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ORIGINAL PAPER

Production of phytohormones by root-associated saprophytic actinomycetes isolated from the actinorhizal plant *Ochetophila trinervis*

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Abstract The aim of the present study was to evaluate phytohormone production by symbiotic and saprophytic actinomycetes isolated from the actinorhizal plant *Ochetophila trinervis* which had previously proved to stimulate nodulation by *Frankia*. Three saprophytic strains out of 122, isolated from the rhizosphere of this plant with multiple enzymatic activities were selected for plant growth experiments in pots: *Streptomyces* sp. (BCRU-MM40), *Actinoplanes* sp. (BCRU-ME3) and *Micromonospora* sp. (BCRU-MM18). For experiments, the symbiotic N_2-fixing strain *Frankia* (BCU110501), isolated from nodules of the same actinorhizal plant was used. Phytohormone production was evaluated in supernatant of non-inoculated and inoculated culture media in exponential growth phase. Indole 3-acetic acid (IAA) and gibberellic acid (GA_3) were analyzed by gas chromatography-mass spectrometry (GC–MS), while zeatine (Z) production was determined by gas chromatography-flame ionization detector and high performance liquid chromatography (HPLC fluorescent and UV). The levels of the three phytohormones produced by the saprophytic rhizoactinomycetes were higher than that produced by the symbiotic *Frankia* strain. Zeatine biosynthesis was higher (μg ml^{-1}) than IAA and GA_3 (ng ml^{-1}), and *Micromonospora* strain produced the highest levels of these phytohormones. Although *O. trinervis* has been shown to be intercellularly infected by *Frankia* without mediation of root hair deformation, when plants were co-inoculated with actinomycetes' culture, some root hair deformation was observed. This is the first report on identification of IAA, GA_3 and Z in saprophytic actinomycetes and their potential role in plant–microbe interaction.

Keywords Auxin · Gibberellin · Zeatine · *Streptomyces* · *Actinoplanes* · *Micromonospora*

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Introduction

Simple organic molecules, such as phytohormones auxins and cytokinins play a crucial role in the elicitation of "totipotency" in several types of plant cells and in the regulation of plant physiological process. The balance between auxins and cytokinins levels controls cellular differentiation and organogenesis in tissue and organ culture, ranging from shoot proliferation to root formation as the auxin/cytokinins ratio increases (Costacurta and Vanderleyden 1995). Interestingly, these phytohormones have been found to be synthesized not only by plants but also by microorganisms, including fungi and bacteria (Glick 1995). Beneficial *Azospirillum*, *Klebsiella*, *Acetobacter, Pseudomonas* and *Xanthomonas* spp., symbiotic *Rhizobium a*nd *Bradyrhizobium* produce indole 3-acetic acid (IAA) in

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culture media and rhizopheric conditions. It is interesting to study IAA production by bacteria, not only because of the unidirectional physiological effect that this phytohormone has on plants, but also in view of its possible role in plant-bacteria interactions. Bacteria that inhabit the rhizosphere may influence plant growth by contributing to a host plant's endogenous pool of phytohormones. Production of the IAA is widespread among plant-associated bacteria (Patten and Glick 1996). However, the role of bacterial IAA in plant growth promotion remains undetermined (Patten and Glick 2002).

Under natural conditions, plants interact continuously with soil microorganisms. This interaction exists primarily at the root level and may be harmful, neutral or beneficial (Glick et al. 1999). Conceptually, Plant Growth-Promoting Rhizobacteria (PGPR) could modify plant growth and development either directly or indirectly, when the microorganism decreases, or prevent some effects of the phytopathogens through production of antibiotics or phytotoxins. On the other hand, bacteria either facilitate the incorporation of nutrients through nitrogen fixation or phosphoros solubilization, or directly provide the plant with different active compounds such as enzymes or phytohormones. In this regard, production and metabolism of phytohormones like auxins, cytokinins and gibberellins (Bottini et al. 2004; Cassán et al. 2001) are the most widely used mechanism by PGPR to promote plant growth. It is well known that a typical PGPR like *Azospirillum* spp. synthesizes auxins, especially IAA, and a variety of other auxins like indole-3-pyruvic acid or indole-3-butyric acid (Crozier et al. 1988; Costacurta et al. 1994; Martínez-Morales et al. 2003). These bacterial compounds contribute to the endogenous plant auxin "pool" in such a way that the effect of *Azospirillum* inoculation can be mimicked by exogenous application of the same compound (Glick et al. 1999).

Some plant hormones have been assumed to be part of the nodulation process in legumes ever since Thimann (1936) reported that pea nodules contain elevated levels of auxin. Hirsch and Fang (1994) reported that auxin and cytokinin frequently interact in antagonistic ways. The production of plant hormones by rhizobia is well documented, as far as *Frankia* is concerned, which produces both, auxin and cytokinin (Stevens and Berry 1988; Berry et al. 1989). Several reports have suggested that the balance of hormones, particularly that between auxin and cytokinin, is part of the nodulation stimulus, but a priori it is not obvious in which direction the balance is shifted (Hirsch et al. 1997). Recently, Giraud et al. (2007) established that the presence of Nod factors (LCOs) was not necessary in legumes inoculated with *Bradyrhizobium* sp. strain capable of using a purine (cytocine) to trigger nodule formation.

Tokala et al. (2002) reported that the soil bacterial genus *Streptomyces,* is a root-colonizing actinomycete, an antifungal biocontrol agent and a biodegradative enzymes, siderophores and phytohormones producer. Not only *Streptomyces*, but also other genera of saprophytic actinomycetes are known to be common rhizoplane and rhizosphere-colonizing bacteria (Frioni 2006; Solans and Vobis 2003), which have a high capacity to synthesize enzymes, antibiotics, phytohormones and antifungal metabolites (Goodfellow and Cross 1974; Takana and Omura 1990). Recent studies showed that saprophytic strains of actinomycetes belonging to *Streptomyces*, *Actinoplanes* and *Micromonospora* genera act as "helper" bacteria on actinorhizal and rhizobial N_2-fixing symbioses in culture tubes and pouches (Solans 2007; Solans et al. 2009), but the responsible metabolites are still unknown. The aim of the present study was to complete these results on *Ochetophila trinervis-Frankia* actinorhizal symbiosis in pots cultures, and to test the hypothesis that phytohormones produced by actinomycetes may interact in the microbe-plant interactions.

Materials and methods

Actinomycete strains

Saprophytic root-associated strains BCRU-MM40 (*Streptomyces* sp.), BCRU-ME3 (*Actinoplanes* sp.) and BCRU-MM18 (*Micromonospora* sp.) were originally isolated from the rhizosphere and rhizoplane of *O. trinervis* (Solans and Vobis 2003) (nucleotide sequense data reported are available in the GenBank databases under the following accesion numbers: FJ771041, FJ771040 and FJ771042). The symbiotic *Frankia* strain BCU110501, an isolate from *O. trinervis* nodules, which has been shown to be infective (Nod+) and effective (Fix+) in its proper host plant species (Chaia 1998), was used as inoculum as symbiotic N_2-fixing bacteria. All strains are stored at the culture collection of the BCRU Herbarium, Department of Botany, Centro Regional Universitario Bariloche, Universidad Nacional del Comahue, S. C. de Bariloche, Argentina. Website: http://www.sciweb.nybg.org/science2/IndexHerbarium.asp.

Bacterial growth

The saprophytic strains were grown in 250 ml Erlenmeyer containing liquid YpSs media (g l^{-1}): yeast extract (4.0); starch (15.0); K_2HPO_4 (1.0) and $MgSO_4 \times 7\ H_2O$ (0.5) according to Emerson (1958). Cultures were shaken in shaker at 120 rpm for 8 days at 22°C and harvested during the exponential growth period. Symbiotic strain was grown

in a shaker at 120 rpm, at 22°C for 4 weeks in 250 ml Erlenmeyer containing liquid BAP minimal media (ml l^{-1}): $MgSO_4 \times 7\ H_2O$ (2.5); $Ca_2Cl.2\ H_2O$ (1.0); micronutrients stock solution (1.0); NH_4Cl (5.0); Fe-EDTA (1.0); vitamins stock solution (1.0) and phosphate buffer solution (10.0) according to Murry et al. (1984) and modified by addition of 55 mM glucose.

Inoculation assay with *Ochetophila trinervis* plants

Germination of seeds and plant growth took place in a growth chamber with 16 h photoperiod (photosynthetic photon flux density = 318 µmol $m^{-2}s^{-1}$) at 26°C temperature and 35% relative humidity. Seedlings were grown in pots (75 ml) containing sand-vermiculite (1:1) as substrate and fertilized with Evans nutrient solution (Huss-Danell 1978). One plant per pot with 15 days old was inoculated with 1 ml of culture medium containing 5×10^6 cfu ml^{-1} of each saprophytic actinomycete, and 0.2 ml of the symbiotic strain *Frankia* (containing 9.4 ml of packet cells), either separately or in combination (Solans 2007). Thus, ten different treatments were applied: non-inoculated plants (C); single inoculation with *Frankia* (F), *Streptomyces* MM40 (S), *Actinoplanes* (A), *Micromonospora* (M); co-inoculation with *Frankia* and each saprophytic strain (FS, FA, FM); co-inoculation of *Streptomyces* and *Actinoplanes* (SA); and triple inoculation with *Frankia*, *Streptomyces* and *Actinoplanes* (FSA). Twelve weeks post-inoculation, plants were completely harvested and the following parameters were measured: (1) shoot and root length, (2) shoot and root dry weight, (3) number of nodules per plant, and (4) nodule dry weight.

Identification and quantification of indole 3-acetic acid (IAA), gibberellic acid (GA_3) and zeatine (Z)

Bacterial cultures in exponential growth phase and uncultured media were separated into several 20 ml fractions for IAA, GA_3 and Z evaluation. Fractions were centrifuged at 8,000 rpm for 20 min at 4°C, and supernatants were acidified at pH 2.5 with acetic acid solution (1% v/v). Individual samples were then added 100 ng of corresponding $^2H_5$-IAA, or $^2H_2$-GA_3 (OlChemIm, Czech Republic) deuterated internal standard and kept at 4°C for 2 h. No deuterated internal standard was used for Z. Each sample was partitioned four times with the same volume of acetic-acid-saturated ethyl acetate (1% v/v). After the last partition, acidic ethyl acetate was evaporated to dryness at 36° C. Dried samples were diluted in 100 µl acetic acid/acetonitrile/water (1:15:85) for IAA determination, and methanol/water (30:70) for GA_3 and Z determination. They were injected into a reverse phase C_{18} HPLC column (µBondapack, 300 × 3.9 mm, Waters Associates, Milford, MA) in a Konik 500 (Konic Instruments) system coupled to a UV–Vis Konik 3000 diode-array spectrometer. For each sample, elution was performed at 1 ml min^{-1} flow rate, and fractions eluting at the retention time corresponding to each pure standard were collected. Z was identified and quantified by HPLC–UV at 254 nm (Tien et al. 1979). IAA, ABA, and GA_3 were identified and quantified by gas chromatography–mass spectrometry with selective ion monitoring (GC–MS-SIM). UV-absorbing fractions at 254 and 220 nm were grouped for IAA, and GA_3 determination, respectively, then methylated with ethereal diazomethane and silylated with 1:1 pyridine/BSTFA [bis (trimethylsilyl) trifluoroacetamide] plus 1% trimethylchlorosilane (Fluka Chemika, Switzerland) to obtain methyl-trimethylsilyl derivatives of IAA and GA_3. Aliquots of each sample were injected directly into a DB1-15 N (15 m × 0.25 mm, 0.25 µM methyl silicone) capillary column (J&W Scientific) fitted in a Hewlett-Packard 5890 Series II GC with a capillary direct interface to a 5970B Mass Selective Detector. The GC temperature program was 60 to 195°C at 20°C min^{-1}, then 4°C min^{-1} to 260°C. Carrier gas (He) flow rate was 1 ml min^{-1}, interface temperature was 280°C, and data acquisition was controlled by an HP 300 Series computer. The amount of free IAA was calculated by comparison of peak areas for the parent ion (m/z) 194 and (m/z) 189 and subtracting the value obtained in the non-inoculated media. In the same way, amount of free GA_3 was calculated by comparison of peak areas for parent ion (m/z) 506 and (m/z) 504 (Cassán et al. 2009).

Morpho-anatomical studies of *O. trinervis* root hairs

Seedlings were grown in the same conditions described above. To evaluate the microbial and phytohormonal effect of inoculation, plants were separately inoculated with 1 ml of actinomycetes (mycelium + supernatant), and 0.2 ml of the *Frankia* culture containing 9.4 ml of *Frankia* packet cells was used, in accordance to Solans (2007). Twelve weeks post-inoculation, the whole root system was carefully cleared of adhering substrate by rinsing with water for microscopical observations. For these studies, the optic microscope Olympus BX50 with Nomarski DIC equipment was used. The observations were made in water, in glycerine or in lactophenol cotton blue according to Amman (Gams et al. 1980). The morphological and anatomical studies were registered by microphotographies and qualitative observations.

Statistical analysis

Treatments were run with six replicates. Values represent mean ± standart error (SE). Data were analyzed by

one-way variance analysis (ANOVA), followed by Tukey's test *post hoc* analysis at $P < 0.05$. Analysis was performed using STATISTICA V6 software.

Results and discussion

Effects of actinomycetes on *O. trinervis* growth and nodulation

Effects of rhizoactinomycetes and *Frankia* on growth and nodulation of the actinorhizal plant growing in pots, are shown in Table 1. In general, the single inoculations with saprophytic strains *Streptomyces* (S), *Actinoplanes* (A) and *Micromonospora* (M) showed a similar growth rate to control plants (C). Double inoculation with *Streptomyces* and *Actinoplanes* (SA) presented a slightly higher growth rate than control plants (Table 1). However, the final growth values were not significantly different in these treatments with the exception of the plants inoculated with S, which presented higher root length than control plants (C) ($P < 0.05$). In this assay in pots, the plants were fertilized with Evans solution with 0.07 mM N and 0.7 mM N as NO_3NH_4, and the saprophytic strains in single inoculations without *Frankia* never showed a promoting effect on *O. trinervis* growth.

On the other hand, the plants inoculated with the fixing-N symbiotic strain *Frankia* (F), in single or double inoculations, presented a higher growth rate compared with control plants (C). Triple-inoculated plants with *Frankia, Streptomyces* and *Actinoplanes* (FSA) showed a significant increase in shoot and root dry weight, and in symbiosis-related parameters, as nodule dry weight ($P < 0.05$), compared with single inoculated plants with *Frankia* (F) ($P < 0.05$). Also, the co- inoculation with *Frankia* and *Micromonospora* (FM) presented a positive effect on root biomass ($P < 0.05$) (Table 1).

Another important effect on *O. trinervis-Frankia* symbiosis shown in previous works, was the stimulation of nodulation when the plants were coinoculated with the supernatant of saprophytic strains together with *Frankia* (Solans 2007), suggesting a helper effect on symbiosis by growth factors present in the supernatant produced by rhizoactinomycetes (Solans 2008). These promoting effects on the actinorhizal *O. trinervis-Frankia* symbiosis, suggest that nodulation and consequently growth rates may involve growth regulators present in the culture medium and maybe a synergic effect of these two strains together, acting in some way in the nodulation process.

Recent studies on nodulation kinetics analysis in *Frankia–O. trinervis* symbiosis revealed different factors involved in the nodulation process suggesting the existence of more than one signal of bacterial origin involved in the process (Gabbarini and Wall 2008).

Phytohormones production in liquid culture media

Identification and quantification of phytohormones by actinomycetes strains indicated that they produce indole 3-acetic acid (IAA), giberellic acid (GA_3) and zeatine (Z) in liquid culture media. The latter, identified as trans-zeatine-ribose, was synthesized in higher level (μg ml^{-1}) than AIA and GA_3 (ng ml^{-1}) for all strains. The phytohormone production is summarized in Table 2. IAA production was higher (eight times higher) for *Micromonospora* sp., with 9.03 ng ml^{-1} than for *Actinoplanes* sp. (0.27 ng ml^{-1}),

Table 1 Effects of actinomycetes on growth and nodulation of the actinorhizal plant *Ochetophila trinervis* cultived in pots, 12 weeks post-inoculation

Treatments	Shoot length (cm)	Shoot dry weight (mg)	Root length (cm)	Root dry weight (mg)	Number of nodules (p.plant^{-1})	Nodule dry weight (mg)
F	6.65 (1.0)	44.04 (10.7)	19.65 (4.1)	74.26 (12.5)	5.5 (0.7)	3.6 (0.8)
FS	6.78 (2.4)	53.07 (19.8)	17.86 (2.6)	84.07 (21.6)	6.4 (2.9)	3.47 (0.7)
FA	7.38 (1.6)	50.94 (11.6)	15.95 (1.2)	84.0 (15.4)	3.0 (0.7)	2.5 (0.5)
FM	8.30 (0.7)	58.32 (5.3)	22.66 (7.4)	121.2 (8.7)*	4.6 (0.9)	3.97 (0.4)
FSA	12.25 (2.1)	90.52 (7.7)*	19.35 (3.4)	120.4 (8.7)*	8.16 (1.5)	6.64 (0.5)*
S	2.55 (0.1)	8.14 (0.1)	25.7 (3.0)**	42.96 (2.7)	0.0 (0.0)	0.0 (0.0)
A	2.25 (0.2)	6.60 (0.5)	18.9 (2.1)	40.72 (4.9)	0.0 (0.0)	0.0 (0.0)
M	2.55 (0.2)	8.12 (0.7)	20.6 (1.6)	38.24 (3.7)	0.0 (0.0)	0.0 (0.0)
SA	2.65 (0.3)	9.59 (1.1)	20.78 (2.7)	50.68 (2.4)	0.0 (0.0)	0.0 (0.0)
C	2.00 (0.2)	6.98 (0.9)	17.53 (1.5)	38.84 (5.8)	0.0 (0.0)	0.0 (0.0)

Values are the means ± error standard, n = 6

F, *Frankia*; FS, *Frankia* + *Streptomyces*; FA, *Frankia* + *Actinoplanes;* FM, *Frankia* + *Micromonospora*; FSA, *Frankia* + *Streptomyces* + *Actinoplanes*; S, *Streptomyces*; A, *Actinoplanes*; M, *Micromonospora;* SA, *Streptomyces* + *Actinoplanes*; C, control

* Denote significant differences respect to *Frankia*; ** Denote significant differences respect to control ($P < 0.05$)

Table 2 IAA, GA_3 and Z production by saprophytic actinomycetes strains and *Frankia* in exponential growth phase of liquid culture medium

Strains	IAA (ng ml^{-1})	GA_3 (ng ml^{-1})	Z (μg ml^{-1})
F	0.92	1.76	15
S	0.75	0.96	240
A	0.27	1.53	310
M	9.03	3.73	270

F, *Frankia*; S, *Streptomyces*; A, *Actinoplanes*; M, *Micromonospora*

Streptomyces sp. (0.75 ng ml^{-1}) and *Frankia* sp. (0.92 ng ml^{-1}). GA_3 production was also higher (almost three times higher) for *Micromonospora* sp. (3.73 ng ml^{-1}) compared with *Streptomyces* sp., which produced 0.96 ng ml^{-1}; *Actinoplanes* sp. 1.53 ng ml^{-1} and *Frankia* sp., 1.76 ng ml^{-1}. Z production was higher for *Actinoplanes* sp. (310 μg ml^{-1}), *Micromonospora* sp. (270 μg ml^{-1}) and *Streptomyces* sp. (240 μg ml^{-1}) than *Frankia* sp. (15 μg ml^{-1}) (Table 2).

Differential capacity of bacterial strains to produce and release plant-growth-regulating compounds in culture media could lead to diverse plant growth response. Biological activity of phytohormones may greatly influence processes such as early germination, early seedling growth, plant colonization and bacterial establishment (Cassán et al. 2009). Results of the present study show that diverse genera of actinomycetes *Streptomyces* sp. MM40, *Actinoplanes* sp. ME3, *Micromonospora* sp. MM18 and *Frankia* sp. 110501 possess the capacity to produce and release plant-growth-promoting substances such as IAA, GA_3 and Z in chemically defined media without precursors. In our study, IAA and GA_3 production for actinomycetes strains were significantly inferior, comparable to Z production for these strains or to that reported by other authors for *Azospirillum lipoferum*, *A. brasilense* and *A. brasilense* Az39 in chemically defined media (Crozier et al. 1988; Perrig et al. 2007; Cassán et al. 2009).

Dobbelaere et al. (1999) determined that inoculation of wheat with 10^8 cfu ml^{-1} of *A. brasilense* sp. 245 was comparable to exogenous application of IAA, which improves root hair density. A similar effect on promotion of root hair development and density was reported in tomato plants inoculated with *A. brasilense*, but there is evidence that in this case ethylene is an intermediate in a signaling pathway, and ethylene positively regulates root hair development in response to PGPR inoculation (Ribaudo et al. 2006). Regarding ethylene, this hormone was not determined and identified in this study. However, its production by these actinomycetes strains can not be ruled out. A recent study on ethylene and the actinorhizal plant *O. trinervis* showed that this hormone is involved in modulating the susceptibility of the seedling roots for nodulation by *Frankia* (Valverde and Wall 2005). In this case, *O. trinervis* roots were able to respond to higher ethylene concentrations, reducing elongation rate, increasing root hair development and root diameter and increasing lateral root formation (Valverde and Wall 2005). These results are similar to those of the present study, where we could observe an increase in root hair density, and high root dry weight in plants inoculated with saprophytic actinomycetes, compared with control plants (Solans 2007).

It is well known that IAA can trigger, *per se*, biochemical signals leading to plant growth improvement and, on the other hand, it can stimulate ethylene production through the activation of the 1-aminocyclopropane 1-carboxylate synthase (ACS) (Ribaudo et al. 2006). The involvement of auxins and ethylene as important plant growth and development regulators has been widely studied (Glick et al. 1999). Also, ethylene has shown that it mediates plant autoregulation of nodulation in both, in actinorhizal plants such as *O. trinervis* (Valverde and Wall 2005) and in legumes such as Alfalfa (Peters and Crist-Estes 1989).

Some reports have been published on cytokinins production by microorganisms. Barea et al. (1976) reported in vivo, that about 90% of rhizobacteria could be considered as potential cytokinin producers such as, *Bacillus* sp., *Erwinia amylovora*, and *Pseudomona putida*. Interestingly, in our study, the actinomycetyes strains presented the highest level of Z production (270–310 μg ml^{-1}). Tien et al. (1979) showed that inoculated pearl millet (*Penisetum americanum* L.) had an increased number of lateral hair roots compared with noninoculated plants, and attributed this response in part to *Azospirillum* cytokine production and release. In recent studies on coinoculated *O. trinervis* and Alfalfa plants with these rhizoactinomycetes strains, it was possible to observe an increase in shoot weight and dry biomass, root dry weight and an increase in nodule number and dry biomass (Solans 2007, 2008; Solans et al. 2009).

It has been shown that in the interaction between plant and free-living plant growth promoting rhizobacteria, bacterial phytohormones are involved (Liste 1993; Höflich et al. 1994; Glick 1995), IAA and cytokinins being the most important ones. Cytokinins and auxins may act synergistically to iniciate cell division and nodule primordial formation, mediating plant responses to rhizobia, integrating the pathway signaling mediated by nod factors (Mudler et al. 2005). Moreover, a pathway for nodulation that is independent of nod factors has recently been described and the signals involved seem to be phytohormone related (Giraud et al. 2007). In previous works, the nodulation kinetics of plants co-inoculated with actinomycetes obtained in pouches culture system, either *O. trinervis*-*Frankia* (Solans 2008) or *Medicago sativa*-*Sinorhizobium*

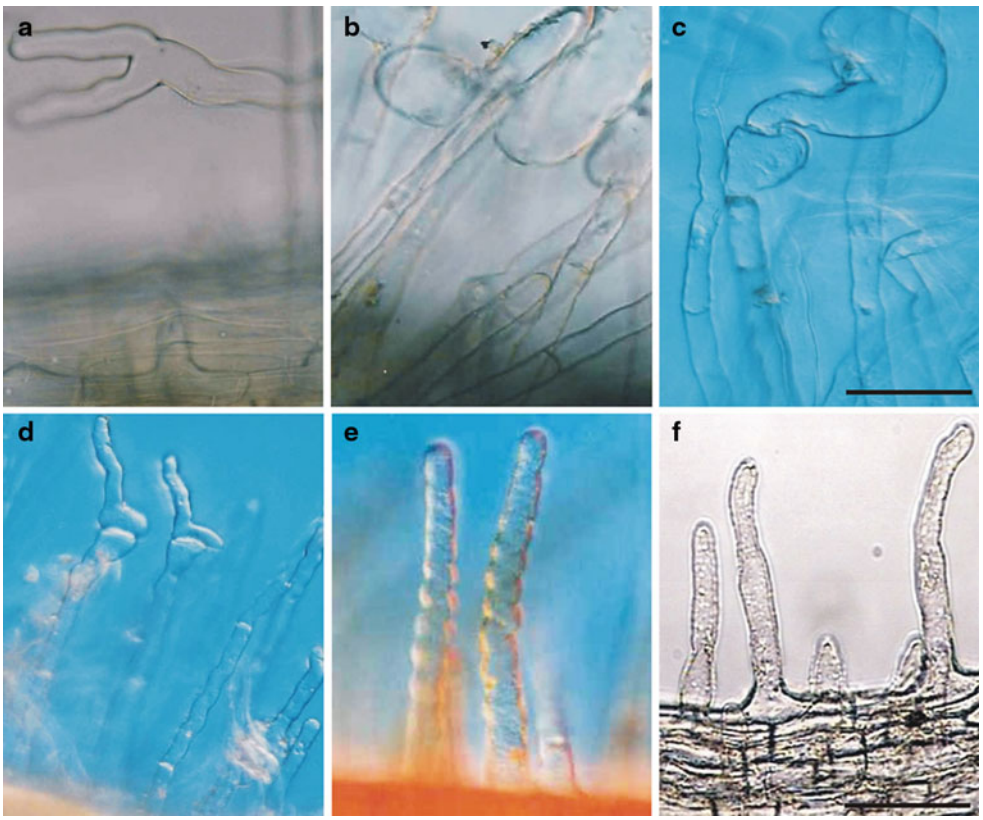

Fig. 1 Development of "deformed" hairs in *O. trinervis* co-inoculated with *Frankia* and actinomycetes (mycelia + supernatant) strains. **a** Bifurcated hair observed in co-inoculated plants with *Micromonospora* (FM). **b** Sub-globose hairs with *Streptomyces* strain (S). **c–d** Irregular claviforme hairs with septa in co-inoculated plants with *Actinoplanes* (FA). **e** Ondulate hairs in plants inoculated with *Actinoplanes* strain (A). **f** Straight hairs in control plants (C). *Scale*: **a–c** 50 μm. **d–f** 70 μm

meliloti symbiosis (Solans et al. 2009), suggested that the effect of rhizoactinomycetes would operate at the beginning of the infection and nodule development, sustaining the nodule initiation and development for longer time periods.

Morpho-anatomical observations of root hairs

These studies were made through qualitative observations and microphotografies. As regards the development of root hairs in *O. trinervis* plants, it was possible to observe the presence of slightly deformed root hairs when plants were inoculated with a culture suspension of saprophytic actinomycetes strains. The root hairs presented different shapes, such as ondulate, vesiculose, subglobose, claviforme and forked, in comparison with the normal (straight) hairs in control plants (Fig. 1). These shapes were observed in plants coinoculated with *Frankia* and saprophytic strains and in plants with saprophytic strains alone (Fig. 1). The microphotografies presented in Fig. 1 showed the different shapes of root hairs in *O. trinervis* plants, such as bifurcated hair in coinoculated plants with *Frankia* + *Micromonospora* (Fig. 1a); subglobose hairs in plants with *Streptomyces* (Fig. 1b); irregular claviforme hairs with septa in coinoculated plants with *Frankia* + *Actinoplanes* (Fig. 1c,d); ondulate hairs in plants inoculated with *Actinoplanes* alone (Fig. 1e); and straight hairs in control plants (Fig. 1f).

Actinomycetes-inoculated plants presented higher development of dense zones of root hairs than control plants without inoculation or with *Frankia* alone (data not shown). The deformed hair phenomenon observed in plants inoculated with rhizoactinomycetes strains could be related to the presence of mycelium in direct contact with roots and/or the presence of metabolites produced by these strains.

It is worth noting that infection towards nodulation of *O. trinervis* by *Frankia* occurs via intercellular invasion without any root hair deformation (Valverde and Wall 1999). However, the root hair deformed by co-inoculation with actinomycetes, or by the addition of actinomycete culture supernatant, could be the expression of a change in the primary plant cell wall structure induced by these substances, which probably could be related to the enhanced infection and nodulation by *Frankia* induced by helper rhizoactinomycetes. A similar phenomenon was

observed by Berry and Torrey (1983) in *Alnus* sp., with intracellularly infection pathway, when inoculated with "helper" *Pseudomonas*. It is important to point out that plants co-inoculated with *Frankia* and either actinomycete's culture suspension or only the addition of culture supernatant without bacteria showed a promotion of nodulation and plant growth, and also presented high root hair density and deformed hair, compared with control plants or plants inoculated with *Frankia* alone (Solans 2007, 2008).

The results presented here support the idea that phythormones or related substances of bacterial origin may take part in the infection and nodulation process, especially in nature where the interaction between *Frankia* and the host roots takes place in the presence of a bacterial diversity in the rhizosphere.

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