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ORIGINAL PAPER

Dendrobium nobile Lindl. seed germination in co-cultures with diverse associated bacteria

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Abstract The conservation of orchids is challenging due to their strong biotic relations and tiny seeds, requiring mycorrhiza for germination. This is aggravated when tropical plants are maintained in artificial conditions of greenhouses. We aimed to select the plant growth promoting rhizobacteria (PGPR) for orchid seed germination, to study plant–microbial interactions, and to determine whether there is any specificity between two species of *Dendrobium* plants in choosing bacterial partners. By the isolation of rhizoplane and endophytic rhizobacteria from *Dendrobium moschatum* roots, the known PGPR (*Azospirillum*, *Enterobacter*, *Streptomyces*) and less popular (*Roseomonas*, *Agrococcus*) strains were tested for the production of biologically active auxin. The bacterization of another orchid, *D. noblie*, with several newly selected strains and previously isolated ones (*Mycobacterium* sp., *Bacillus pumilus*) revealed that the orchids did not express evident specificity in relations with favorable bacteria, but refused to establish associations with *Streptomyces* and *Azospirillum*. Endophytic *Agrococcus* and *Sphingomonas* strains showed significant promotion of orchid germination. *Mycobacterium* and *B. pumilus* were also stable in their positive influence on the acceleration of *D. noblie* seed development. The active colonization of the seed surface and the inner tissues by associated bacteria was observed under electron microscopy. The analysis of orchid–bacteria relations was made. Altogether, the data shows that selection provides a good strategy for choosing the active strains for orchid seeds' bacterization, since not all known PGPR are useful and successful in building associative frameworks with orchid seeds. The stable activity of the strains guarantees their long-term and effective application in orchid in vitro biotechnology.

Keywords Orchid-associated rhizobacteria · Endophytes · Orchid seed bacterization · Auxin production · Scanning electron microscopy

Introduction

The genus *Dendrobium* is one of the largest genera of the *Orchidaceae* Juss. family. Although orchid seeds are produced in thousands and millions per one seed capsule, they are the smallest by size (0.05–6 mm) or weight (0.31–24 μg) among the seed-bearing plants (Roberts and Dixon 2008), they have no endosperm, and they only germinate in symbiosis with an appropriate fungus (Smith and Read 2008). A symbiotic in vitro orchid germination requires complex nutrient media supplemented with various substances, vitamins and plant growth stimulators (Teixeira da Silva et al. 2015). The stable relations between the host-plant and plant growth promoting rhizobacteria (PGPR) are determined due to nitrogen fixation, production of antimicrobials and plant growth regulators, solubilization of

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minerals, and such bacterial activities as lowering the ethylene level in plants or enhancing plant resistance under diverse abiotic stress conditions (Ryan et al. 2008; Saharan and Nehra 2011; Ahemad and Kibret 2014; Passari et al. 2015). A number of PGPR capable of auxin (indole-3-acetic acid, IAA) production has been reported among different species of bacteria (Khalid et al. 2004; Tsavkelova et al. 2006; Spaepen et al. 2007; Ryan et al. 2008; Ahemad and Kibret 2014; Habibi et al. 2014).

Previously we showed the differences in bacterial communities colonizing the aerial and substrate roots of several epiphytic and terrestrial greenhouse and wild grown orchids (Tsavkelova et al. 2004, 2007a; reviewed in Teixeira da Silva et al. 2015). The first mention of the orchid–bacteria interactions was made by Knudson (1922). Wilkinson et al. (1989, 1994) showed that mycorrhizal fungus co-inoculated with *Pseudomonas* and *Bacillus* strains promoted the germination of the terrestrial *Pterostylis vittata* seeds. We showed the formation of microbial consortia consisting of fungi, heterotrophic bacteria and cyanobacteria on the roots of epiphytic orchids as well as the capacity of pure bacterial cultures to promote orchid seed germination (Tsavkelova et al. 2007b).

Nowadays, the interest in this subject is rising due to investigation of orchid-associated bacteria from tropical (Galdiano Júnior et al. 2011; Faria et al. 2013; Yang et al. 2014) and temperate regions (Shekhovtsova et al. 2013). Faria et al. (2013) showed that several *Paenibacillus* strains promoted the development and growth of *Cattleya loddigesii* Lindl. seedlings. The strains of *Bacillus* sp. and *Enterobacter* sp. improved acclimatization and plantlet survival of *Cattleya walkeriana* Gardn. (Galdiano Júnior et al. 2011). While the above-mentioned studies described the treatment of already germinated plantlets, we focused on the role of rhizobacteria in the first phases of seed germination. There is much data on the successful application of diverse beneficial microbial strains promoting growth of different crop plants (e.g. reviewed in Tsavkelova et al. 2006; Ahemad and Kibret 2014). However, the specificity of the orchid biology restricts the number of possible PGPR strains that can be used in orchid–microbial biotechnology. Moreover, orchid seed germination is a long term process, and it requires selective strategies for choosing the optimal species and the strains of bacteria.

In this study, our purpose was to study whether there is any specificity between two *Dendrobium* species in choosing their bacterial partners among several previously (Tsavkelova et al. 2007b) and newly (endophytes) isolated strains. Bacteria isolated from the roots of the *Dendrobium moschatum* (Buch.–Ham.) Swartz were taken for seed bacterization of another *Dendrobium*, *D. nobile* Lindl. Together with the search for potentially rarely used PGPR, we aimed to test the strains, usually recognized as PGPR of the crop plants, for their capacity in orchid seed germination. In order to examine the direct influence of orchid-associated bacteria on acceleration of seed development, we analyzed the strains for the production of indole-3-acetic acid under supplementation of different sources of exogenous tryptophan as a potential inducer of microbial auxin biosynthesis. We also wanted to show the stability of selected strains in their long-termed application for orchid germination and thus, plant conservation. To estimate if the beneficial bacteria establish the endophytic lifestyle, their preferred localization when interacting with the seeds and nascent sprouts was examined by electron microscopy.

Materials and methods

Sampling of the roots and isolation of orchid-associated bacteria

For the selection of bacteria that might be used for orchid seed bacterization technique, the cultivable microbial cultures can only be used. Thus, all of the investigated strains were isolated by using the standard methods of single colony isolation. *Mycobacterium* sp. and *Bacillus* sp. isolated previously (Kolomeitseva et al. 2002; Tsavkelova et al. 2004), were active in the promotion of *D. moschatum* seed germination. We wonder if the bacterial cultures selected from one *Dendrobium* species were effective to another species, *D. nobile*. Both plants are held in the Stock greenhouse of the Main Botanical Garden (Moscow) and they are cultivated as pot plants. The bacteria were isolated on the day of sampling. For the isolation of rhizoplane bacteria, the roots were cleaned from the pine bark, rinsed in the sterile tap water, grounded with pestle in the phosphate buffer saline to prepare initial suspension and the tenfold dilutions (Tsavkelova et al. 2007a); the aliquot of 0.1 ml was plated onto the two selective media with nystatin (50 mg ml^{-1}) to prevent the fungal growth. Tryptic soy agar (Oxoid, UK) and modified Czapek agar supplemented with yeast extract were used. For the isolation of endophytic bacteria, the roots were sterilized by immersing for 5 min in 10 % household bleach, followed with triple rinsing in distilled water. After incubation under 28 °C until single colonies could be detected, each different colony was isolated. On the basis of differences in colony characteristics, such as size, color, shape, texture, consistency, and mucilage formation, a total of 100 isolates were taken for cultivation.

Identification of the strains

Bacterial cultures tested for auxin biosynthesis (see below) and producing enough biomass on the examined nutrient media,

were preliminary analyzed based on such routine morphological and biochemical characteristics as cell morphology, Gram reaction, the presence of oxidase and catalase activities, mycelium (for *Actinomycetes*) and spore formation. The analysis of the double strand 16S rRNA gene sequences has been performed for the cultures that showed active production of IAA. For this aim, the freeze-dried bacterial biomass was homogenized with a Mini-BeadBeater-8 (BioSpec Products, USA). DNA extraction was made by standard phenol–chloroform procedure; PCR amplification of 16S rRNA was performed by using reagents of primers designed in Sintol (Moscow, Russia) and using the "Biometra Tpersonal" thermocycler (Germany). Two pair of primers were used: the first one amplifying nearly full-length of 16S ribosomal DNA (rDNA) B63f (5′-CAG GCC TAA CAC ATG CAA GTC-3) and B1387r (5-GGGCGGWGT GTA CAA GGC-3′) (Marchesi et al. 1998), and partial-length UNIV 515F (5′-GTGBCAGCMGCCGCGGTAA-3′, Kublanov et al. 2009) and BACT 907R (5′-CCGTCAATTCMTTTGAGTTT-3′, Muyzer et al. 1998). Purified genomic DNA was used as a template in the PCR reactions. The DNA amplification was performed in 25 µl mixtures using 10 ng genomic DNA, PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.25 mM dNTPs, and 0.06 U μl^{-1} Taq polymerase (Sintol, Russia). PCR conditions were as follows: 1 cycle of 3 min at 94 °C; 30 cycles of 20 s at 90 °C, 30 s at 55 °C, and 1 min at 72 °C; and a final elongation for 4 min at 72 °C. 2–4 µl of the PCR product were analyzed by electrophoresis in a 1 % agarose gel with TAE 1× buffer at 80 V for 30 min. DNA GeneRuler™ 1 Kb DNA ladder (MassRuler DNA Ladder Mix, Thermo scientific, Germany) was used as control. The amplified fragment of the 16S rRNA gene was purified using Agencourt AMPure XP (Beckman Coulter) and sequenced on an ABI 3730 automated DNA sequencer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The standard procedures recommended by the manufacturer were performed, and primers mentioned above were used. The analysis of the sequences of the 16S rRNA genes was done with software Lasergene (DNASTAR) and VectorNTI (Invitrogen). For comparative analysis and homologous sequence searches the NCBI (National Center for Biotechnology Information website; http://www.ncbi.nlm.nih.gov/blast) and Ribosomal Database Project (RDP, http://rdp.cme.msu.edu) databases were used.

Bacterial auxin production and colorimetric assay for indole-3-acetic acid determination

To estimate the differences in influence of exogenous tryptophan, we tested it in concentrations of 200 and 400 µg ml^{-1}, as well as using different tryptophan (Trp) forms—its optical active L-form and racemic DL-mixture. All combinations were added to the mineral (Modified Czapek medium, MCM) and to the organic (triple diluted TSB medium) nutrient media. The measurements of produced IAA were taken in dynamics (every 24 h) till the maximal concentration was reached. Indoles content was estimated by the Salkowski method (Gordon and Weber 1951) as we previously described (Tsavkelova et al. 2007a, b). Uninoculated medium served as the control. The standard curve was prepared from the serial dilutions of 100 mM IAA stock solution (ICN, Germany). Bacterial growth was determined by the optical density (OD) at 590 nm.

Evaluation of microbial IAA biological activity by plant assay

In order to verify the presence of IAA and its biological activity, a biotest with the bean (*Phaseolus vulgaris*) cuttings has been carried out. This plant assay is an easy model demonstrating rhizogenesis induction, which only occurs under exogenous auxin impact. The formation of the adventitious roots and its topography correlates to the IAA concentration, thus making visual and clear the auxin effects that express in appearance of new supplementary roots along the stem of the cuttings. The procedure was described earlier by Kefeli and Kutacek (1977). After that the roots and the basal part of 14 day old bean sprouts were cut off, they were submerged for 6 h in the sterile tap water (control) and bacterial culture fluids; then they were rinsed, transferred to new vials with tap water, and incubated under room temperature. The height of root formation and the number of emerging roots were analyzed after 8–10 days. All experiments included five replications and were repeated three times.

Bacterization of orchid seeds

A quantity assay for the estimation of bacterial influence on the orchid seed germination was performed by using plate counting. For this aim, the fresh mature seeds of *Dendrobium nobile* were used. After the seed boll was rubbed with 70 % (v/v) ethanol and opened, the orchid seeds were surface-sterilized by soaking in 10 % domestic bleach for 15 min, and then three times rinsed in sterile distilled water. About 1000 seeds were transferred aseptically with inoculation loop onto the surface of Murashige and Skoog (MS) solid medium (35 ml). Tested bacteria were cultivated for 48 h in TSB medium. An aliquot of 0.5 ml (10^8 CFU ml^{-1}) of aseptically rinsed bacterial culture was added to the surface. The flasks were incubated at 23 ± 2 °C for 2 weeks in the dark, and then with a photoperiod duration of 12 h.

The estimation of seed development by counting the germinated seeds was made by analyzing the time of seed germination and the stages of plant development, such as: 0—seed coat intact, dormant embryo; 1—embryo swollen and seed coat split; 2—massive swelling, developed rhizoids; 3—emergence of leaf-like organ; 4—development of the subsequent leaves and roots. As a control, seeds were incubated with no bacterial inoculum under the same conditions. The percentage of the germinated seeds was calculated after 2 months of incubation (not swollen seeds considered as ungerminated) by counting several randomized area containing 100 seeds/seedlings (in 4–6 replicates for each variant).

Analysis of orchid–microbial interactions by scanning and transmission electron microscopy

Germinated seeds were analyzed under scanning electron-microscopy (SEM) and transmission electron microscopy (TEM). They were fixed for 30 min with a 2.5 % solution of glutaraldehyde in phosphate buffered saline and dehydrated in ethanol solutions of increasing concentrations; after the final dehydration in absolute ethanol and overnight soaking in 100 % acetone, the samples were dried by critical point using an HCP-2 device (Hitachi, Japan), coated with Au–Pd (Eiko IB-3 Ion Coater, Hitachi, Japan), and examined with an JSM-6380LA scanning electron microscope (Jeol, Japan).

For transmission electron microscopy, the seedlings were fixed in 2.5 % solution of glutaraldehyde in phosphate buffered saline (0.1 M, pH 7.2), postfixed in 1 % osmium tetroxide (overnight), rinsed with distilled water, followed by dehydration in ethanol solutions of increasing concentrations (30 and 60 %). The samples were treated with 2 % uranyl acetate in 70 % ethanol (overnight). After the final dehydration in absolute ethanol and 100 % acetone the samples were embedded in epoxy resin. Embedding was carried out by using a mixture of the resin (Epon 812) and hardeners (DDSA and MNA) in the ratio 13:8:7, supplemented with 1 part of catalyst (DNP). After drying at 60 °C overnight, the sections were cut with a diamond knife on a LKB Ultratome V and stained with lead citrate and examined in a transmission electron microscope JEM-100B (Jeol, Japan).

Statistics

All experiments were made in three to five repetitions. The data performed as the mean ± SD. The values were separated by Student's *t* test and considered to be significant at $p \leq 0.05$. The data was analyzed with Microcal Origin program, OriginLab (http://www.originlab.com).

Results

Isolation of *Dendrobium*-associated bacteria and microbial IAA production

Considering that our final goal was to apply the bacterial strains for co-cultivation with the orchid seeds on the solid nutrient (MS) medium, the chosen species should have produced enough biomass and been active in auxin biosynthesis. Bacterial cultures that were able to proliferate and produce enough biomass after two passages of subculturing were screened for auxin (IAA) production. Under supplementation of 100 μg ml^{-1} of exogenous L-tryptophan (Trp), there were several strains producing higher than 20 μg ml^{-1} of auxin, while the majority of the isolates produced about 2–10 μg IAA ml^{-1}. Several strains were selected: three of them were isolated from the aerial roots (*Agrococcus* sp., *Roseomonas* sp., *Sphingomonas* sp.), and *Azospirillum* sp., *Caulobacter* sp., *Enterobacter* sp., *Streptomyces* sp.—from the substrate roots of *D. moschatum*. These strains were firstly grouped by using classical microbiological methods, and identified based on analysis of the 16S rRNA gene sequences (Table 1). Among the isolated bacteria, there were known PGPR strains, such as *Azospirillum*, *Bacillus*, *Sphingomonas*, and *Streptomyces*, as well as less popular *Roseomonas* and *Agrococcus*. *Sphingomonas* sp. and *Agrococcus* sp. were isolated as endophytes.

In order to estimate microbial auxin production, we tested different sources and concentrations of exogenous tryptophan. Such optimization is needed considering that the in vitro orchid germination is a long-term process; the supplementation of the plant growth nutrient medium with the appropriate form of Trp might assure the constant stimulation of the IAA production by symbiotic (associative) bacteria. For *Roseomonas* sp., *Sphingomonas* sp., *Bacillus pumilus*, and *Mycobacterium* sp., maxim of IAA production corresponds to the addition of 400 μg ml^{-1} of L-tryptophan to the mineral medium (MCM); they produced 31.6, 52.2, 22.4, and 69.8 μg ml^{-1} of auxin, respectively (Table 2). For *Bacillus* sp. and *Mycobacterium* sp. cultures, the difference was significant between the two tested media: in TSB the IAA amount did not exceed 3–4 μg ml^{-1}, while in MCM IAA content reached about 20 and 70 μg ml^{-1}, respectively. The cultures of *Caulobacter* sp. and *Enterobacter* sp. showed the opposite effect with about 20 μg ml^{-1} of auxin in MCM and 60 and 70 μg of IAA ml^{-1} in TSB (under supplementation of 400 μg ml^{-1} of L-Trp). However, biomass accumulation for *Caulobacter* sp. was much higher in the mineral medium. The stimulating effect of L-tryptophan on IAA production by *Streptomyces* sp. culture was visible only when

Table 1 Phylogenetic affiliation of the selected orchid-associated bacterial strains, producing auxin

Clone sequenced (GenBank acc. no)	Best match with database	Identity (%)	Microbial group affiliation
KP794603	*Caulobacter vibrioides* (GU459216.1)	98	*Caulobacter* sp. *SR*[a]*-C13*
KP794604	*Roseomonas cervicalis* (NR_114672.1)	98	*Roseomonas* sp. *AR-1*
KP794605	*Streptomyces* sp. (JN187861.1)	96	*Streptomyces* sp. *SR-C12*
KP794606	*Azospirillum irakense* (HQ018753.2)	95	*Azospirillum* sp. *SR-C10*
KP794607	*Enterobacter cloacae* (GQ260073.1)	94	*Enterobacter* sp. *SR-C20*
KP794608	*Agrococcus iejuensis* (HQ896931.1)	98	*Agrococcus* sp. *AR-II5*
KP794609	*Sphingomonas* sp. (JQ660212.1)	96	*Sphingomonas* sp. *AR-II14*
KP7946010	*Bacillus pumilus* (KP713764.1)	100	*Bacillus pumilus GBS-1*

Bacillus pumilus GBS-1 was isolated previously from the roots of *D. leonis* (Kolomeitseva et al. 2002). Strains of *Agrococcus* sp. and *Sphingomonas* sp. are endophytic

[a] Bacteria, designated as SR were isolated from the substrate roots, and designated as AR—from the aerial roots of *Dendrobium moschatum*

the strain was cultivated in MCM but when it was transferred in TSB, the medium had smoothed the difference between L- and DL-Trp effects (Table 2). Despite the common fact that natural L-form of tryptophan is better assimilated and thus, stimulates the IAA biosynthesis with more effectiveness, our results show that the difference between concentrations and isomeric forms is less obvious than the medium composition. The amounts of IAA produced by *Roseomonas* sp. with 200 and 400 μg ml^{-1} of L-Trp in TSB differed slightly, while the difference in auxin production in mineral medium was significant, although the biomass amounts were similar. The stimulative effect of exogenous Trp on auxin biosynthesis confirms the Trp-dependent way of IAA biosynthesis in all investigated bacteria. Under supplementation of MCM with 200 μg ml^{-1} of L-Trp, *Azospirillum* sp. and *Agrococcus* sp. produced 31.8 ± 1.3 and 12.4 ± 1.8 μg of IAA ml^{-1}, respectively (data not shown). Additionally, we showed that *Mycobacterium* and *Bacillus* did not much reduce their capacity for auxin production: *Mycobacterium* sp. kept it on the level of 47.7 and 69.8 μg IAA ml^{-1} in MCM-L200 and MCM-L400, respectively. *Bacillus pumilus* produced 7.5 (MCM-L200) and 20.4 (MCM-L400) μg IAA ml^{-1}.

Evaluation of microbial IAA biological activity

Although bacteria can produce diverse indolic compounds, only IAA is active in the rhizogenesis of plants. To show that the produced microbial IAA is biologically active, we tested the cultural broth on the bean cuttings, susceptible to exogenous IAA (pure standard compound) that we reported previously (Tsavkelova et al. 2007a). Each treatment of the cuttings resulted in the increased number of the roots and their length; the root formation height exceeded the control sample values up to 3–17-fold. The most indicative results of the assay are summarized in Table 3. The tested strains did not suppress the plant growth except *Enterobacter* sp., which provoked the negative symptoms, such as rotting of the stem.

Bacterization of the *Dendrobium nobile* seeds with orchid-associated bacteria

In this assay, we pursued several goals: to test the application of the strains isolated from one orchid species (*D. moschatum*) to promote germination of another species (*D. nobile*); to compare the capacities of the previously and newly isolated bacterial strains in seed bacterization by using fresh mature seeds and complex Murashige and Skoog (MS) medium with no addition of any plant growth stimulators; to confirm the necessity of the selection process among the potentially beneficial bacteria that are usually recognized as PGPR. None synthetic plant growth stimulators were used, but supplementation with L-Trp for favoring IAA production by chosen bacteria.

We changed the list of selected bacteria by discarding *Enterobacter* sp. and *Caulobacter* sp. cultures: the first strain did not seem promising due to rotting effect caused on kidney bean cuttings. *Caulobacter* sp. and *Roseomonas* sp. cultures failed to grow, and they produced insufficient biomass on MS medium used for orchid seed cultivation. Thus, *Agrococcus* sp., *Streptomyces* sp., *Azospirillum* sp. and *Sphingomonas* sp. were selected as potential PGPR strains.

Under the tested conditions, *Azospirillum* sp. and *Streptomyces* sp. provoked obvious negative effect on the seed germination. The abundant extracellular mucus, produced by *Azospirillum* sp. completely drowned the seeds, whereas *Streptomyces* sp. suppressed the germination and obstructed the growth of already germinated seedlings (additional data are given in Online Resource 1). On the

Table 2 Microbial IAA production in dependence of nutrient media, concentration and optical isomers of tryptophan by selected bacteria

Bacterial culture[a]	Nutrient medium tryptophan (L-, DL-form)/tryptophan concentration μg ml^{-1}[b]	IAA (μg ml^{-1})	OD^{c}_{600}
Roseomonas sp. AR	MCM-L200	1.3 ± 0.08	0.5 ± 0.04
	MCM-L400	31.6 ± 1.60	0.4 ± 0.08
	MCM-DL200	0.8 ± 0.10	0.4 ± 0.00
	MCM-DL400	20.3 ± 0.94	0.8 ± 0.02
	TSB-L200	12.5 ± 0.20	0.7 ± 0.06
	TSB-L400	19.7 ± 1.02	0.7 ± 0.00
	TSB-DL200	10.3 ± 0.40	0.7 ± 0.10
	TSB-DL400	19.9 ± 0.30	0.6 ± 0.13
Sphingomonas sp. AR	MCM-L 200	29.4 ± 1.30	1.6 ± 0.00
	MCM-L400	52.2 ± 2.84	1.0 ± 0.00
	MCM-DL200	12.0 ± 0.38	1.3 ± 0.04
	MCM-DL400	26.1 ± 0.81	1.8 ± 0.08
	TSB-L200	6.8 ± 0.14	0.9 ± 0.04
	TSB-L400	11.3 ± 0.12	1.6 ± 0.02
	TSB-DL200	10.3 ± 0.36	1.2 ± 0.05
	TSB-DL400	14.5 ± 0.70	1.1 ± 0.00
Caulobacter sp. SR	MCM-L200	18.8 ± 0.90	5.6 ± 0.33
	MCM-L400	23.6 ± 0.95	5.1 ± 0.18
	MCM-DL200	5.1 ± 0.07	3.3 ± 0.08
	MCM-DL400	8.5 ± 0.38	3.5 ± 0.24
	TSB-L200	46.4 ± 2.16	0.5 ± 0.00
	TSB-L400	60.9 ± 2.64	0.6 ± 0.06
	TSB-DL200	23.3 ± 1.05	0.6 ± 0.10
	TSB-DL400	34.8 ± 1.50	0.7 ± 0.00
Enterobacter sp. SR	MCM-L200	4.2 ± 0.12	0.6 ± 0.00
	MCM-L400	20.7 ± 1.12	0.5 ± 0.20
	MCM-DL200	2.4 ± 0.09	0.6 ± 0.03
	MCM-DL400	14.5 ± 0.87	0.2 ± 0.00
	TSB5-L200	59.4 ± 3.50	1.1 ± 0.04
	TSB-L400	71.5 ± 3.82	1.2 ± 0.02
	TSB-DL200	47.0 ± 2.28	1.2 ± 0.05
	TSB-DL400	56.7 ± 1.13	0.6 ± 0.08
Streptomyces sp. SR 1	MCM-L200	7.7 ± 0.07	2.8 ± 0.05
	MCM-L400	14.3 ± 0.54	3.0 ± 0.05
	MCM-DL200	4.3 ± 0.15	3.0 ± 0.02
	MCM-DL400	10.6 ± 0.64	3.0 ± 0.06
	TSB-L200	14.5 ± 1.20	3.5 ± 0.02
	TSB-L400	19.7 ± 0.02	3.8 ± 0.33
	TSB-DL200	20.3 ± 1.05	3.8 ± 0.08
	TSB-DL400	22.6 ± 0.85	3.9 ± 0.24
Bacillus pumilus	MCM-L200	7.5 ± 0.34	0.9 ± 0.03
	MCM-L400	20.4 ± 0.05	0.9 ± 0.07
	MCM-DL200	6.8 ± 0.05	0.8 ± 0.00
	MCM-DL400	18.2 ± 0.73	0.9 ± 0.12
	TSB-L200	3.3 ± 0.18	1.2 ± 0.02
	TSB-L400	2.6 ± 0.06	1.4 ± 0.29
	TSB-DL200	2.4 ± 0.04	1.4 ± 0.31
	TSB-DL400	2.5 ± 0.16	0.9 ± 0.23

Table 2 continued

Bacterial culture[a]	Nutrient medium tryptophan (L-, DL-form)/tryptophan concentration μg ml^{-1}[b]	IAA (μg ml^{-1})	OD^{c}_{600}
Mycobacterium sp.	MCM-L200	47.7 ± 2.47	2.9 ± 0.35
	MCM-L400	69.8 ± 3.20	3.5 ± 0.22
	MCM-DL200	14.3 ± 0.36	3.4 ± 0.08
	MCM-DL400	39.1 ± 1.55	3.3 ± 0.26
	TSB-L200	4.0 ± 0.22	1.8 ± 0.04
	TSB-L400	3.9 ± 0.08	1.2 ± 0.00
	TSB-DL200	1.8 ± 0.10	1.5 ± 0.02
	TSB-DL400	2.7 ± 0.06	1.2 ± 0.03

Values are the means of three replicates of three independent experiments ± SD

[a] Bacterial strains designated as: SR—isolated from substrate roots, and AR—isolated from aerial roots of *Dendrobium moschatum*; *Bacillus pumilus* was isolated previously (Kolomeitseva et al., 2002) and sequenced in this study; *Mycobacterium* sp. was isolated and sequenced previously (Tsavkelova et al. 2007a, b)

[b] For assessment of IAA production, cultures were grown for 72–96 h. Auxin content was estimated with Salkowski reagent. *MCM* modified Czapek medium, *TSB* trice diluted tryptic-soy broth

[c] OD600 corresponds to maximal IAA production

Table 3 Effects of bacterial culture fluids on rooting of kidney bean cuttings

Test variant	Auxin content in cultural broth		Rhizogenesis	
	Medium	(μg ml^{-1})	Stem height (mm)	Number of roots per one cutting
Control (water)	–	0	5[a] ± 1.3	5 ± 2.2
Control (MCM)	–	0	4[b] ± 0.7	7[b] ± 4.6
Streptomyces sp.	TSB-DL400	22.6	25.0 ± 2.7	38 ± 5.0
Enterobacter sp.	TSB-L400	71.5	49.0 ± 1.8	16 ± 3.2
Roseomonas sp.	MCM-L400	31.6	42,6 ± 2.1	20 ± 5.7
Sphingomonas sp.	MCM-L400	52.2	47,0 ± 3.3	38 ± 3.2
Caulobacter sp.	TSB-DL400	34.8	30.0 ± 2.3	29 ± 4.0
	MCM-L400	23.6	19.0 ± 2.0	38 ± 6.2
Mycobacterium sp.	MCM-L400	69.8	60.0 ± 2.8	83 ± 6.0
Bacillus pumilus	MCM-DL400	18.2	17.0 ± 1.5	21 ± 4.5

[a] Values are the mean of 3–5 replicates of three independent experiments

[b] Not significantly different from the control ($p \leq 0.05$); other values are significantly different ($p \leq 0.05$) from the control

contrary, the prominent positive influence among the newly isolated strains was observed with *Sphingomonas* and *Agrococcus* cultures (Table 4). Photosynthetic activity of the protocorms could be observed after 2.5 weeks of incubation. In 9 weeks of incubation, all the seedlings treated with the bacteria had one to two well-developed leaves and rhizoids, whereas the control plants possessed only one well-developed leave-like organ. The highest percentage of the germinated seeds (94 %) was revealed in co-culture with *Agrococcus* sp. (Table 4), the genus that has not been previously shown as a PGPR strain. Another bacteria, *Sphingomonas* sp., promoted 92 % of seeds to germinate and to switch from the just swollen embryo to the following phases. Also noteworthy, both PGPR strains were isolated as endophytes.

The cultures of *Mycobacterium* sp. and *B. pumilus* also promoted seed germination, confirming their effectiveness by stable capacity to stimulate the growth and development of the orchid seeds in vitro. In our first experiments, *Mycobacterium* sp. derived from *D. moschatum* promoted the germination of 1.2 % of its seeds (with none of the seeds germinated in the control, Tsavkelova et al. 2007b). In this study, by using complex MS medium and fresh mature seeds of another *Dendrobium* plant, *Mycobacterium* sp. enhanced its activity, promoting 12 % seeds to grow; in the co-culture with *B. pumilus* allowed 14 % more *D. nobile* seeds to germinate (Table 4).

Thus, no evident specificity between two different species of *Dendrobium* orchids and their associative PGPR was observed: isolated from *D. moschatum*, they

Table 4 Influence of the orchid-associated bacteria on germination of *Dendrobium nobile* seeds

Test variant	3 weeks	6 weeks	9 weeks	Germinated seeds (%)[b]
Control (seeds + water)	1 + 2[a]	2	3	76 ± 3.8
Sphingomonas sp.	2	2	3 + 4	92 ± 4.2
Mycobacterium sp.	2	2 + 3	3 + 4	88 ± 4.5
Azospirillum sp.	0	0	0	0
Bacillus pumilus	2	2 + 3	3 + 4	90 ± 3.5
Agrococcus sp.	1 + 2	2 + 3	3 + 4	94 ± 2.3
Streptomyces sp.	0 + 1	0 + 1 + 2	0 + 1 + 2	≤50[c]

[a] Seed germination and protocorm developmental stages: 0—seed coat intact, dormant embryo; 1—embryo swollen and seed coat split; 2—massive swelling, developed rhizoids; 3—emergence of leaf-like organ; 4—developed second leaf-like organ. The numbers characterize the presence of ≥10 % of each developmental stage in total population

[b] Germinated seeds (photosynthetically active seedlings) were calculated after 2 months of incubation. The data are the mean of 4–6 replicates ± SD

[c] The percentage is an approximate value, since many of the initially developed protocorms died during the observation period

beneficially promoted the germination of *D. nobile*. However, the peculiarity of orchid seed biology (the minute size, no endosperm) as well as the necessity of using complex carbohydrate-rich nutrient media appear to narrow the application of only known PGPR, particularly those producing abundant extracellular polysaccharide matrix.

Scanning and transmission electron microscopy of the *Dendrobium nobile* seeds, germinated in vitro in co-cultures with bacteria

We used SEM and TEM techniques in order to observe whether and how the PGPR strains interact with the orchid seeds. We found that PGPR extensively attach to the surface of the seed coat, preferring to colonize the stria on the wrinkled and ribbed seed surface. They spread across the surface, embedding in the furrows and grooves (Fig. 1a–c). No bacteria were detected in the control samples (Fig. 1g), while the inoculated seeds were massively covered with bacteria. The microorganisms multiply and form microcolonies and new micropopulations on the rhizoids. The prominent role in the colonization process is lead by extracellular matrix (EM) excreted by bacterial cells that favors the formation of bacterial agglomerates and clusters, particularly within the surface furrows. At the same time, together with bacterial clusters, joined by EM, many individual cells were also seen on the seed surface, settling the new territories and engendering new clusters and microcolonies (Fig. 1a–d). In contrast to the seed growth promoting bacteria, *Streptomyces* sp. that provoked a negative effect on the germination did not colonize the seeds until they were alive (Fig. 1e). Gradually, the growth of germinated seeds slowed down and their resistance decreased, then the mycelia braided the seed (Fig. 1f).

The PGPR strain of *Mycobacterium*, on the contrary, immediately colonized the rhizoids, followed by populating the developed roots (Fig. 1a). Thick EM entrapped the cells by covering them like a blanket that was distinctly seen in the case of *Sphingomonas* sp. (Fig. 1c). Together with the vegetative cells, *B. pumilus* abundantly produced spores kept in chains and clusters within the microcolonies and spread over the seed surface (Fig. 1d). By an example of the seedlings, treated with cultures of *Mycobacterium* and *Sphingomonas* sp. (Fig. 2a–c), we revealed the bacteria penetrating inside the testa (Fig. 2a, b), and in the velamen cells of the roots of the 6 month old plantlet (Fig. 2c). TEM observations confirmed the preferable endophytic localization of previously isolated from the rhizoplane of *D. moschatum*, *Mycobacterium* sp. (Fig. 3a) as single cells or micro colonies, surrounded by EM. Bacteria entered inside the apoplast of the root cortex of *Dendrobium nobile* seedlings, actively colonizing the space between the cortical cells of root parenchyma. The bacterial cells were shown to actively multiply (different stages of cell division were noticed, Fig. 3b). In the seedlings inoculated with *Mycobacterium* sp., we noticed that plastids stored big starch grains, and additionally osmophilic (lipid) globules (Fig. 3c).

Discussion

The plant surfaces of various tropical plants provide a favorable habitat for microbial colonization (Baldotto and Olivares 2008). In this study, we aimed to isolate associative bacteria not only from the root surface of *D. moschatum*, as we previously reported (Tsavkelova et al. 2004), but also those from the inner root tissues. Such endophytic microorganisms colonize plant tissues, usually

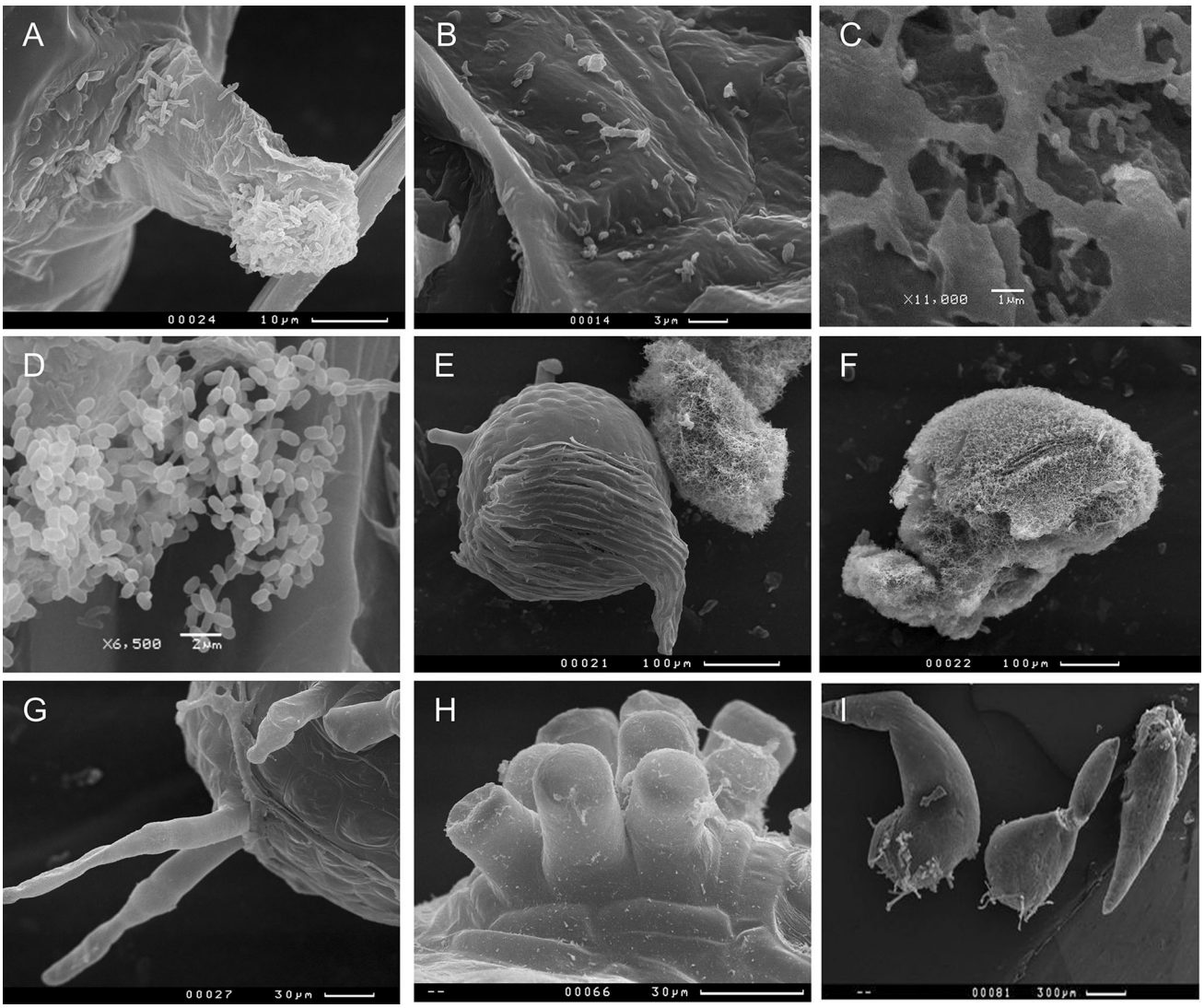

Fig. 1 SEM of the *Dendrobium nobile* germinated seeds, inoculated with rhizobacteria (4–8 weeks after inoculation): **a** *Mycobacterium* sp., actively colonizing the nascent rhizoid (*Bar* 10 µm), **b** *Agrococcus* sp. individual cells and microcolonies on the ribbed surface (*Bar* 3 µm), **c** *Sphingomonas* sp. clusters and individual cells, covered with exopolysaccharide matrix (*Bar* 1 µm), **d** *Bacillus pumilus* vegetative cells and spores on the seed surface (*Bar* 2 µm), **e**, **f** *Streptomyces* sp. microcolony next to the germinating alive embryo (**e**), and the seed, covered with mycelium (**f**, *Bar* 100 µm), **g** control—untreated embryo with the rhizoids (*Bar* 30 µm), **h** the nascent rhizoids at 2 weeks (*Bar* 30 µm), **i** emergence and enlongation of the first leaf at 8 weeks (*Bar* 30 µm)

showing no external sign of infection or negative influence on their host (Ryan et al. 2008). In order to find the appropriate bacterial cultures to promote orchid seed germination, we tested them for auxin production, and several strains, ranging in IAA biosynthesis from 1.3 to 47.7 µg IAA ml^{-1} were selected. The known auxin producing bacteria, which are able to improve plant growth, vary in IAA biosynthesis capacity between 5 and 10 up to 200 µg IAA ml^{-1} and higher (e.g. Khalid et al. 2004; Shahab et al. 2009; Habibi et al. 2014). The microbial production of a key auxin substance, IAA, is mostly Trp-dependent (Spaepen et al. 2007). The IAA biosynthesis by the investigated bacteria was also stimulated by Trp, although the strains significantly differed in auxin production feedback to the addition of L- and DL-Trp, supplemented to the mineral MCM or organic TSB media (Table 2). Such specific reaction supports the idea that optimization of cultivation conditions for IAA production is species-specific. Bharucha and Patel (2008) reported that the IAA biosynthesis of *Pseudomonas putida* UB1 increased when the L-Trp (200 µg ml^{-1}) medium was supplemented with 0.5 % of sucrose and 10 mg ml^{-1} of $(NH_4)_2SO_4$, whereas the best conditions for the nitrogen-fixing bacteria were when the yeast extract mannitol medium was supplemented with 300 µg ml^{-1} of L-Trp (Shokri and Emtiazi 2010), and the organic broth (8 g L^{-1} of meat extract and

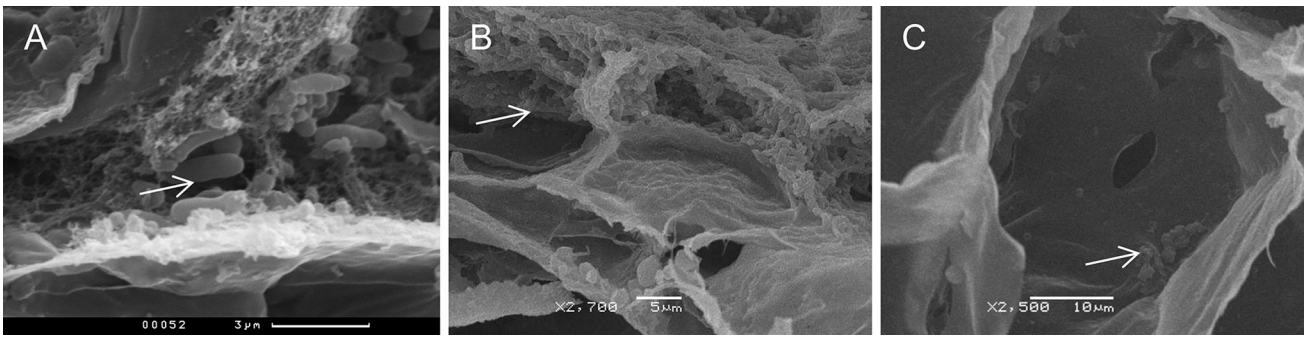

Fig. 2 SEM of the cross-sections of *Dendrobium nobile* seedlings. **a**, **b** *Mycobacterium* sp. on the surface and inside the testa of 9 week old seedlings. **c** *Sphingomonas* sp. within the velamen cells of the roots of 6 month old plantlet; the round native perforations of the velamen structure can be seen. Bacterial cells are marked with the *arrows*

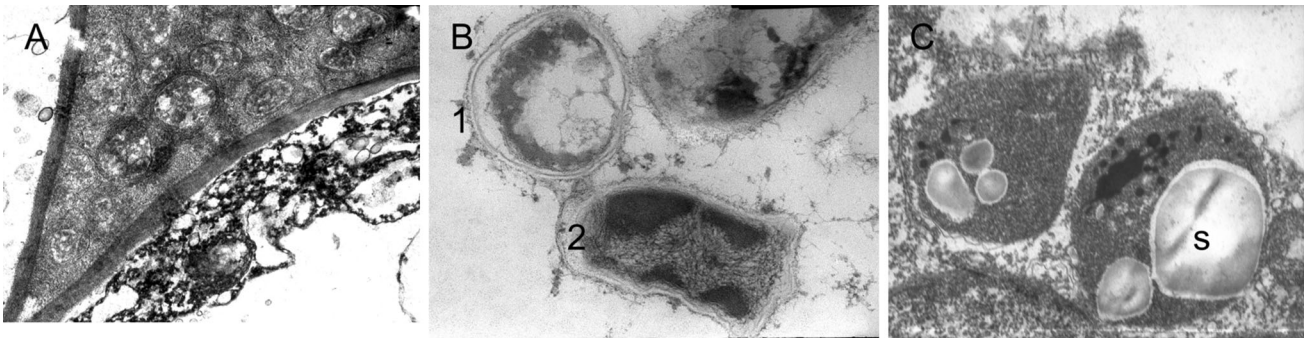

Fig. 3 TEM of *Dendrobium nobile* seedlings' roots (6 months after inoculation). **a** *Mycobacterium* sp. cells colonizing the space between the cortical cells of root parenchyma, **b** bacterial cells: cross (*1*) and longitudinal (*2*) sections, **c** plastids with starch (s) grains and black osmophilic globules

100 μg ml^{-1} of L-Trp) was the optimal medium for *Pantoea agglomerans* PVM (Apine and Jadhav 2011).

The plant assay (Table 3) showed that the microbial IAA was active in rhizogenesis, and the bean cuttings were highly susceptible to it. However, the negative outcome was that *Enterobacter* sp. induced rotting of the plant stem. De Melo Pereira et al. (2012) reported that after inoculation of the strawberry plants with endophytic *Enterobacter* strains, some of them increased growth of the plants, whereas the treatment with *E. ludwigii* lead to inhibitory effects on shoot length.

Among the selected bacteria, two endophytic species of *Sphingomonas* sp. and *Agrococcus* sp. turned out to be the most prominent in orchid seed bacterization assay (Table 4). Although there is information on the presence of *Agrococcus versicolor* in phyllosphere of potato plants (Behrendt et al. 2008), this is the first report on *Agrococcus* sp. as an active PGPR strain producing IAA, establishing tight interactions with the orchid-host plant, and promoting its seed germination. On the contrary, *Sphingomonas* strains are the recognized endophytes of sweet corn (McInroy and Kloepper 1995), black cottonwood, willow (Doty et al. 2009), and rice plants (Videira et al. 2009). Apart from nitrogen fixation, sphingomonads synthesize siderophores (Sessitsch et al. 2004), and are capable of gibberellins and IAA production, promoting tomato plant growth (Khan et al. 2014). On the contrary, *Streptomyces* sp. suppressed the orchid germination and protocorm development (Fig. 1f). One of the possible reasons for this could be the production of antibiotics, which are known for their phytotoxic effects, particularly on root elongation (Liu et al. 2009) as well as foliage photosynthesis and photosynthetic pigment content (Opriş et al. 2013; Wang et al. 2015).

The genus *Azospirillum* is considered as one of the most representative PGPR (Cassán et al. 2014) and is widely applied for bacterization of the different crop plants (Mehnaz and Lazarovits 2006). *A. amazonense* was also detected on the roots of several cultivable Brazilian orchids—*Oncidium varicosum*, *Vanda tricolor*, *Dendrobium fimbriatum*, and *D. nobile* (Lange and Moreira 2002). However, under conditions of in vitro orchid seed germination, *Azospirillum* sp. overproduced biomass and extracellular matrix (Online Resource 1), thus making it inappropriate for application in co-cultures with the minute

orchid seeds. Complex solid media used for this aim usually contain high carbohydrate amounts, plant growth stimulators, vitamins, and other various nutrient substances (reviewed in Teixeira da Silva et al. 2015). Such sucrose-rich media contributes to proliferation of the bacterial population and provoke the abundant production of the microbial extracellular polysaccharide-containing matrix. Previously (Tsavkelova et al. 2007b), we showed that no germination of *D. moschatum* seeds happened with another usually considered as PGPR strain of *Rhizobium* sp. due to the same excessive production of extracellular mucus that entirely covered the seeds and deprived them of light and air.

At the same time, extracellular matrix, composed of exopolysaccharides, proteins and DNA, is an inherent part of biofilms that play structuring and communicative roles as well as protect bacterial cells from UV radiation, predation, desiccation, and antibiosis (reviewed in Oleskin et al. 2000; Morris and Monier 2003). Biofilms help rhizobacteria, particularly non-spore-forming species, to colonize plant roots (Rinaudi and Giordano 2010). Vice versa, plant polysaccharides (the components of plant's cell wall) induce the biofilm formation, although microbial colonization capacities vary according to the host-plant, as it was shown by *Bacillus*-tomato and *Bacillus–Arabidopsis* examples (Beauregard et al. 2013).

By using SEM and TEM microscopy (Figs. 1, 2, 3), we showed that the orchid PGPR strains of *Mycobacterium* sp., *Agrococcus* sp., *Sphingomonas* sp., and *Bacillus pumilus* actively colonized the surface of the seeds, formed microcolonies, and penetrated inside the plant tissues as endophytes. Such interactions were also mediated by their capacities for biofilm formation (Figs. 1a–d, 2a–c). Our results agreed with those of Ryan et al. (2008) that successful endophyte colonization involves a compatible host plant, and that bacteria colonize the plant surfaces as solitary cells, microcolonies, or biofilms (Baldotto and Olivares 2008). Endophytes might enter the roots through splits and cracks at the points of root emergence, subsequently colonizing the intercellular spaces, aerenchyma, and cortical cells, as it was shown for the rice PGPR strain, *Herbaspirillum seropedicae* Z67 (James et al. 2002).

Orchid germination differs from all other plants: orchid seeds are capable of swelling due to minimal water uptake and optimal light and temperature conditions, but for the further development of the protocorm, mycorrhizal colonization or exogenous carbohydrate supply is strictly needed (Smith and Read 2008). The stages of *D. nobile* seed germination and seedling developmental growth under in vitro conditions are described in the recently published review (Teixeira da Silva et al. 2015); it can take weeks and even months until the seedlings produce one or more leaves and roots (Fig. 1i). After the testa is ruptured, the emerged rhizoids (Fig. 1a, h) of the photosynthetically active protocorms attract the bacterial cells, apparently by the produced exudates (Fig. 1a). Plant root exudates usually contain sugars, amino acids, and organic acids, in addition to diverse secondary metabolites (Kamilova et al. 2006; Broeckling et al. 2008). We may only assume the more or less similar content of the secreted compounds by the orchid seedlings, since the nascent rhizoids have only about 30–50 μm in length (Fig. 1a, h), and can hardly be analyzed. Thus, further separate studies should focus on identifying the composition of the rhizoid exudates of the orchid protocorms.

Most auxin in the rhizosphere is derived from Trp secreted by the plant roots and effectively converted to IAA by some rhizosphere bacteria (Kamilova et al. 2006). Plant roots are sensitive to the fluctuations in auxin content: low amounts of IAA (between 10^{-9} and 10^{-12} M) stimulate primary root growth, whereas higher IAA levels can inhibit it (Meuwley and Pilet 1991; Patten and Glick 2002). *Paenibacillus lentimorbus* and *P. macerans* isolated from the meristems of *Cymbidium eburneum*, promoted the growth of in vitro micropropagated seedlings of another orchid, *Cattleya loddigesii*, by producing only 1.5–3.6 μg IAA ml^{-1} (Faria et al. 2013). *Bacillus* sp. and *Enterobacter* sp. isolated from the roots of *Cattleya walkeriana*, produced 18 and 32 μg IAA ml^{-1}, respectively (Galdiano Júnior et al. 2011), and promoted the host growth during its ex vitro acclimatization (Galdiano Júnior et al. 2011). Yang et al. (2014) reported on the bacterization of *Dendrobium officinale* seeds with growth-promoting *Sphingomonas paucimobilis* ZJSH1, which increased stem height by 8.6 % and fresh weight by 7.5 %, and produced 11.75 ng ml^{-1} of IAA. In the present study, endophytic strain of *Sphingomonas* sp. produced 29.4 μg IAA ml^{-1} and promoted an extra 16 % of *D. nobile* seeds to germinate (Table 4), although the best results we obtained with a newly reported orchid endophyte, *Agrococcus* sp. with 12.4 μg of IAA ml^{-1} and 18 % more seed germinated.

We previously showed that originally derived from *Dendrobium leonis*, *B. pumilus,* promoted seed germination and development of several epiphytic and terrestrial plants of *Dendrobium, Paphiopedilum, Cranichis*, and *Dactylorhiza* orchids, although its initial IAA production did not exceed 9 μg IAA ml^{-1} (Kolomeitseva et al. 2002). In this study, we confirmed that no obvious direct specificity exists between orchid and selected PGPR partners. Such indifference of the host-plant is curiously a good feature, since the biotechnological application of the IAA-producing bacteria provides an additional advantage for multiple and long-term usage of once selected strains. Nevertheless, the profitable orchid PGPR should satisfy certain basic requirements—to be recognized as cultivable cultures, not over-proliferating either with biomass or

extracellular matrix, and to be active in IAA biosynthesis, since IAA is responsible for the acceleration of rhizogenesis. Other activities, such as nitrogen fixation, phosphorous solubilization or production of antimicrobias are not relevant, when the complex media, supplemented with all needed major nutrients and trace elements, are used. Thus, orchid-associated bacteria provide a successful technique for orchid seed germination and ex situ conservation, playing an important role in the early stages of plant development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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