseudomonas* (SP_15L, SP_26L, SP_29R, SP_40R, SP_41R and SV_63S) caused a reduction of fungal growth, and one completely inhibited T. calospora (SP_30R). Isolates with inhibitory activity on *T. calospora* were derived from all vegetative organs. Interestingly, the NO_103S isolate appeared to induce fungal growth, as the mycelium grew over the bacterium (Figure 3e).

Discussion

We have isolated endophytic bacteria from three species of Mediterranean terrestrial orchids and characterized them by a number of assays in order to elucidate their potential beneficial functions as plant growth promoting (PGP) bacteria, their salt and drought tolerance and their interactions with other components of the orchid microbiota. PGP bacteria can stimulate growth of their host plant by producing phytohormones or by the synthesis of ACC deaminase, which lowers plant ethylene levels (Glick 2015). In addition, PGP bacteria can facilitate plant growth by nitrogen fixation, or

Table 3. Stress–tolerance, antimicrobial activity and interaction with the orchid mycorrhizal fungus *Tulasnella calospora* (AL13/4D) of the bacteria isolated in this study.

	Stress test[#]						
	NaCl (%)			Antimicrobial activity[##]		Growth of mycorrhizal fungus in co-culture[###]	
Isolate	5	7,5	PEG 10%	*Kokuria rhizophila*	*Escherichia coli*	*T. calospora* AL13 / 4D	Taxon Group
SP_1L	+/–	+/–	+	–	–	n.a	*Pseudomonas*
SP_15L	+/–	–	+	–	–	reduced	
SP_20L	+/-	+/-	+	–	–	n.a	
SP_25L	–	–	+	–	–	n.a	
SP_26L	+	+	+	+	+	reduced	
SP_29R	–	–	+	–	–	reduced	
SP_30R	–	–	+	–	–	complete inhibition	
SP_38R	–	–	+	–	–	n.a	
SP_40R	–	–	+/-	–	–	reduced	
SP_41R	+/-	+/-	+	–	–	reduced	
SP_43R	–	–	+	–	–	n.a	
SP_44R	–	–	+/-	–	–	n.a	
SP_46R	+/–	+/–	+	–	–	n.a	
SP_49R	+/–	+/–	+	–	–	n.a	
SP_53R	–	–	+/-	–	–	n.a	
SP_55R	–	–	+/-	–	–	n.a	
SV_60S	–	–	+	–	–	n.a	
SV_63S	–	–	+	–	–	reduced	
SV_69S	–	–	+	–	–	n.a	
SV_82L	–	–	+	–	–	n.a	
SV_87L	–	–	+	–	–	n.a	
NO_101S	–	–	+	–	–	n.a	
NO_103S	+/-	+/-	+/-	–	–	increased	
SP_9L	+/-	+/-	+	–	–	n.a	*Pantoea*
SP_16L	+/-	+/-	+	–	–	n.a	
SP_17L	–	–	+	–	–	n.a	
SP_21L	+/-	+/-	+	–	–	n.a	
SP_22L	+/-	+/-	+	–	–	n.a	
SV_66S	+	+	+	–	–	n.a	
SV_71S	–	–	+	–	–	n.a	
SV_72S	+	+/-	+	–	–	n.a	
SV_73S	+	+	+/-	–	–	n.a	
SV_75S	–	–	+	–	–	n.a	
SV_67S	+/-	+/-	+	–	–	n.a	*Rahnella*
SV_78S	+/-	+/-	+/-	–	–	n.a	
NO_104S	–	–	+/-	–	–	n.a	
SP_37R	+/-	+/-	+/-	–	–	n.a	*Staphylococcus*
SP_48R	+/-	+/-	+/-	–	–	n.a	
SV_92R	+/-	+/-	+/-	–	–	n.a	
SV_99C	+	+	+	–	–	n.a	
NO_102S	+/-	+/-	+/-	–	–	n.a	
SV_94R	–	–	+	–	–	n.a	*Bacillus*
NO_107C	–	–	+	–	–	n.a	
NO_109C	+/-	+/-	+	–	–	n.a	
SP_50R		–	+/-	–	–	n.a	*Streptomyces*
SP_51R	nt	nt	nt	–	–	n.a	*Microbacterium*
SV_R95	nt	nt	nt	–	–	n.a	
SV_98R	nt	nt	nt	–	–	n.a	
SV_91R	–	–	+	–	–	n.a	*Fictibacillus*
SP_1C	–	–	+/-	–	–	n.a	*Sphingomonas*

[#]+ 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.
[###]n.a. not affected.

assist plants in the acquisition of phosphorus and other essential minerals, improve water uptake or act as biocontrol agents by decreasing the inhibitory effects of various phytopathogens (Glick 2015). Despite these important roles, endophytic PGP bacteria have been poorly investigated in terrestrial orchids, where most attention has been focused on the mycorrhizal fungal endophytes colonizing orchid roots (Smith and Read 2008).

Most endophytic bacteria were isolated from the vegetative organs of the three terrestrial orchid species. They belonged to three different phyla: Proteobacteria, Actinobacteria, and Firmicutes. Within the Proteobacteria, thirty-six isolates belonged to the γ-class (*Pseudomonas*, *Pantoea*, *Rahnella*) and one isolate to the α-class (*Sphingomonas*). *Pseudomonas* strains were mainly isolated on King's B, a classical medium for isolation of fluorescent *Pseudomonas* (King et al. 1954). Nine strains grouped within the Firmicutes (*Staphylococcus*, *Bacillus*, *Fictibacillus*) and four within the Actinobacteria (*Streptomyces*, *Microbacterium*).

Pseudomonas is a widespread genus of Gram-negative bacteria commonly associated with the rhizosphere (Lugtenberg and Dekkers 1999). Because of their metabolic versatility and

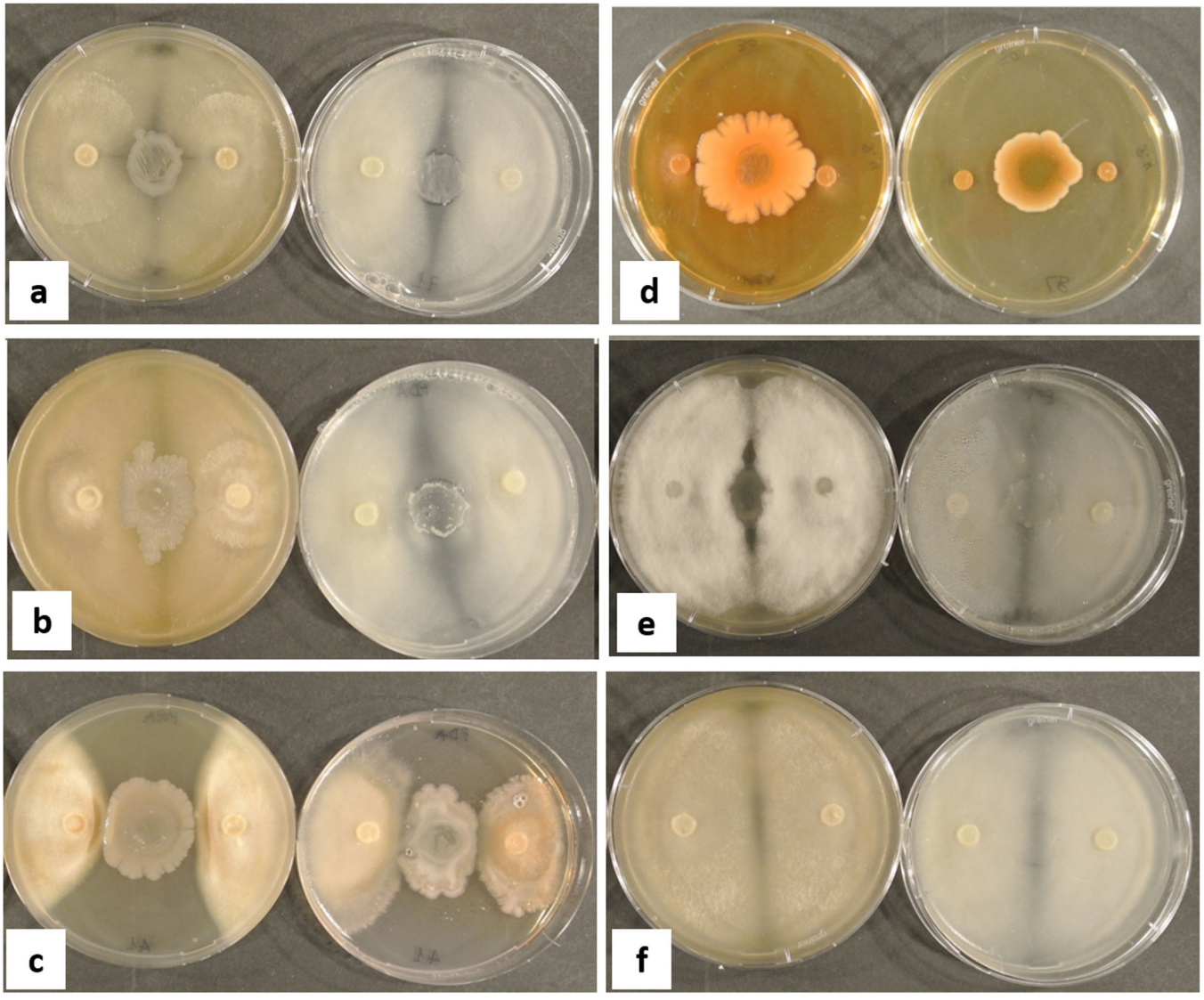

Figure 3. Dual-plate interaction assays of endophytic bacteria (at the center of the plates) and the fungal symbiont (*Tulasnella calospora*) of *Serapias vomeracea* (at the two sides of the same plate). All photos were taken 15 days after inoculation on two different solid media (MEA on the left and PDA on the right for each photograph, see materials and methods). (a) SP_48R isolate showing no inhibition; (b) SV_92R isolate showing no inhibition; (c) SP_41R isolate showing reduced fungal growth, as compared to the control; (d) SP_30R isolate showing complete fungal growth inhibition; (e) NO_103S isolate showing an increase of aerial mycelium, as compared to the control, produced by the fungus on MEA medium; (f) Control conditions, with fungal inocula growing in the absence of bacteria.

ubiquity, *Pseudomonas* species colonize a wide range of natural habitats and adopt a variety of lifestyles. They have been also isolated from a great variety of hosts and ecological niches within the hosts (Mercado-Blanco and Bakker 2007), where they can live as symbionts or as parasites. *Pseudomonas* have been found as endosymbionts in Australian terrestrial orchids (Wilkinson et al. 1989, 1994), as well as in the roots of epiphytic orchids (Tsavkelova et al. 2004). Bacterial endophytes in the genus *Pseudomonas* represented the largest group and were isolated from all three orchid species, although most isolates were found in the roots of *S. spiralis* where they represented the dominant taxonomic group. *Pseudomonas* were not isolated from the roots of the other two orchid species, suggesting some host preference. Some *Pseudomonas* strains associated with the roots of *S. spiralis* were the best siderophore producers among the isolated endophytes. Siderophores are iron chelating compounds that confer competitive advantage to PGP bacteria during the colonization of plant roots, especially in conditions of iron limitation. This mechanism not only ensures iron to the host, but makes this micronutrient less available to different microrganisms, including pathogens (Kloepper et al. 1980; Weller 2007). In addition, most *Pseudomonas* isolates could solubilize insoluble phosphate and potassium compounds and some strains produced indolacetic acid (IAA), a property that stimulates and facilitates the growth of plants. In fact, as demonstrated by Pavlova et al. (2017), P. fluorescence is able to colonize seeds of Dendrobium nobile Lindl (an epiphytic orchid) and induce their germination. Overall our findings suggest that *Pseudomonas* orchid endophytes can use different mechanisms to promote plant growth.

The genus *Pantoea*, in the Enterobacteriaceae, is a highly diversified group of Gram-negative bacteria found in aquatic and terrestrial environments in association with plants and animals (Brady et al. 2008; Völksch et al. 2009; Nadarasah and Stavrinides 2014; Rahman et al. 2018). Endophytes in the

genus *Pantoea* have been commonly isolated from plants such as, grapevine, pea and rice (Elvira-Recuenco and van Vuurde 2000; Mano et al. 2007; Bell et al. 2011). Although *Pantoea* has been isolated from a variety of plant species and organs, we isolated *Pantoea* strains only from the aerial parts of *S. spiralis* and *S. vomeracea*. Interestingly, bacteria belonging to the genus *Pantoea* could not be isolated from the roots of the terrestrial orchid species *Paphiopedilum appletonianum*, whereas they were isolated from the aerial roots of the epiphytic orchid *Pholidota articulata* (Tsavkelova et al. 2007). This finding may indicate a preference for the colonization of aerial parts in orchids irrespective of the plant organ. The PGP effects of *Pantoea* endophytes for the host plants are well documented, including production of auxin and cytokines (Tsavkelova et al. 2007; Chalupowicz et al. 2009), ACC–deaminase activity (Zhang et al. 2011), siderophores production (Loaces et al. 2011), phosphate solubilization (Son et al. 2006; Castagno et al. 2011), induction of systemic resistance (Trotel-Aziz et al. 2008; Rahman et al. 2018). The results obtained for the *Pantoea* endophytes isolated from the two Mediterranean orchids indicate a possible role of orchid endophytic *Pantoea* in both hormone–induced enhancement of plant growth and support of plant mineral nutrition. In fact, most of them showed high (for some isolates very high) production of IAA and different abilities to solubilize insoluble phosphate and potassium forms. All *Pantoea* isolated from leaves of *S. spiralis* were able to grow on semisolid N–free medium, indicating possible nitrogen fixation, as reported for some *Pantoea* species (Loiret et al. 2004).

Although members of the genus *Rahnella* commonly colonize the rhizosphere (Rozhon et al. 2010), they have been isolated as endophytic bacteria from root samples, stems and surface-sterilized seeds of plants grown in contaminated or marginal soils (Cankar et al. 2005; Taghavi et al. 2009). In this work, we isolated three strains belonging to the genus *Rahnella* from stems of *S. vomeracea* and *N. ovata*. Plant-associated *Rahnella* usually showed multiple PGP properties (Kumar et al. 2009). For example, *R. aquatilis* NBRIK3 and *Rahnella* sp. BIHB 783 (Vyas et al. 2010) produced IAA, siderophores, ACC deaminase and solubilized inorganic phosphate. The *Rahnella* endophytes from the two Mediterranean orchids were able to solubilize both organic and inorganic phosphates, as well as potassium.

Most bacterial endophytes could grow on culture media containing NaCl (ca. 50% of all isolates) and 10% PEG (all tested isolates) indicating their ability to tolerate abiotic stress such as salinity and osmotic stress. Whether this indicates an adaptation to the Mediterranean climate (characterized by dry seasons) or whether it represents an additional potential role as abiotic stress alleviators to the host plant, remains to be clarified.

A beneficial interaction between *Pseudomonas* bacteria and mycorrhizal fungi has been described both as a helper effect of the bacterium on fungal growth and as a synergistic mechanism during mycorrhiza formation. For example, some authors suggested for helper *Pseudomonas fluorescens* strains a specific priming effect on growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* (Deveau et al. 2007). Furthermore, *Pseudomonas* sp. pl. isolated from *Tuber borchii* ascocarps (Sbrana et al. 2000) showed antifungal activity against some pathogens. As for orchid mycorrhizal fungi, a number of bacterial taxa were found to be associated with a *Serendipita* sp. isolate by molecular methods, but their interactions with the fungus were not investigated (Novotná and Suárez 2018).

Most of the bacterial endophytes isolated from the three Mediterranean orchid species did not show any antimicrobial activity or particular influence on the growth of the orchid mycorrhizal fungus *T. calospora*, irrespective of the organ of origin, and only the strain (SP_26L) inhibited the growth of bacteria tester (*K. rizophila* and *E. coli*). Only some *Pseudomonas* isolates showed antimicrobial activity and caused a reduction of fungal growth, whereas one strain (NO_103S) induced fungal growth. Although the outcome of in vitro tests may not reflect the situation in planta, these data would suggest a limited antagonistic ability of endophytic bacteria toward other members of the orchid microbiome. However, cultivable microbes are only a minor fraction of the total community and it is therefore possible that important bacterial partners, potentially interacting with the mycorrhizal fungus or with other endophytic bacteria, could not be tested in the current study. A functional traits-based cultivation-independent analysis or a co-occurrence analysis would be necessary to further address this important point. Anyhow, since there are to our knowledge no specific investigations on the interactions between endophytic bacteria and orchid mycorrhizal fungi, our preliminary results pave the way towards new hypotheses on the interactions between endophytic bacteria and symbiotic fungi in orchids. The significance of growth attenuation and inhibition in T. calospora remains to be better clarified

Most bacterial endophytes could grow on culture media containing NaCl (ca. 50% of all isolates) and 10% PEG (all tested isolates) indicating their ability to tolerate abiotic stress such as salinity and osmotic stress. Whether this indicates an adaptation to the Mediterranean climate (characterized by dry seasons) or whether it represents an additional potential role as abiotic stress alleviators to the host plant, remains to be clarified.

In conclusion, although research on endophytic bacteria is still scanty in orchids, the PGP traits identified in this study suggest that these components of the orchid microbiota may potentially play important roles in promoting growth of Mediterranean terrestrial orchids. A demonstration remains to be done in planta during both seed germination and early plant development. If these endophytic bacteria reveal plant growth promotion activities in orchids, they could also be tested on other orchid species to support in vitro growth in the frame of conservation programs.

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Disclosure statement

The authors declare that they have no conflict of interest.

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