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ORIGINAL ARTICLE

Colonization strategy of the endophytic plant growth-promoting strains of *Pseudomonas fluorescens* and *Klebsiella oxytoca* on the seeds, seedlings and roots of the epiphytic orchid, *Dendrobium nobile* Lindl

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Keywords
auxin (indole-3-acetic acid), bacterization of *Dendrobium nobile*, endophytic rhizobacteria, orchid seed germination, *Pseudomonas fluorescens* and *Klebsiella oxytoca*.

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Abstract

Aims: Orchids form strong mycorrhizal associations, but their interactions with bacteria are poorly understood. We aimed to investigate the distribution of plant growth promoting rhizobacteria (PGPR) at different stages of orchid development and to study if there is any selective specificity in choosing PGPR partners.

Methods and Results: Colonization patterns of gfp-tagged *Pseudomonas fluorescens* and *Klebsiella oxytoca* were studied on roots, seeds, and seedlings of *Dendrobium nobile*. Endophytic rhizobacteria rapidly colonized velamen and core parenchyma entering through exodermis and the passage cells, whereas at the early stages, they stayed restricted to the surface and the outer layers of the protocorms and rhizoids. The highest amounts of auxin (indole-3-acetic acid) were produced by *K. oxytoca* and *P. fluorescens* in the nitrogen-limiting and NO_3-containing media respectively. Bacterization of *D. nobile* seeds resulted in promotion of their *in vitro* germination. The plant showed no selective specificity to the tested strains. *Klebsiella oxytoca* demonstrated more intense colonization activity and more efficient growth promoting impact under tryptophan supplementation, while *P. fluorescens* revealed its growth-promoting capacity without tryptophan.

Conclusions: Both strategies are regarded as complementary, improving adaptive potentials of the orchid when different microbial populations colonize the plant.

Significance and Impact of the Study: This study enlarges our knowledge on orchid–microbial interactions, and provides new features on application of the nonorchid PGPR in orchid seed germination and conservation.

Introduction

Orchidaceae Juss. is one of the largest and intriguing plant families with more than 26 500 species. Epiphyte orchids outnumber terrestrial taxa by two to one (Swarts and Dixon 2009) and possess a number of specific morphological and physiological traits, which help them adapt to a quite stressful habitat. One of the peculiar characteristics of their aerial roots is a spongy, usually multilayered, epidermis consisting of dead perforated cells called velamen, and the internal exodermis consisting of both thick-walled cells and so-called passage cells (Pridgeon 1987). The velamen radicum plays a key role in the mechanical and UV light protection and water conservation; it is also involved in transpiration of O_2 and CO_2 exchange; it absorbs water solutions within seconds, while

evaporation from the velamen takes several hours (Zotz and Winkler 2013). Such traits are beneficial not only for the plant but also for its associative micro-organisms. In our previous studies, we reported on the number of rhizobacteria, phototrophic cyanobacteria and fungi (Tsavkelova 2011; Tsavkelova *et al.* 2003, 2007) that abundantly colonized the roots of *Dendrobium* Sw., *Acampe* Lindl., *Phalaenopsis* Blume. and *Pholidota* Benth. The host plants supply the associated microbial communities with the root exudates that usually contain sugars, amino acids and organic acids, as it was shown for a number of the crop plants (Kamilova *et al.* 2006). All together, these factors favour the formation of the balanced plant–microbial network that is needed for better plant adaptation and its ecological stability.

A number of the plant growth-promoting rhizobacteria (PGPR), particularly endophytes, are known to stimulate the plant host growth by phytohormone production or by the biosynthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene levels (Glick 2014). Also, PGPR facilitate the plant growth and adaptive capacities by nitrogen fixation, assistance in acquisition of phosphorus and essential minerals, improvement of the water uptake or by their activity as biocontrol agents through decreasing the inhibitory effects of various phytopathogens (Ahemad and Kibret 2014; Singh and Gaur 2016). Various endophytic bacteria isolated from a large diversity of plants have been reported for their practical applications addressing their capacity for accelerating seedling emergence and promoting further plant growth (Sturz *et al.* 2000).

Nowadays, many wild orchid populations are vulnerable and threatened due to a number of reasons, where the most severe are habitat loss, overexploitation, altered abiotic conditions and climate change impacts, as well as a breakdown in biotic connections with pollinators and mycorrhiza (Swarts and Dixon 2009). The mycorrhizal fungus, the symbiosis with which is required for the germination of the orchid seeds, can be substituted *in vitro* by using complex nutrient media supplemented with various plant extracts, vitamins and plant growth stimulators (Teixeira da Silva *et al.* 2015). Worldwide distributed botanical gardens are considered as the leading centres in orchid horticulture, research and their conservation, including *in situ* reintroduction and ecological restoration programs (Swarts and Dixon 2009). However, one of the obstacles in the orchids' successful cultivation in the greenhouses, as well as the plants' reintroduction into the wild, is that they lack natural biotic partners that make the adaptation of the axenic seedlings to the soil conditions ineffective. Moreover, the specificity and diversity of the mycorrhizal fungi is an additional significant challenge in construction of such artificial associations. Vice versa, a potent tool for the orchid seed germination, conservation and reintroduction could become the orchid seed bacterization with the PGPR strains.

Although it is likely that all plants harbour endophytic bacteria (Rosenblueth and Martínez-Romero 2006), the distribution of microbial populations and the mechanisms of bacterial colonization in orchids are still undiscovered. In our previous studies (Tsavkelova *et al.* 2007, 2016; Tsavkelova 2011), we revealed that orchid-associated bacteria (OAB), isolated and selected for the auxin production, showed their efficiency in the orchid seed germination. The strains belonging to *Sphingomonas, Agrococcus, Mycobacterium* and *Bacillus* produced indole-3-acetc acid (IAA) and favoured the stimulation of the host-orchid germination *in vitro*. Moreover, the bacterial strains isolated from one orchid were shown to promote seed germination and plant adaptation of other tested orchid plants (Faria *et al.* 2013; Tsavkelova *et al.* 2016). Surprisingly, the known PGPR, such as *Rhizobium* and *Azospirillum* that were also isolated among the orchid-associated rhizobacteria, appeared to be inappropriate for the orchid germination due to the overproduction of the biomass and extracellular polysaccharide-containing matrix, when they were cultivated on the complex carbohydrate-rich Knudson-C and Murashige and Skoog media, respectively (Tsavkelova *et al.* 2016). Nevertheless, the orchids did not show any specificity towards the tested orchid-associated PGPR, and both the positive and the negative outcome of the orchid-bacteria cocultivation were derived from the bacterial growth and capacity for producing secondary metabolites.

The general idea of the present research was to test an alternative approach for using PGPR strains isolated from nonorchid plants that would enable the elimination of the labour-intensive and time-consuming, as well as challenging and costly procedure of isolation, identification and selection of the orchid-associated PGPR from the host-orchids. In order to investigate the interactions of the orchid host plant of *D. nobile* with the nonorchid PGPR, we focused on Gram-negative bacteria such as *Pseudomonas* spp. and *Klebsiella* spp., which were reported among the OAB (Wilkinson *et al.* 1989; Wilkinson *et al.* 1994; Tsavkelova 2011; Yu *et al.* 2013), as well as among the known PGPR of various crop plants (Jha and Kumar 2007; Ahemad and Kibret 2014; Kifle and Laing 2016). Additional aims of this research were to compare the bacterial localization and distribution within the tissues of the adult orchid plant and its germinating seeds; to estimate the growth-promoting capacity of the tested strains of *Klebsiella oxytoca* and *Pseudomonas fluorescens* under different conditions of tryptophan supplementation; and to study if the orchid host plant expresses any species-specific responses to the nonorchid PGPR.

Materials and methods

Organisms

The tropical epiphytic orchid, *Dendrobium nobile* Lindl., cultivated in the Stock greenhouse of the Main Botanical Garden (Moscow, Russia), and the strains of *K. oxytoca* and *P. fluorescens* were studied. The adult mature orchid plants were grown in containers (pots) filled with pine bark under the temperature and humidity conditions described elsewhere (Tsavkelova *et al.* 2003). The wild type of *P. fluorescens* was isolated from the roots of wheat, and it was previously transformed with VSP61TIR plasmid, carrying GFP gene (van Bruggen *et al.* 2008). The strain of *P. fluorescens*-32-gfp was kindly provided by Dr. A. Semenov (Dept. of Microbiology, MSU). The wild type of *Klebsiella* sp. was originally isolated from the rhizosphere of the cucumber plants (Emtsev 1994) and kindly provided by Dr. J. Blinkov.

Identification of *Klebsiella* sp

The wild strain of *Klebsiella* sp. was previously identified as *K. planticola* (Emtsev 1994) based on its morphological and biochemical characteristics. In this study, we perform its molecular re-identification, based on the analysis of the 16S rRNA gene sequences. The conditions, technique and reagents as well as the protocols of DNA extraction, PCR and sequencing are described previously (Tsavkelova *et al.* 2016). The primers B63f (5′-CAG GCC TAA CAC ATG CAA GTC-3) and B1387r (5-GGGCGGWGT GTA CAA GGC-3′) (Marchesi *et al.* 1998) were used. A preliminary analysis of the sequences was done with software Lasergene (DNASTAR) and VectorNTI (Invitrogen, Waltham, MA, USA). 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.

Tagging *Klebsiella oxytoca* with gfp

Plasmid DNA carrying gfp reporter gene (pPROBE KT-Kan) was kindly provided by Prof. Dr S. Lindow, Berkley, USA (Miller *et al.* 2000). It was isolated from *Escherichia coli* cultivated overnight in LB, supplemented with 25 μg of kanamycin according to the Qiagen miniprep kit manufacturer's protocol. For the transformation, the aliquot of the competent *Klebsiella* cells, prepared according to the modified protocol (Merrick *et al.* 1987), was thawed by hand and put into ice, mixed with 1 μg of pPROBE KT-Kan plasmid, frozen in liquid nitrogen for 1 min and then thawed for 1 min. The freeze-thawing process was repeated three times, after which the content was transferred into 3 ml of warm LB (antibiotic free), incubated for 2 h at 32°C, plated onto LB agar with 100 μg ml^{-1} of kanamycyn and incubated overnight. No colonies were visualized in control variant (cells without plasmid), whereas transformed colonies possessed blue-green fluorescence under the UV light. The transformation and the storage procedure, using DMSO, has been described elsewhere (Chung *et al.* 1989).

Growth conditions of gfp-tagged *Klebsiella oxytoca* and *Pseudomonas fluoresc*ens

Both gfp-tagged strains were maintained on LB agar, supplemented with kanamycin (100 μg ml^{-1} for *K. oxytoca*, and 60 μg ml^{-1} for *P. fluorescens*) at 30°C. The liquid LB and mineral media, which were modified according to the nitrogen source, were used: the basic medium (K2-N_0) of (g l^{-1}): K_2HPO_4—0·5; KH_2PO_4—0·3; $MgSO_4{\cdot}7H_2O$—0·1; NaCl – 0·75; $CaCl_2{\cdot}6H_2O$—0·03; sucrose—6·0; trace elements—1 ml. To the mineral 'ammonium' medium (K2-NH_4) yeast extract (0·1) and $(NH_4)_2SO_4$ (1·5), and to the 'nitrate' medium (K2-NO_3)—yeast extract (0·1) and $NaNO_3$ (1·5) were added. Nitrogen-free (basic) medium was used only for the cultivation of diazotrophic strain of *K. oxytoca*. The optical density (OD) was determined at 590 nm, and the CFU (colony-forming units) were counted on the LB agar.

Bacterial auxin production

In order to analyse a capacity of the tested strains to produce auxin (IAA), the media was supplemented with 200 μg ml^{-1} of L-Trp. The measurements of produced IAA were taken in dynamics (every 24 h) until the maximal amount was reached. Relative auxin content was estimated by the Salkowski method (Gordon and Weber 1951); samples were analysed as previously described (Tsavkelova *et al.* 2007, 2016). All experiments on auxin production were repeated with three replicates. The data on auxin content, estimated by the colorimetric method, were performed as a calculation of the mean values of repetitions ± standard deviation.

Indoles extraction and thin-layer chromatography

Bacterial cultures were centrifuged, supernatant adjusted to pH 3·0 (with 2N HCl) and 800 μl aliquots were extracted with 1 ml of ethyl acetate by vigorous shaking for 5 min. For the thin layer chromatography (TLC) analysis, the two closest variables from each repetition were taken for the analysis. After phase separation, the ethyl acetate fraction was removed and evaporated, and the solid residue was dissolved in 30 μl of methanol.

Samples were spotted onto a silica gel "Macherey-Nagel GmbH&Co. KG" (Germany) plates with UV (254) indicator, and developed with chlorophorm : ethyl acetate : formic acid (50 : 40 : 10) running solvent. After development, the plates were dried, sprayed with van Ehmann's reagent (a 3 : 1 mixture of Salkowski's and Ehrich's reagents; Ehmann 1977), and heated to 90°C until the spots were visualized. The R_fs and colours of the bands were compared with those of the standards. The stock solutions of the standard compounds of IAA, indole-3-acetamide (IAM), indole-3-pyruvic acid (IPyA) and indole-3-lactic acid (ILA) were prepared in concentrations of 87·6, 87·1, 101·6 and 102·6 mg, respectively, and dissolved in 5 ml of ethanol. The 100-fold dilutions were used for loading onto a plate. Another system of running solvent was *n*-hexane : ethyl acetate : isopropanol : glacial acetic acid (40 : 20 : 5 : 1). The plates were dried and visualized under UV light; the spots were identified, scratched off from the plate, transferred into a new tube and additionally extracted with methanol. After its evaporation, the samples were dissolved in 30 μl of methanol, spotted on the new plate, developed with the same phase, dried and sprayed with van Ehmann's reagent as mentioned above.

Evaluation of IAA biological activity by the plant assay

A bio-assay with the bean (*Phaseolus vulgaris*) cuttings as a model plant for IAA-induced rhizogenesis, was carried out as previously described (Kefeli and Kutacek 1977; Tsavkelova *et al.* 2016). The formation of the adventitious roots and their topography correlates to the exogenous IAA concentration, making visual the auxin effects that appear in active growth of the nascent roots along the stem of the bean cuttings. Thus, this bioassay is used as evidence for IAA-induced plant growth responses. The height of root formation and the number of emerging roots were analysed after 10 days. The plant assay with the *P. vulgaris* cuttings was repeated with five replicates. The data were performed as means ± standard error, and subjected to a one-way analysis of variance. The statistical differences between control and treatments were compared with the Fisher's least significant difference (LSD) test at $\alpha = 0{\cdot}05$.

Bacterization of *Dendrobium nobile* seeds

The percentage of the viable *D. nobile* seeds was comparatively estimated by the topographic tetrazolium test (Lakon 1942) and by the direct calculation of the germinated seeds; the advantage of the first technique is that it helps to evaluate the capacity of the seeds that require a long period to germinate. For this test, 75 mg of 2,3,5-tri-phenyl-tetrazolium chloride (TTC) was solved in 15 ml of phosphate buffer saline (pH = 7·5), and the seeds were incubated in this solution for 18–24 h at 24°C. Viable seeds with red colour, due to the formation of trifenylformazan, were counted, while nonviable seeds remain uncoloured. Another similar indicator, 2,3,5-triphenyl-tetrazolium bromide (TTB), was also tested.

The seeds were sterilized in 10% house-hold bleach containing NaOCl for 20 min, followed with three times rinsing in distilled water. The seeds (800–1000 per plate) were plated on the Knudson-C medium, g l^{-1}: $Ca(NO_3)_2{\cdot}4H_2O$—1·0; $(NH_4)_2SO_4$—0·5; KH_2PO_4—0·25; $MgSO_4{\cdot}7H_2O$—0·25; $FeSO_4{\cdot}7H_2O$—0·025; $MnSO_4{\cdot}7H_2O$—0·0075; sucrose—20·0; agar– 8·0; pH = 6·5. As a fungicide, nystatin (1000 U ml^{-1}) was added. The medium in experimental variables was also supplemented with 200 μg ml^{-1} of tryptophan for the induction of auxin biosynthesis by rhizobacteria. Bacteria were cultivated in LB +kan for 48 h and centrifuged at 4000 rev min^{-1} for 10 min; the sediment diluted up to 10^6 CFU per ml; and an aliquot (0·2 ml) was smeared onto the surface of the medium. The final calculation of the ungerminated seeds was made after 2 months of cultivation. The seeds were incubated for 2 weeks in the dark at 24°C and afterwards, at room temperature with a 12-h photoperiod. As a control, the seeds were incubated with no bacterial inoculum under the same conditions. The estimation of the orchid seed growth and calculation of the germinated seeds was made by analysing the time of germination, seedling's length and the stages of plant development, such as: (i) seed coat intact, dormant embryo; (ii) embryo swollen and photosynthetically active (green); (iii) massive swelling, developed rhizoids and emergence of leaf-like organ; and (iv) development of the subsequent leaves and roots. The percentage of the seeds at each stage was calculated in four repetitions by counting three randomized areas in each variable containing 60 seeds/embryos/plantlets in each one. The length of the *D. nobile* plantlets was counted in three repetitions by measuring 20 plantlets in three randomly selected areas in each repetition. To evaluate the influence of the microbial IAA on plant rhizogenesis, the values were separated by the Student's *t*-test. The data on the orchid *in vitro* bacterization was performed as the means ± standard error. The values were separated by the Student's *t*-test and considered to be significant at $P \leq 0{\cdot}05$.

Microscopic visualization of bacteria

Confocal laser scanning *microscopy* (CLSM)*: In* order to investigate plant–microbial interactions, the substrate and aerial roots of *D. nobile* were submerged for 30 min into suspensions of *gfp*-tagged *K. oxytoca* and *Pseudomonas*

fluorescence, cultivated in LB + kan for 48 h. After the treatment, the roots were cut and dissected for longitudinal and the cross-sections. The samples were examined by Nikon A1 confocal laser scanning microscope with ×20/0·45 objective. For excitation, 488 and 532 nm lasers were used for green and orange channels respectively. Images were collected using DU4 detector with 525/50 or 595/50 emission filters; Z-series collected from 40 to 120 optical sections ranging from 1·3 to 7·2 μm in thickness. *Scanning electron microscopy* (SEM): Germinated seeds and seedlings of bacterized *D. nobile* (as described above) 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 drying method with CO_2 on HCP-2 device (Hitachi Ltd., Tokyo, Japan), coated with Au–Pd (Eiko IB-3 Ion Coater; Hitachi), and examined with an JSM – 6380LA scanning electron microscope (Jeol Ltd., Tokyo, Japan). *Transmission electron microscopy* (TEM) was performed as previously described (Tsavkelova *et al.* 2016) with the 4-month-old bacterized *D. nobile* plantlets.

Results

Localization and distribution of *Klebsiella oxytoca* TSKhA-gfp and *Pseudomonas fluorescens* 32-gfp in the roots and seedlings of *Dendrobium nobile*

After 30 min of incubation of the *D. nobile*'s roots with the *K. oxytoca* TSKhA-gfp, bacterial cells were detected in both substrate and aerial roots, accumulating mostly in the velamen tissue (Fig. 1a). The further colonization of the core parenchyma also occurred through the passage cells, facilitating their penetration inside the root. Twenty-four hours after incubation, *K. oxytoca* cells were detected within the vascular cylinder (stele) of the substrate roots, whereas in the aerial roots, bacteria were only colonizing the parenchyma cells. Nevertheless, a week after the treatment, bacteria were visualized within the vascular tissues of the aerial roots as well. Since the bacterial cells reached the vascular bundles, we assumed that they would go upstream by tracheid cells and sieve tubes. However, after 2 weeks of incubation, no bacteria were observed in the aerial root samples taken from 1 to 3 cm above the treatment area (data not shown), whereas within the treated area, the gfp-tagged bacteria have been revealed during the month after inoculation.

Pseudomonas fluorescens was also shown to actively colonize the root tissues immediately after the bacterization. After 30 min of incubation, the bacterial cells were detected in the parenchyma of both aerial and substrate roots (Fig. 1b). They also entered through the velamen and exodermis. Although some unique bacterial cells were detected in the stele, no active colonization of this vascular tissue took place. In their majority, pseudomonads preferred to stay in the velamen and the cortical parenchyma cells. Notably, the colonization of the aerial roots was more intense, whereas minor cells were detected in the substrate roots after 7 days of incubation. Apart from localization in the roots of the adult orchid plant, distribution of rhizobacteria was also examined on the seeds, protocorms and 1-year-old plantlets of *D. nobile*, germinated and cultivated *in vitro* on MS-agar medium. In contrast to the mature roots, no passive entrance of the bacterial cells was observed when the seeds and protocorms were treated. Bacteria massively colonized the surface of the rhizoids as well as the protocorm itself, attaching and accumulating in the hollows and wrinkles (Figs 2 and 3). To study the bacterization at the early stages of *D. nobile* development, we used the seeds germinated with the cocultures of *K. oxytoca* and *P. fluorescens*. The results of the scanning electron microscopy are given after 21 and 49 days of the bacterization (Fig. 2). Both strains were shown to preferably colonize the seedling's surface and the nascent rhizoids, forming microcolonies and aggregating within the cavities, bowls and wrinkles. The bacterial extracellular matrix (EM) anchors the single cells and sticks the micro-populations together; *P. fluorescens* formed evident biofilm-like structures on the plant surface (Fig. 2f). However, at this early stage of the orchid germination, in contrast to the roots of the mature plant, both bacterial strains were shown not to enter in the vascular system of the seedlings (Fig. 2d,h), remaining only within the outer layers of the meristematic region.

The CLSM (Fig. S1) of the 1-year-old seedlings, incubated with the tested rhizobacteria for 7 days, confirmed that the bacterial cells were abundantly present on the surface of the seedlings as well as on their roots (Fig. S1a, c,d). Although for the majority of the examined variables both tested strains did not enter deeper than the external layers, in the samples incubated with *K. oxytoca* TSKhA-gfp, bacteria were observed within the inner tissues of the seedling (Fig. S1b).

The transmission electron microscopy revealed that when the plantlets were inoculated with *P. fluorescens* 32-gfp, a lot of bacterial cells submerged in the EM were swarming in the immediate vicinity of the plant cell wall. After 24 h of inoculation (Fig. S1a–d), the bacterial cells showed cellulolytic activity, mediating a localized digestion of the plant cell wall polymers, thus provoking deformation of the epidermal cell wall beneath the bacterial cell and an active detachment of the cuticle and cleavage of the plant cell coat. Such a consequence of

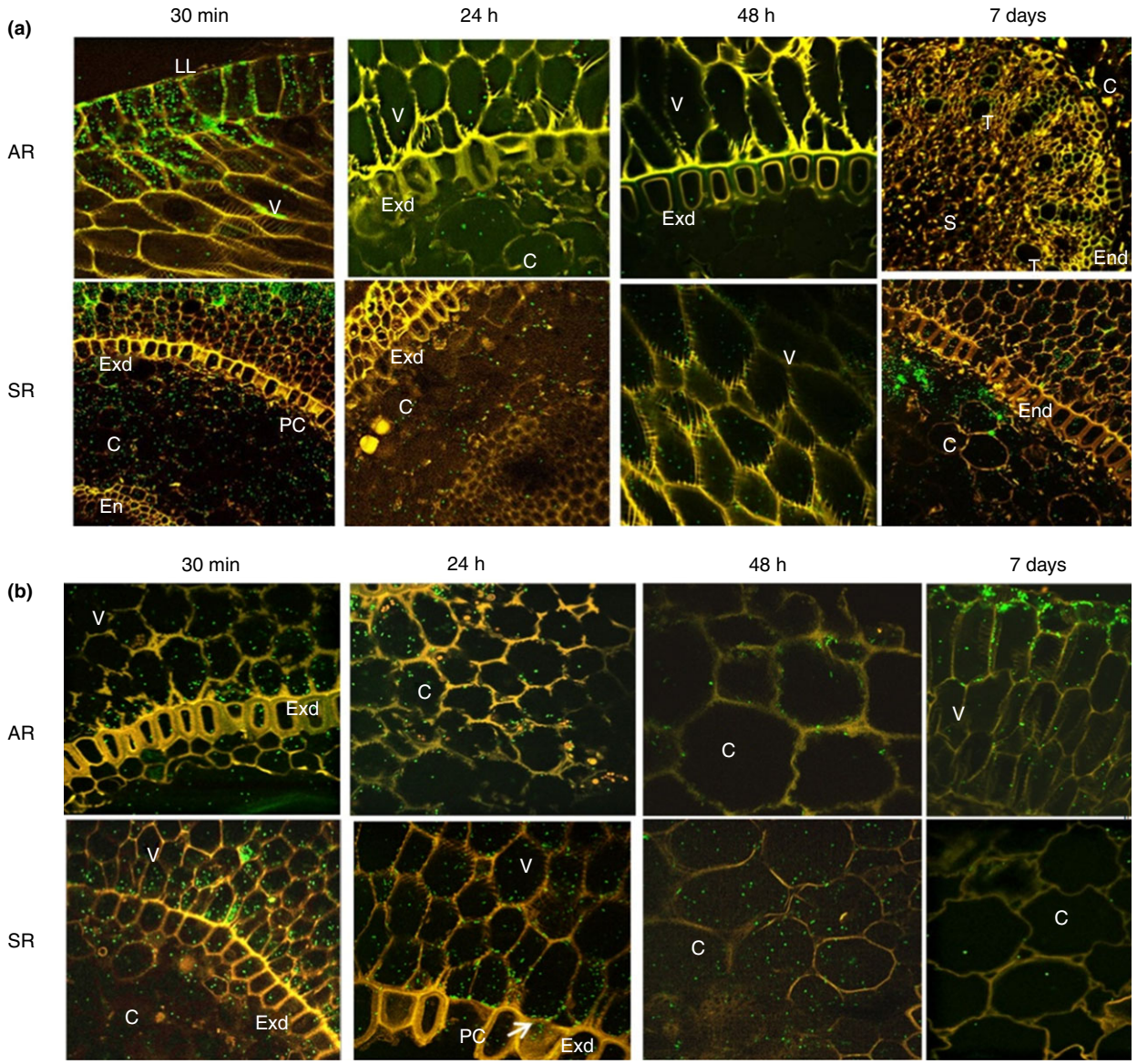


Figure 1 CLSM images of the *Dendrobium nobile* root cross-sections inoculated with rhizobacteria. The treatment of the aerial (AR) and substrate roots (SR) was made with the GFP-tagged *Klebsiella oxytoca* TSKhA-gfp (a) and *Pseudomonas fluorescens* 32-gfp (b), as described in the Materials and Methods. LL—limiting layer of the velamen; V—velamen; Exd—exodermis; C—cortical parenchyma; PC—passage cells; End—endodermis; S – stele; T—tracheids. Magnitude—200–400×. [Colour figure can be viewed at wileyonlinelibrary.com]

events is known for the formation of plant–microbial associations and symbiosis with endophytic bacteria. When the samples treated with *K. oxytoca* TSKhA-gfp were studied, the bacterial cells were shown to inhabit the intercellular space in cortex (Fig. S1e,f), a week after inoculation.

Production of the plant growth stimulator, IAA

In this study, we re-identified *Klebsiella* sp. based on the analysis of the 16S rRNA gene sequences as *K. oxytoca* TSKhA-gfp; GenBank acc. no KX397297 (identity of 99% with the best match to *K. oxytoca* strain CFRB1, acc. no JX848323.1 and to *K. oxytoca* strain JKo3, complete genome sequence, acc. no AP014951.1). Previously (Blinkov *et al.* 2014), we showed that the wild type of *K. oxytoca* (former *K. planticola* TSKhA-91) produced the highest IAA yield at the stationary phase of growth under the tryptophan supplementation, producing up to 85·5 μg ml^{-1} of auxins in the nitrogen-free medium. In order to estimate the IAA production by its gfp-tagged transformant, *K. oxytoca* TSKhA-gfp as well as

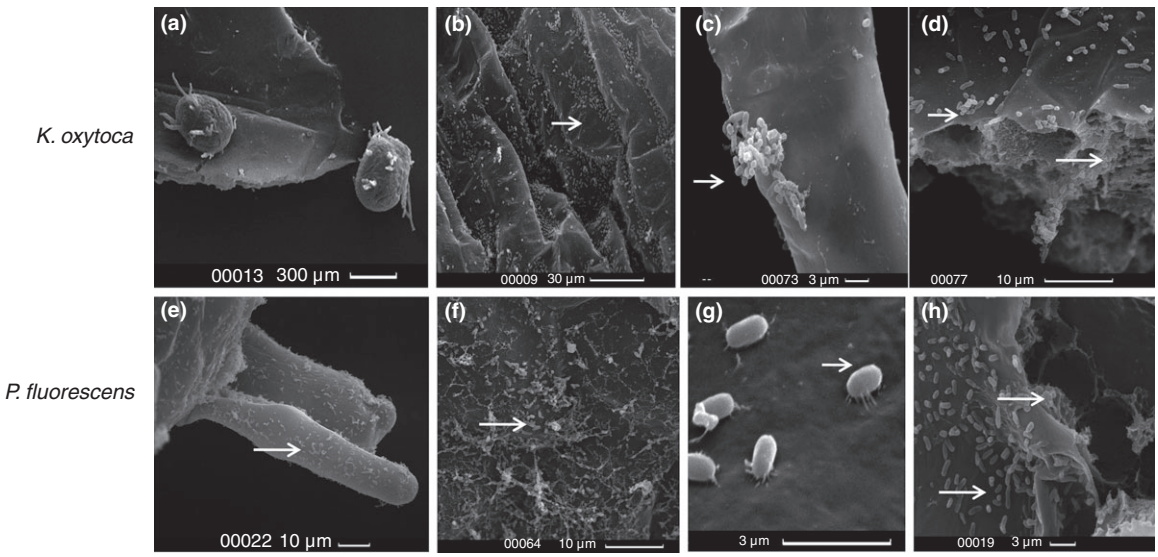


Figure 2 SEM micrographs of the microbial colonization of *Dendrobium nobile* seeds and protocorms. The localization of rhizobacteria after 21 days (a–d) and 49 (e–h) days of seed germination in cocultures with *Klebsiella oxytoca* TSKhA-gfp and *Pseudomonas fluorescens* 32-gfp. (a, e) Germinated seeds with the nascent rhizoids; (b, f) The seedling's surface with the bacterial cells on it (arrows); (c, g) microcolony on the rhizoid and *P. fluorescens* bacterial cells anchoring to the plant surface by extracellular polymers; (d, h) bacterial cells on the surface and in the epidermis and outer layers of the protocorm's basal part. Scale bars: 300 μm (a); 30 μm (b); 3 μm (c); 10 μm (d); 10 μm (e); 10 μm (f); 3 μm (g); 3 μm (h).

P. fluorescens 32-gfp, were also tested for their capacity of auxin production with various nitrogen sources. Both cultures reached their maximum biomass accumulation in LB medium during the first (*Klebsilella*) and second (*Pseudomonas*) days of cultivation (Fig. S2). In comparison to LB, the biomass yield of *K. oxytoca* TSKhA-gfp reduced by 1·5-, 2·0- and 7·5-fold when it was cultivated in the mineral media supplemented with nitrate, ammonium or no nitrogen respectively (Fig. S2a,b). The addition of exogenous Trp to the media did not influence the biomass accumulation by *K. oxytoca*, however, in the nitrogen-free medium, Trp on the contrary, enhanced its biomass accumulation by 20%. For *P. fluorescens* 32-gfp, the stimulative effect of Trp was observed when the K2-NO_3 medium was used (Fig. S2c,d).

Based on the bacterial growth dynamics, we analysed auxin production during the first 96 h of cultivation. Without Trp supplementation, biosynthesis of IAA by *K. oxytoca* TSKhA-gfp did not exceed 3 (in K2-NO_3) and 4 (in LB) μg ml^{-1}, whereas no production (0 μg ml^{-1}) was detected in K2-NH_4 medium (Fig. 4a,b). The addition of 200 μg ml^{-1} of Trp resulted in the enchancement of auxin production by 4·0-, 7·7- and 30-fold in LB, K2-NH_4 and K2-NO_3 respectively. Maximal effect was detected in nitrogen-free media with a more than 400-fold stimulation and production of 120 μg ml^{-1} of IAA, whereas only 7·5 μg ml^{-1} of IAA were produced in K2-NH_4 medium. LB medium, rich in organic peptides and amino acids, including Trp, had almost no stimulative effect on the IAA biosynthesis, whereas induction of the auxin production was clearly seen under stress conditions with the limiting nitrogen supplementation. On the contrary, *P. fluorescens* did not produce IAA in the absence of the exogenous Trp when it was cultivated in the mineral media; 12 μg ml^{-1} of IAA was detected within 48 h in the LB medium only (Fig. 4c). Supplementation with Trp stimulated auxin biosynthesis up to 20 and 50 μg ml^{-1} in K2-NO_3 and LB respectively (Fig. 4d). Nevertheless, the IAA biosynthesis in K2-NH_4 medium remained still minimal (2 μg ml^{-1}), showing the inhibitory effect of ammonium on the IAA biosynthesis.

Identification of the indolic compounds by TLC

With no Trp supplementation, only the slight spots corresponding to IAA, were detected when bacteria were cultivated in LB organic medium (Figs S3d and S4c). The addition of the exogenous Trp resulted in the enhancement of the IAA production (intensified the spots). The largest variety of indoles was shown for *K. oxytoca*, cultivated in K2-nitrate and nitrogen-free media (Fig. S3a,c). The results of TLC analysis correspond to the data on relative auxin estimation by Salkowski reagent; ammonium inhibited the IAA biosynthesis and significantly reduced the amounts of produced IAA.

We did not reveal any visible IAA spots when *P. fluorescens* 32-gfp was cultivated in K2-NH_4; it was scarcely

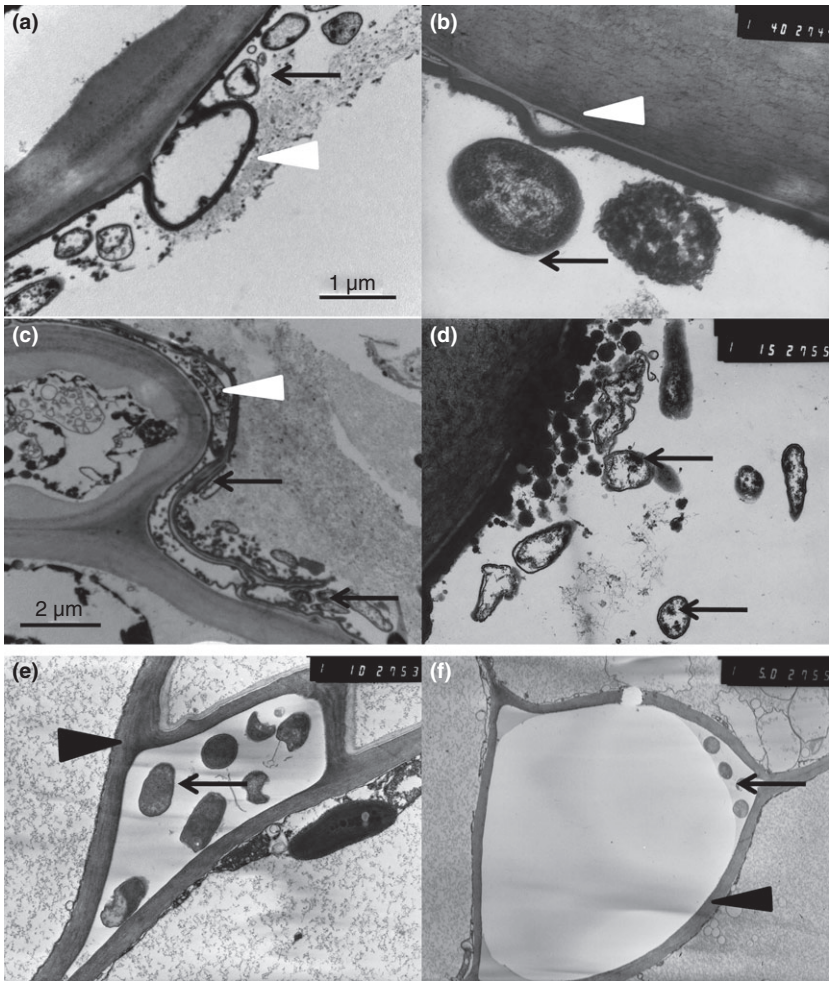


Figure 3 Transmission electron micrographs of *Dendrobium nobile* plantlets inoculated with rhizobacteria. *Pseudomonas fluorescens* 32-gfp (a–d) and *Klebsiella oxytoca* TSKhA-gfp (e, f) after 24 h (*P. fluorescens*) and 7 days (*K. oxytoca*) of inoculation. Bacterial cells are marked with black arrows, detachment of the epidermal cell wall is marked with a white triangle. *Klebsiella oxytoca* inhabit the intercellular space between the cells of core parenchyma; black triangles are the cell walls of the parenchyma cells.

detected in K2-NO_3 (Fig. S4a) by 96 h. Nevertheless, the spots corresponding to IAA were clearly seen on the TLC plates, when ethyl acetate extracts were originated from the LB medium (Fig. S4c). The culture broths (CBs) of the gfp-tagged *P. fluorescens* (Fig. S4d) and *K. oxytoca* (Fig. S4e,f) contain the compounds corresponding to ILA and IPyA that are typical for indole-pyruvic way of IAA biosynthesis, whereas no spot of IAM characterizing another way of IAA biosynthesis, was detected.

The biological activity of microbial IAA

To confirm the biological activity of microbial auxins produced by *K. oxytoca* and *P. fluorescens*, we used the bean cuttings, which are susceptible to exogenous IAA. The results of this bio-assay (Fig. S5, Table 1) showed that microbial IAA significantly and considerably stimulated the rooting of cuttings by the enhancement of the stem height with the nascent roots abundance. The differences between control and treated cuttings were found significant in the case of stem height (LSD = 0·37), as well as for number of roots (LSD = 5·4). The control cuttings were poorly rooted with only three to four roots per cutting; the formation of the adventitious roots was detected at a height of only 0·4 cm. When the cuttings were treated with the CB of *K. oxytoca* containing 60 and 80 μg ml^{-1} of auxins, the rhizogenesis occurred at a stem height of 5·7 and 6·4 cm respectively. In comparison to the pure IAA standard solution of 60 μg ml^{-1}, the cuttings treated with *K. oxytoca*'s CB of the same concentration formed the adventitious roots lower on the stem, whereas their number was higher (Table 1). When the CB of *K. oxytoca* with 80 μg ml^{-1} of IAA was used, the stem height and the root number enhanced by 1·1 and 1·5 fold more respectively. The activity of IAA produced by *P. fluorescens* was less efficient due to its fewer amounts in the CB. Nevertheless, both bacterial CBs promoted the rhizogenesis of the bean cuttings, thus providing the evidence for the IAA-induced plant growth responses.

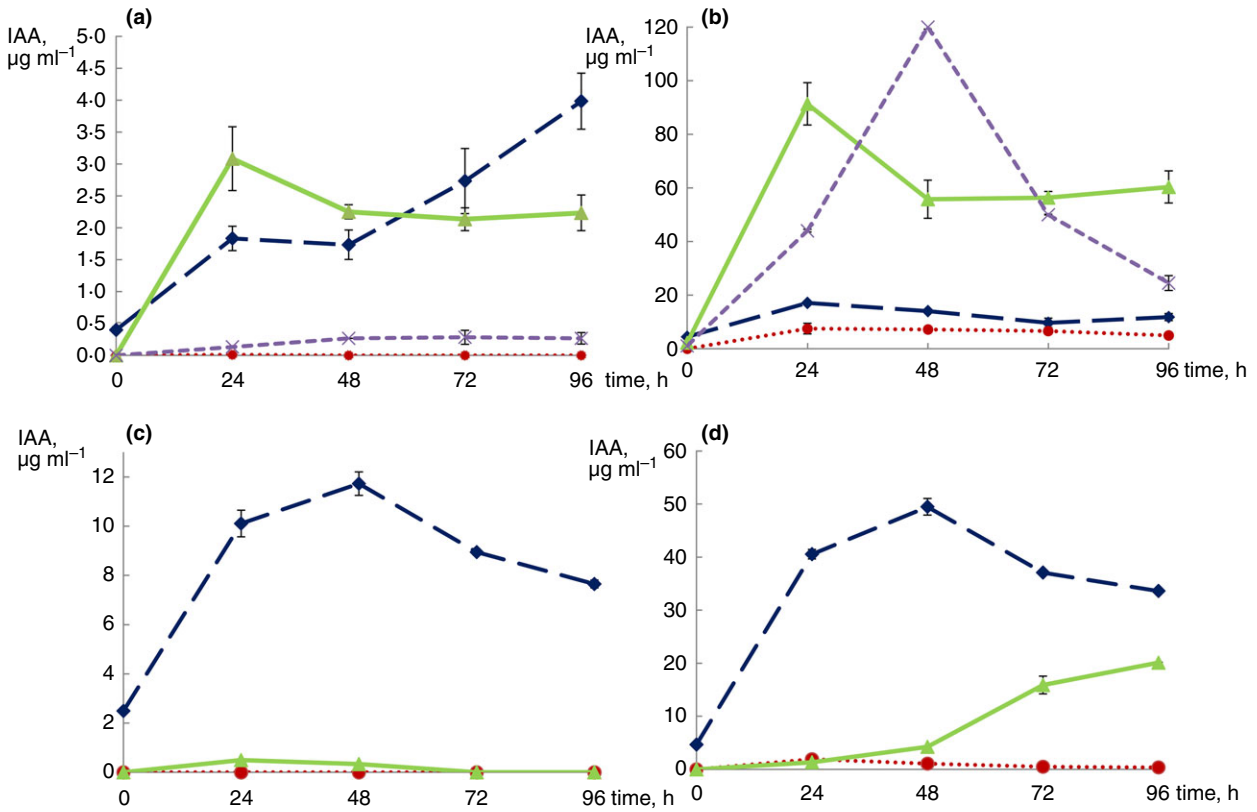


Figure 4 Microbial auxin (indole-3-acetic acid, IAA) production by gfp-tagged *Klebsiella oxytoca* TSKhA-gfp (a, b) and *Pseudomonas fluorescens* 32-gfp (c, d). Rhizobacteria were cultivated in LB (filled square, dashed line) and mineral media: K2-N_0 (cross, densely dashed line), K2-NO_3 (filled triangle, solid line), and K2-NH_4 (filled circle, dotted line), supplemented with different nitrogen sources without Trp (a, c) and with 200 μg ml^{-1} of L-Trp (b, d). The basic medium (K2-N_0) consists of (g l^{-1}): K_2HPO_4—0·5; KH_2PO_4—0·3; $MgSO_4{\cdot}7H_2O$—0·1; NaCl—0·75; $CaCl_2{\cdot}6H_2O$—0·03; sucrose—6·0; trace elements—1 ml. K2-NH_4 medium is supplemented with yeast extract and $(NH_4)_2SO_4$, and the 'nitrate' medium (K2-NO_3) is supplemented with yeast extract and $NaNO_3$. Nitrogen-free (basic) medium was used only for cultivation of the diazotrophic strain of *K. oxytoca*. For the details, see the Materials and Methods section. The experiments were repeated with three replicates; the results are given as mean value, and the error bars indicating the standard deviation. [Colour figure can be viewed at wileyonlinelibrary.com]

Bacterization of the *Dendrobium nobile* seeds with *Klebsiella oxytoca* TSKhA-gfp and *Pseudomonas fluorescens* 32-gfp cultures

In order to confirm our suggestion that orchids do not develop strain-specificity to PGPR, the bacterization of the *D. nobile* seeds was made on the Knudson-C medium with no addition of any plant growth stimulators but supplemented with L-Trp for the possible induction of the microbial auxin production. The number of the viable seeds estimated with TTC and TTB varied and comprised 47·5% ± 3·4 and 71·0% ± 4·7 respectively (the data are the average of three replicates with a calculation of 150–200 seeds per each replicate). Both cultures, *P. fluorescens* and *K. oxytoca*, showed no inhibitory effect on the early growth of the *D. nobile* seeds. On the contrary, they enhanced the number of the germinated seeds and accelerated the stages of the seed development (Figs 5 and 6). With no exogenous tryptophan addition, after 39 days of inoculation, tested rhizobacteria significantly ($P \leq 0{\cdot}05$) augmented the number of the germinated seeds with developed rhizoids and formed the first leaf-like organ. Although the positive influence of the *P. fluorescens* strain on the orchid germination has been observed through this bioassay study, the statistically significant difference ($P \leq 0{\cdot}05$) between the control and the seeds treated with *P. fluorescens* 32-gfp was detected only after 94 days of inoculation. The growth-promoting influence of the *K. oxytoca* TSKhA-gfp strain was observed in both tested conditions (without and with addition of the exogenous tryptophan). By day 101 of bacterization, the inoculation of the seeds with this rhizobacterium significantly ($P \leq 0{\cdot}05$) enhanced the number of the seedlings with two and more leaves by 17% (−Trp) and by 22% (+Trp). Even without tryptophan supplementation, this bacterial culture showed the

Table 1 Influence of the microbial culture broth on the rooting of the bean cuttings

Test variant	Auxin content (μg ml^{-1})	Rhizogenesis	
		Stem height with the nascent roots (cm)	Number of roots per one cutting
Control (water)	–	0·42 ± 0·30	3·9 ± 0·06
Auxin (IAA)	60	7·06 ± 0·06	47·7 ± 5·20
Klebsiella oxytoca	60	5·67 ± 0·33	63·8 ± 3·15
Klebsiella oxytoca	80	6·38 ± 0·52	96·3 ± 6·86
Pseudomonas fluorescens	30	2·00 ± 0·07	18·3 ± 2·90

Values are the mean of five replicates ± standard error.
All treatments were significantly different from the control according to one-way analysis of variance (ANOVA); the stem height was higher, *F*-test ($P < 0·001$), and the root number increased, *F*-test ($P < 0·001$).

evident ($P \leq 0·05$) stimulation of the orchid seeds germination by the gradual reduction of the ungerminated seeds and raising the number of the developed embryos (the ones with rhizoids and first leaf at 39 and 54 days after the inoculation, and those with two and more leaves after 94 days after the inoculation). The supplementation of tryptophan has slightly smoothed the plant growth-promoting effect at the early stages (39, 54 and 73 days) of the seed development (the difference between the treated seeds and the control variables was not statistically significant ($P \leq 0·05$)). Nevertheless, the most evident promoting effect was observed after 94 days of bacterization, when the plantlets passed to the juvenile stage of their ontogenesis with an active photosynthesis, growth and differentiation by forming additional leaves and roots. Exactly at this stage, *K. oxytoca*, as the active producer of IAA, showed the most prominent stimulation.

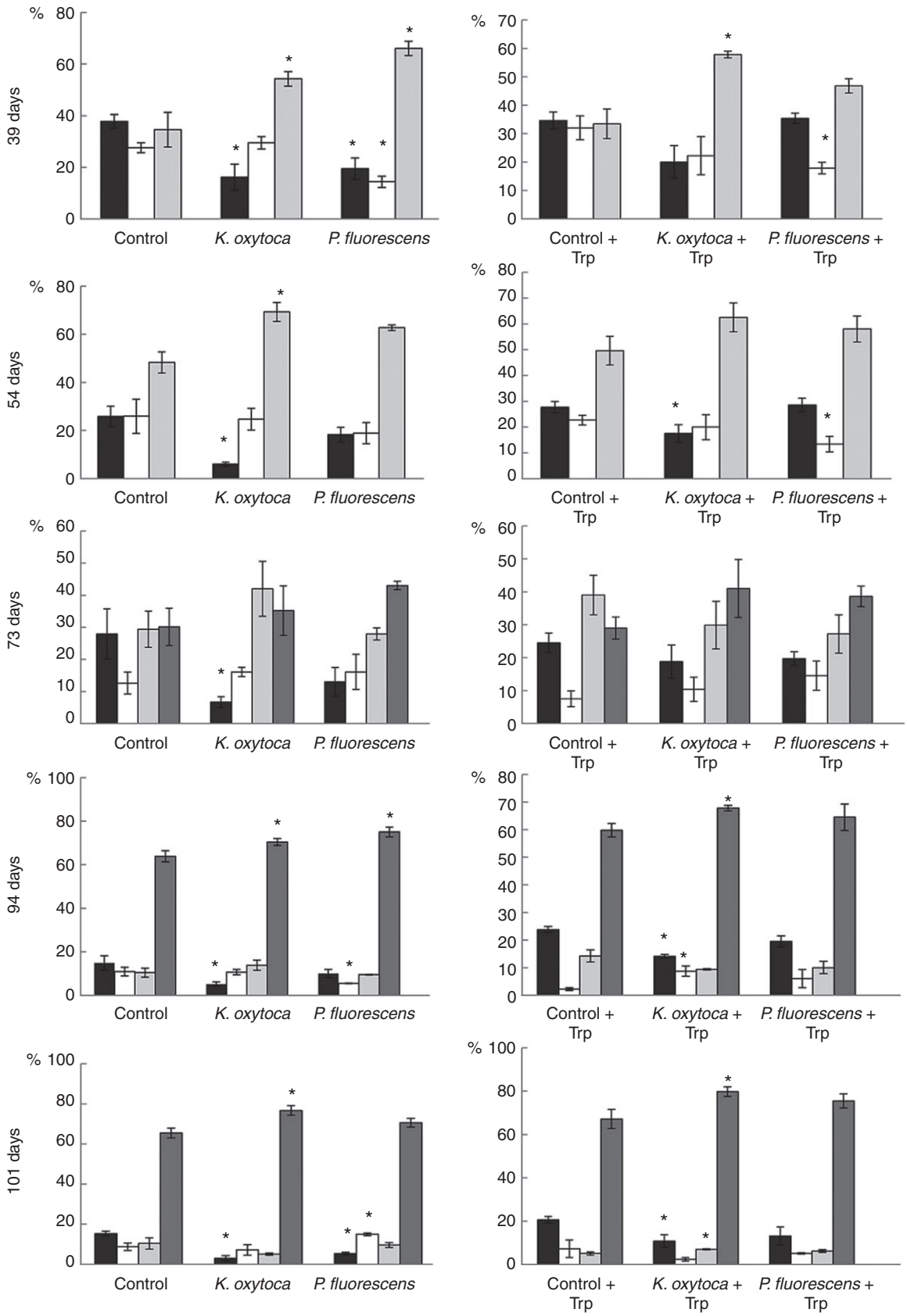
It was also noticed that without tryptophan addition, the length of the plantlets was at 73, 94 and 101 days after bacterization 40, 25 and 27% more with *K. oxytoca* treatment, and 14, 29 and 43% more with *P. fluorescens* treatment (Fig. 6). The supplementation with Trp reduced the effectiveness of *P. fluorescens* 32-gfp and the difference became not statistically significant. On the contrary, the most effective influence (with significant difference at $P \leq 0·05$) was detected when the seeds were inoculated with *K. oxytoca* TSKhA-gfp under tryptophan supplementation; the dynamics of the length enhancement at 54, 73, 94 and 101 days of bacterization comprised 25, 45, 36 and 35% respectively. The addition of exogenous tryptophan resulted in the most pronounced elongation of the plantlets with an active IAA producer, *K. oxytoca*, that also confirmed the effective conversion of Trp into biologically active auxin.

Thus, both rhizobacteria promoted *in vitro D. nobile* seed germination at the early stages of the orchid development, and the host plant showed no selective specificity to the tested bacterial cultures under the experimental conditions, although the PGPR strains showed different patterns in orchid–microbial relations: *K. oxytoca* demonstrated more intense root colonization activity (Fig. 1) and more efficient growth promoting impact under tryptophan supplementation (Figs 5 and 6), whereas the *P. fluorescens* strain with a much less level of produced IAA (Fig. 4), showed its growth promoting capacity in -Trp conditions. Both strategies, however, can be regarded as complementary and improving adaptive potentials of the host-orchid when different microbial populations, particularly endophytic, are colonizing the plant.

Discussion

It is generally accepted that beneficial PGPR are effective only when they successfully colonize and persist in the plant rhizosphere. Endophytes are also considered as ubiquitous, colonizing the plant tissues latently (Hallmann *et al.* 1997), showing no external sign of infection or negative influence on their host (Ryan *et al.* 2008). Among the OAB, diverse rhizobacteria were isolated as endophytes, such as *Burkholderia*, *Sphingomonas*, *Novosphingobium* and *Pseudomonas* from *Dendrobium officinale* (Yu *et al.* 2013); *Paenibacillus lentimorbus* and *P. macerans* were isolated from the meristems of *Cymbidium eburneum* (Faria *et al.* 2013); pseudomonads and bacilli strains were isolated from the terrestrial Australian orchids (Wilkinson *et al.* 1989). Previously we showed that *Dendrobium moschatum*'s endophytic strains of *Sphingomonas* sp., *Agrococcus* sp. and *Mycobacterium* sp. effectively promoted *in vitro* orchid seed germination, and they actively attached to the seed surface and formed microcolonies with the subsequent colonization of the rhizoids and developed roots (Tsavkelova *et al.* 2007, 2016). In this study, nonorchid strains of *K. oxytoca*

Figure 5 Stages of the *Dendrobium nobile* seed germination and development without (control) and inoculated with *Klebsiella oxytoca* TSKhA-gfp and *Pseudomonas fluorescens* 32-gfp rhizobacteria. The bacterization was made on the Knudson-C medium without exogenous tryptophan and under supplementation of 200 μg ml^{-1} of L-tryptophan (+Trp); dormant or swallowed but not photosynthesizing embryo (in black); green photosynthetically active embryo (in white); developed rhizoids and first leaf-like organ (in light grey); two and more leaves (in dark grey). The values are the mean of four repetitions (60 seeds/embryos/plantlets were counted in three randomized areas in each repetition) ± standard error. Asterisks indicate significant difference between treatments and control by Student's *t*-test ($n = 4$; $P \leq 0·05$).


39 days
54 days
73 days
94 days
101 days
%
Control
K. oxytoca
P. fluorescens
Control + Trp
K. oxytoca + Trp
P. fluorescens + Trp

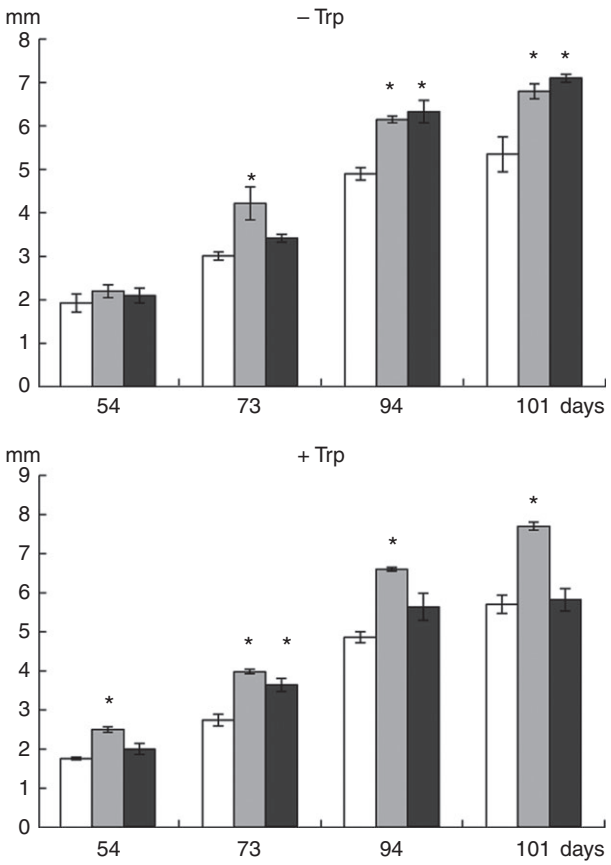


Figure 6 The length of the *Dendrobium nobile* embryos and plantlet without bacterization (in white), and inoculated with PGPR cultures of *Klebsiella oxytoca* TSKhA-gfp (in light grey) and *Pseudomonas fluorescens* 32-gfp (in dark grey) without (−Trp) and supplemented with L-tryptophan (+Trp). The values are the average of three repetitions (20 plantlets were measured in three randomly selected areas in each repetition: 60 plantlets in total for one repetition) ± standard error. Asterisks indicate significant difference between treatments and control by Student's *t*-test ($P \leq 0{\cdot}05$).

and *P. fluorescens* also showed the same tendency in forming associations with *D. nobile* seeds by colonizing their surface as solitary cells and microcolonies, preferring to spread along the nascent rhizoids (Fig. 2). In *Brassica napus* associations with *P. fluorescens*, bacteria were mostly detected on the proximal region of the plant roots, closest to the seed then on the mid and distal portions (Pallai *et al.* 2012).

Within 24 h of inoculation, the *P. fluorescens* strain showed its activity in the digestion of plant cell wall polymers (Fig. 3). Similar cellulolytic and pectinolytic enzyme activities were reported in *P. fluorescens* (Benhamou *et al.* 1996; Quadt-Hallmann *et al.* 1997). Moreover, the enzymatic degradation of plant cell walls was only noticed when endophytes colonized the root epidermis but not after colonizing intercellular spaces of the root cortex (Lodewyckx *et al.* 2002). Pectate lyase production by *Klebsiella* sp. strains has also been reported during plant colonization (Kovtunovych *et al.* 1999). This is the first study to report the localization and the ways of *in vitro* colonization, penetration and persistence of two PGPR strains inside the orchid roots, its seeds and plantlets. Both investigated bacteria behaved as endophytes when they were colonizing the aerial and substrate roots of the adult orchids. By the CLSM analysis we observed that gfp-tagged *K. oxytoca* and *P. fluorescens* actively populated the roots of the adult *D. nobile* plant, and they easily entered through the velamen into the core parenchyma (Fig. 1) facing no evident resistant barriers from the orchid. Such an active colonization of the inner tissues might be explained by the beneficial plant growth-promoting capacities of the tested strains; moreover, they belong to the well-known PGPR of the genera *Klebsiella* and *Pseudomonas*, the representatives of which have also been reported as the orchid-associated endophytes (Wilkinson *et al.* 1989; Wilkinson *et al.* 1994; Tsavkelova 2011; Yu *et al.* 2013). However, new studies are needed to investigate the strategies of bacterial colonization by the gfp-tagged orchid-associated PGPR both *in vitro* and *in situ* to provide better knowledge and understanding of the orchid–microbial interactions.

The modern investigations on the orchid–mycorrhizae relations incline to the idea that the tropical epiphytic orchids, as well as European orchids, appear to vary dramatically in degree of specificity in their mycorrhizal interactions (Otero *et al.* 2002); there are orchids strictly specific and generalist in their choice of the fungal symbiotic partner. As for the OAB, it becomes obvious that the orchids do not show any strict specificity to the chosen bacterial partners. Nevertheless, the selected strains should possess neutral or beneficial traits that might enlarge the adaptive capacities of the host-orchids and not to provoke direct pathogenicity. At the same time, some acknowledged PGPR strains, such as *Rhizobium* sp. or *Azospirillum* sp. could be beneficial to the adult plant when colonizing the roots, but absolutely unsuccessful in germination of their tiny seeds (Tsavkelova *et al.* 2007, 2016). Among the strategies for the host plant colonization, the PGPR strains use invasion through the root hair cells, as it was shown for the *Azospirillum brasilense*-wheat association (Schloter and Hartmann 1998) or entering through splits and cracks at the points of lateral root emergence with the subsequent colonization of the intercellular spaces and cortical cells of the rice plants by *Herbaspirillum seropedicae* Z67 (James *et al.* 2002). *Klebsiella oxytoca* GR-3, the endophytic strain of *Typha australis*, prefers the apoplastic localization in the intercellular spaces of cortical and vascular zones (Jha and Kumar 2007). In this study, both investigated rhizobateria were also observed not only on the root surface but in the cortex and stele tissues (Fig. 1). The density of the endophytic microbial populations inside the roots differed

between the strains; unlike the abundantly present *K. oxytoca* cells, the number of the *P. fluorescens* cells decreased and was hardly detected after a week of inoculation, particularly within the substrate roots. The tested strains of *K. oxytoca* and *P. fluorescnes* demonstrated the typical epiphytic and endophytic colonization patterns. Nevertheless, both rhizobacteria showed different strategies in establishing their endophytic lifestyle with the juvenile and adult orchid plants. When the roots of the mature *D. nobile* orchid were inoculated with the bacterial cultures, they easily entered over the root surface through the velamen inside the root cortex (Fig. 1). When the young 1-year-old plantlets were treated with the rhizobacteria, we observed that the micro-organisms could penetrate inside by the micro-splits at the emergence of the growing roots with the microbial activity in localized digestion of the plant cell wall by *P. fluorescence* and colonization of the intercellular space by *K. oxytoca* in the root cortex (Fig. 3). However, when the germinating seeds and protocorms were bacterized, *K. oxytoca* and *P. fluorescnes* actively colonized the surface and formed microcolonies on the nascent rhizoids, but they did not enter inside the germinating embryos except for a couple of the outer layers (Fig. 2). Such resistance of the plant seeds to the microbial invasion could be explained by the production of the lytic enzymes and antimicrobials. Orchids are known for biosynthesis of various phenolic and flavonoid compounds; the pronounced production of alkaloids in *Dendrobium candidum* protocorms and phytoalexins in *Phalaenopsis* sp. young plants was induced by a fungal elicitor (Chen *et al.* 2006) and infection of *Botryces cinerea* and *Rhizoctonia* spp. (Reinecke and Kindl 1994) respectively. Significant increase in kinsenosides and flavonoids in *Anoectochilus formosanus* was also reported as a response to the co-inoculation with F-23 *Mycena* mycorrhizal fungus (Zhang *et al.* 2013). Such strategy is needed to control the fungal infection in the early stages of the orchid symbiotic development.

The strains of *K. oxytoca* and *P. fluorescens* are known for their beneficial influence on plant growth due to the nitrogen fixation (*Klebsiella* sp.), production of the antimicrobials or plant growth stimulators, and improvement of the mineral nutrition and siderophore biosynthesis (*Pseudomonas* sp.) (Spaepen *et al.* 2007; Glick 2014). Exogenous tryptophan is a major precursor in microbial auxin biosynthesis; 100 μg ml^{-1} of Trp enhanced IAA biosynthesis by 2·2-fold in *Klebsiella pneumoniae* (Sachdev *et al.* 2009). Among pseudomonads, there are strains with significant stimulation of IAA production, such as *Pseudomonas* ssp. with up to 230·2 μg IAA per ml (Parray *et al.* 2013), and the strains with the minimal tryptophan influence, such as *P. fluorescens* strain ACC_{14} with only 3·0 μg IAA per ml (Shaharoona *et al.* 2007). In this study, we have also shown Trp-dependent biosynthesis of IAA by the tested gfp-tagged bacteria (Figs 4 and S3). The model plant test showed that the bean cuttings were highly susceptible to the microbial auxin, contained in the CB (Table 1, Fig. 5), confirming the rooting responses to IAA and thus, the biological activity of the IAA produced by *P. fluorescens* and *K. oxytoca*. The highest amounts of auxin for *K. oxytoca* and *P. fluorescens* (91 μg ml^{-1} and 20 μg ml^{-1} respectively) were detected in the mineral medium with the nitrate source of nitrogen. Ammonium presence suppressed auxin biosynthesis, and IAA concentration did not exceed 8 μg ml^{-1} and 2 μg ml^{-1} respectively. The inhibitory effect most probably occurs at the deamination stage of IAA biosynthesis. Other authors (Mishra and Kumat 2015) showed that decreasing IAA levels produced by *K. pneumoniae* were observed in the presence of vitamins and amino acids, as compared to the control. Our results also correspond to the previous data (Shokri and Emtiazi 2010; Mohite 2013) reporting that nitrate is the optimal nitrogen source for the microbial IAA biosynthesis. The maximal IAA production for diazotrophic *K. oxytoca* TSKhA-gfp was observed in nitrogen free medium (120 μg ml^{-1}); rhizobacteria are known to produce high amounts of auxin in unfavourable conditions that might induce the formation of plant–microbial associations (Oljunina and Shabaev 1996). The wild strain of the tested *Klebsiella* also demonstrated the highest production in the same nitrogen-free conditions (Blinkov *et al.* 2014) in amounts of around 80 μg ml^{-1}. In this previous study, the feeding experiments with exogenous IAM supplementation did not show any consumption or its transformation into IAA by the wild *Klebsiella* strain. Here, we confirmed the absence of IAM intermediate in both tested cultures, whereas the intermediates of the indole-pyruvic IAA pathway, such as ILA and IPyA (Figs S3 and 4) were detected by TLC. IPyA pathway of auxin biosynthesis (Trp-IpyA-IAAld-IAA) is widely reported in plant-associated klebsiellas and nonphytopathogenic pseudomonads (Spaepen *et al.* 2007). Indole pyruvate decarboxylase catalysing formation of indole-3-acetaldehyde from IPyA was detected in *K. pneumonia* (El-Khawas and Adachi 1999; Mishra and Kumat 2015). Pseudomonads are the most important PGPR due to the auxin production (Khakipour *et al.* 2008); the authors showed that 48% of the *P. putida* synthesized IAA via IAM, 41% of them used both paths, and 7% of the strains produced IAA via IPyA. *Pseudomonas fluorescens* 6-8, a rhizosphere isolate, enhancing root elongation of canola (*B. napus* L.), produced cytokinins (isopentenyl adenosine, zeatin riboside and dihydroxyzeatin riboside), but low concentrations of IAA (Pallai *et al.* 2012); in the presence of Trp (200 μg ml^{-1}) it reached production of 8–10 μg ml^{-1} through IPyA biosynthetic pathway. The inoculation of the *Gossypium hirsutum* L. seeds with *K. oxytoca* Rs-5 enhanced their germination under salinity stress (Liu *et al.* 2013); a

significant increase in root and shoot length of *T. australis* was reported for the endophytic strain of *K. oxytoca* GR-3 (Jha and Kumar 2007). Inoculation of the maize seeds with *Pseudomonas* spp. and *Klebsiella variicola* enhanced stomatal conductance and chlorophyll content (Kifle and Laing 2016), and the application of the culture supernatants of *K. pneumoniae* increased rice root elongation, root dry matter and development of the lateral roots and root hairs (El-Khawas and Adachi 1999).

In this study, we clearly observed a positive plant growth response upon inoculation with the endophytic *P. fluorescens* and *K. oxytoca* strains, which showed different strategies in plant–microbial interactions with the seeds, young plantlets and adult *D. nobile* orchid. The plant did not reveal any strict specificity towards the tested PGPR partners. Our data provide new evidence regarding the ways endophytes interact with the orchids and establish the tight beneficial associations. Although the strains responded differently to the addition of the exogenous tryptophan, the inoculation of *D. nobile* seeds with *K. oxytoca* and *P. fluorescens* promoted *in vitro* seed germination and development, thus highlighting the successful application of the nonorchid PGPR in biotechnology and conservation of the endangered orchids.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CLSM images of the bacterized *Dendrobium nobile* 1-year-old seedlings. The surface and the sections of the seedlings treated with the gfp-tagged *Klebsiella oxytoca* TSKhA-gfp (A–C) and *Pseudomonas fluorescens* 32-gfp (d, f); R—roots. Magnitude—200–400×.

Figure S2. The optical density (OD_{590}) of *Klebsiella oxytoca* TSKhA-gfp (a, b) and *Pseudomonas fluorescens* 32-gfp (c, d). Rhizobacteria were cultivated in LB and several mineral media supplemented with a different nitrogen source without Trp (a, c) and with 200 μg ml^{-1} of L-Trp (b, d). The basic medium (K2-N_0) consists of (g l^{-1}): K_2HPO_4—0·5; KH_2PO_4—0·3; $MgSO_4 \cdot 7H_2O$—0·1; NaCl—0·75; $CaCl_2 \cdot 6H_2O$—0·03; sucrose—6·0; trace elements—1 ml. K2-NH_4 medium is supplemented with yeast extract and $(NH_4)_2SO_4$, and the 'nitrate' medium (K2-NO_3) is supplemented with yeast extract and $NaNO_3$. Nitrogen-free (basic) medium was used only for cultivation of the diazotrophic strain of *K. oxytoca*. The experiments were repeated with three replicates; the results are given as mean OD value, and the error bars indicate standard deviation.

Figure S3. Production of IAA by *Klebsiella oxytoca* TSKhA-gfp. The cultivation media were the mineral nitrate (a), ammonium (b) and no nitrogen medium (c) as well as LB (d) without (-Trp) and with 200 μg ml^{-1} of Trp (+Trp). The culture broth was sampled at 1, 24, 48, and 72 h, and total indoles were extracted and analysed by TLC in chlorophorm: ethyl acetate: formiat (50 : 40: 10) running solvent. The variation of the indolic compounds in the culture broth (CB) of *K. oxytoca* under UV light (e, f). (e) TLC in chlorophorm : ethyl acetate : formiat (50 : 40 : 10) running solvent; (f) rechromatography of the scratched off and eluted in MeOH corresponding spots in the second running solvent (*n*-hexane : ethyl acetate : isopropanol : acetate (40 : 20 :5 : 1)). IAA—standard of indole-3-acetic acid, IAM—indole-3-acetamide, ILA—indole-3-lactic acid, IPyA—indole-3-pyruvic acid.

Figure S4. Production of IAA by *Pseudomonas fluorescens* 32-gfp. The cultivation media were the mineral nitrate (a), ammonium (b) and LB (c) without (-Trp) and with 200 μg ml^{-1} of Trp (+Trp). The culture broth was sampled at 1, 24, 48, 72 and 96 h (a–c), and total indoles were extracted and analysed by TLC. (d) TLC of the culture broth (CB) of *P. fluorescens* 32-gfp, cultivated in LB and itrate (NO_3) media for 2 (t2), 5 (t5) and 7 (t7) days. The CB from the 'NO_3' medium was taken in ×3 concentration. IAA—standard of indole-3-acetic acid; IAM—indole-3-acetamide; ILA—indole-3-lactic acid; IPyA—indole-3-pyruvic acid.

Figure S5. The rhizogenesis of the kidney bean (*Phaseolus vulgaris* L.) cuttings under the treatment with bacterial IAA. The cuttings were submerged in water (control), auxin (IAA) of 60 μg ml^{-1}, and microbial culture broth (CB) of different concentrations (60 and 80 μg ml^{-1} for *Klebsiella oxytoca*, and 30 μg ml^{-1} for *Pseudomonas fluorescens*). Relative auxin content was estimated by the Salkowski method. (a) *Klebsiella oxytoca* TSKhA-gfp; (b) *Pseudomonas fluorescens* 32-gfp.