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Pseudomonads Isolated from within Fruit Bodies of *Tuber borchii* are Capable of Producing Biological Control or Phytostimulatory Compounds in Pure Culture

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Abstract

From the core of surface-sterilized sporocarps of *Tuber borchii* collected in Spring 1997, 600 bacterial strains were isolated, among which were about 300 pseudomonads. Counts revealed a consistent presence of *Pseudomonas* spp., at 10^5–10^8 cfu per gram of dry weight. Identification showed the isolates mainly representing species of *Pseudomonas fluorescens*, *P. corrugata* and *P. tolaasii*. Using a monoclonal antibody against indole-3-acetic acid (IAA) all isolates studied were found to be phytohormone-producing *ex-planta*. Some isolates were examined and found to be producers of biocontrol substances (2,4 diacetylphloroglucinol, HCN, proteases and fluorescent siderophores) and active in carboxy-methyl cellulase and

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collagenase activities. Biological assays revealed weak biocontrol activity *ex-planta* against *Botrytis cinerea*, *Cryphonectria parasitica*, *Diplodia mutila*, *Phytophthora cactorum* and *Phytophthora cinnamomi*, whereas *Fusarium oxysporum* f.sp. *dianthii* was unaffected by the strains tested.

Keywords: *Tuber borchii*, pseudomonads, fruit bodies, biocontrol, indole-3-acetic acid

1. Introduction

Bacterial cells have frequently been found associated with mycorrhizal roots, and their presence has been shown to affect both plant and fungal behaviour by different means. Some bacterial species, i.e. the mycorrhizal helper bacteria (MHB), are able to sustain mycorrhizal infection establishment or functioning (Garbaye and Bowen, 1989; Garbaye et al., 1990; Garbaye, 1994), while other species are involved in the supply of essential nutrients or behave as antagonists of plant pathogens (Défago et al., 1990; Jayaswal et al., 1990; Li et al., 1992; Barea, 1997).

The isolation of bacterial strains from within fruiting bodies of different ectomycorrhizal fungi, such as *Cantharellus cibarius*, *Laccaria laccata*, *Hebeloma crustuliniforme* and *Rhizopogon vinicolor* has been reported (Swartz, 1929; Li and Castellano, 1987; Garbaye et al., 1990; Danell et al., 1993; Varese et al., 1996). Strains of *Pseudomonas*, *Bacillus* and *Corynebacterium*, isolated from sporocarps, have been shown to affect, either positively or negatively, propagule germination or mycelial growth of ectomycorrhizal fungi (Garbaye et al., 1990; Danell et al., 1993; Varese et al., 1996). Nitrogen-fixing bacteria and/or nitrogenase activity have been detected in the fruit bodies of some ectomycorrhizal fungi (Larsen et al., 1978; Spano et al., 1982; Li and Castellano, 1987), suggesting that associated microorganisms may play a role in fungal growth and nutrition during sporocarp development. Mycelial growth and fungal morphogenesis may be also affected by bacterial metabolites, as it has been reported that unidentified metabolites from *Pseudomonas* sp. were possibly involved in basidiome initiation by *Agaricus bisporus* mycelium in axenic culture (Hayes et al., 1969; Wood, 1976; Rainey et al., 1990).

The presence of associated bacteria inside the fruit body of truffles (*Tuber* spp.) has been described in previous studies (Citterio et al., 1995); however, the functional activity of these bacteria remains unclear. In this study we report the results on the characterization of bacteria isolated from within *Tuber borchii* sporocarps.

2. Materials and Methods

Culturable bacteria isolation and counts

Thirty sporocarps of *Tuber borchii,* freshly collected in different areas of Marche (central Italy) and stored at –82°C, were washed and gently brushed in running tap water, then washed three times in a solution of Tween 20 (100 μl/l) in NaCl 0.85% under continuous shaking (150 rpm) for 20 min. The surface of sporocarps was briefly flamed, before collecting 1-gram sample fragments from the inner tissue. Fresh weights of samples were recorded, and the remaining sporocarp tissue was used for dry weight determination. The inner tissue samples were mortar-blended in sterile physiological solution (NaCl 0.85%) and serial dilutions (10^{-1} to 10^{-8}) were plated onto different culture media. Pseudomonads were enumerated after plating samples on ACC Medium according to Simon and Ridge (1974); strains were tentatively referred to genus *Pseudomonas* after Gram stain and performing oxydase test (DrySlide, Difco) and to the fluorescent group after observation under Wood light. Spore-forming bacillaceae were counted by plating on Tryptic Soy Agar after treating samples at 80°C for 10 min. Actinobacteria were assessed by plating samples on starch-casein agar (Kuster and Williams, 1964). The number of colonies formed by chitinolytic bacteria was evaluated through the clearing of halo after plating samples on agar supplemented with colloidal chitin 0.4% (Lingappa and Lockwood, 1962).

Taxonomic identification

GN Biolog microplates (Biolog Inc., Hayward, CA, USA) were inoculated with 24 h culture suspensions of the pseudomonads isolated from inside *T. borchii.* Incubation was at 27°C for 24 h, according to the manufacturer's protocol. Metabolic patterns were entered in the Biolog software and compared with the Biolog Database release 3.70. An identification was acknowledged when the Biolog similarity index was 0.5 or more. As control strains *Pseudomonas tolaasii* ATTC 33618 and *Pseudomonas fluorescens* F113 (Fenton et al., 1992; Fedi et al., 1997) were used.

Scanning electron microscopy (SEM)

Sporocarps of *T. borchii* were sectioned into 5 mm wide blocks, briefly rinsed in 0.05 M phosphate buffer, pH 6.8, and fixed in glutaraldehyde 3% in phosphate buffer for 5 h at room temperature. After four washes, samples were treated with 1% OsO_4 in the same buffer overnight at 4°C, repeatedly washed and dehydrated before treating with routine "critical point" method. Samples

were then radial-fractured, metal-coated and observed under scanning electron microscope (Philips 505 at 9.9 kV) and photographed using Polaroid films.

Production of indole-3-acetic acid

Filtrate (2 ml) of 72 h culture of 90 randomly chosen strains of *Pseudomonas* sp. isolated from inside *T. borchii* were acidified to pH 2.5 with 1 N HCl, extracted three times with diethyl ether and dried under a stream of nitrogen. Then the samples were methylated with diazomethane and finally solubilized in Tris buffer saline 25 mM pH 7.5 added with 10% methanol. IAA assay was performed by using the enzyme immunoassay test "Phytodetek®-IAA" (IDEXX Laboratories Inc., ME, USA) according to the manufacturer's instructions. The monoclonal antibody contained in this kit (IAA-17-II-A) shows, as reported in the accompanying documentation, a high specificity for IAA in the methylated form.

Plate assays for lytic activities

Chitinolytic activity was assessed using two different solid media containing (a) K_2HPO_4 (0.8 g/l), KH_2PO_4 (0.2 g/l), $(NH_4)_2SO_4$ (0.5 g/l), $MgSO_4 \bullet 7H_2O$ (0.2 g/l), $CaCl_2 \bullet 2H_2O$ (10 mg/l), $FeCl_3 \bullet 6H_2O$ (10 mg/l), $ZnSO_4 \bullet 7H_2O$ (1 mg/l), casamino acids (2 g/l), purified agar (15 g/l) and an equal volume of a 1% (w/v) colloidal chitin suspension (Sigma, St. Louis, USA); (b) K_2HPO_4 (0.7g/l), KH_2PO_4 (0.7g/l), $MgSO_4 \bullet 5H_2O$ (0.5g/l), $FeSO_4 \bullet 7H_2O$ (0.01 g/l), $ZnSO_4$ (0.001 g/l), $MnCl_2$ (0.001 g/l), Agar (20 g/l), colloidal chitin (4 g/l), pH 8 (Hsu and Lockwood, 1975). Plates were incubated at 28°C and observed after 7 and 14 days.

Proteolytic activity was studied on plates containing skim milk (100 g/l), yeast extract (1.5 g/l), and technical agar (15 g/l).

Bacteria were streaked directly onto the plates or, alternatively, 100 µl of filter-sterilized cell-free supernatant of bacterial cultures (in SA or LB), obtained after concentration (×500) using an Amicon ultrafiltration unit (Amicon Corp., USA), were spotted onto the centre of the plates. The plates were incubated at 28°C and observed after 7 and 14 days. Both chitin- and skim milk-containing plates are opaque and enzymatic activity was identified by the development of a zone of clearing (halo) around the colonies and the cell-free supernatant spots. As a reference strain *Stenotrophomonas maltophilia* strain W81, producing both proteolytic and chitinolytic enzymes (Dunne et al., 1996, 1997, 1998) was used.

In addition, proteases were identified as follows using the fluorimetric assay described by Twinning (1984): 80 µl of FPLC-derived samples were incubated in

the presence of 80 μl of FITC (fluorescein isothiocyanate)-labelled casein at 28°C for 2 h. Insoluble material was then removed by centrifugation (3000 g). An 80 μl aliquot of each supernatant were treated with 300 μl of 5% TCA and protein allowed to precipitate at room temperature for a minimum of 1 h and removed by centrifugation. Sixty μl of supernatant were neutralized by addition of 3 ml of 0.5 M Tris, pH 8.5. Fluorescence was measured at 490 nm using a Beckman DU640 spectrophotometer and cuvettes of 1 cm pathlength. One thousand units of enzyme activity were arbitrarily equated to 1 unit of fluorescence. The W81-produced protease was identified through the single addition of 10 individual protease inhibitors (Cat. No. 1206 893; Boehringer Mannheim) each capable of inhibiting the activity of a specific proteolytic enzyme and assessment of the remaining enzymatic activity.

Cellulase activity assay was carried out using a modified method from that of Teather and Wood (1982). The medium contained yeast extract (Oxoid L21) 1 g l^{-1}, carboxymethyl cellulose (Sigma C-4888) 5 g l^{-1}, ammonium sulphate 1 g l^{-1}, P.V. salts ($MgSO_4 \cdot 7H_2O$ 3.0 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 0.7 g l^{-1}, $ZnSO_4 \cdot 7H_2O$ 1.7 g l^{-1}, $MnSO_4 \cdot H_2O$ 1.6 g l^{-1}, deionised water 100 ml) 1 ml l^{-1}, purified agar 15g l^{-1}, pH 7.0. Plates were inoculated either as streaks or cell suspensions, incubated for 72 hours at 29°C, and then treated with Congo red (1 mg ml^{-1}) for 30 min, NaCl 1 M for 15 min (yellow halo around positive colonies) and HCl 1 N (to detect slight degradation activity).

Collagenase activity was assessed on a medium modified from Dhigra and Sinclair (1986), containing the same compounds as above but collagen (Sigma) 5 g/l instead of carboxymethyl cellulose.

Production of PHL, HCN and other metabolites

Assay for the production of 2,4-diacetylphloroglucinol (PHL) was performed according to Fenton et al. (1992) and Dunne et al. (1998); HCN production was determined as described by Castric and Castric (1983); fluorescent siderophores release was tested by fluorescence of culture medium after growing strains on Sucrose asparagine agar (SA: sucrose 20 g l^{-1}, asparagine 2 g/l, K_2HPO_4 1 g l^{-1}, $MgSO_2 \cdot 7H_2O$ 1 M 2 ml l^{-1}, $FeCl_3$ 0.1 M in HCl 0.1 N 1 ml l^{-1}, purified agar 15 g l^{-1}). As a reference strain *Pseudomonas fluorescens* strain F113, producing a fluorescent siderophore, HCN and PHL (Fenton et al., 1992) was used.

Biocontrol assays

16 h cultures of *Pseudomonas* sp. grown in Nutrient Broth (Difco) were used to obtain four perpendicular streakes on Sucrose asparagine plates (SA: sucrose 20 g/l, asparagine 2 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 1 M 2 ml/l, $FeCl_3$ 0.1 M in

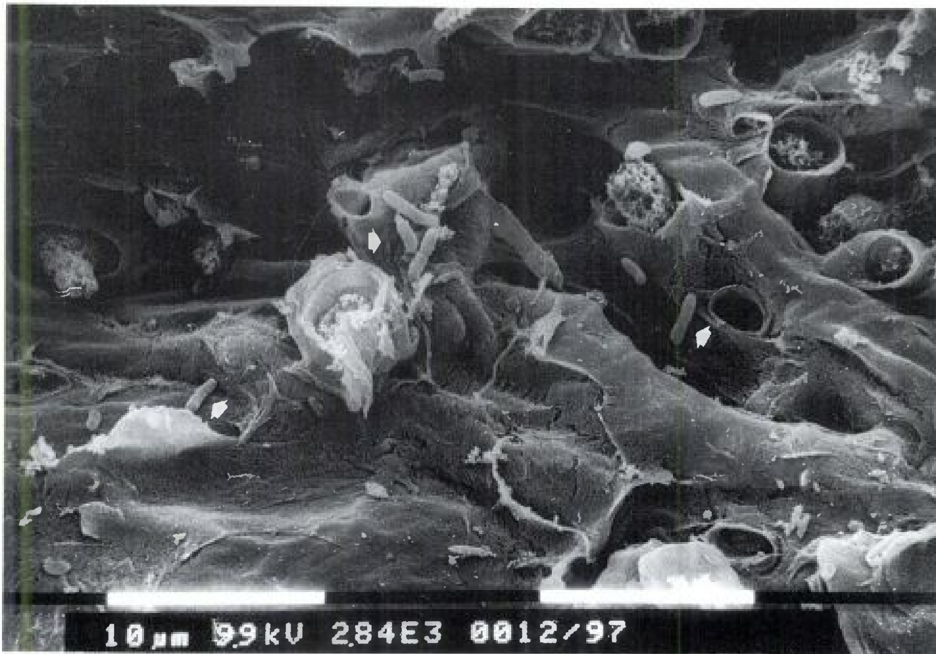

Figure 1. Scanning electron micrograph of a sporocarp core of *Tuber magnatum*. The arrows indicate bacterial cells, mostly (dividing) rods, adhering to the hyphal matrix. Bar = 10 µm.

HCl 0.1 N 1 ml/l, purified agar 15 g/l). Reference biocontrol strain was *Pseudomonas fluorescens* F113 (Fenton et al., 1992). After 24 h of bacterial growth, *Botrytis cinerea*, *Cryphonectria parasitica*, *Diplodia mutila*, *Phytophthora cactorum*, *Phytophthora cinnamomi* and *Fusarium oxysporum* f.sp. *dianthii* were inoculated as mycelial squares 4×4 mm wide. Biocontrol activity of each strain was recorded after 10 days incubation at 26°C in the dark.

3. Results

SEM observations carried out on *Tuber borchii* sporocarp inner tissues showed the presence of single or clustered bacterial cells widespread in the whole sporocarp and densely populating hyphal and spore surfaces in the fertile area (Fig. 1). The cells were apparently dividing and consisted mainly of short rods.

Counts of microbial groups tested have shown that the fruit bodies analysed contained *Pseudomonas* spp. and spore-forming Gram-positive bacillaceae,

whereas actinobacteria and chitinolytic bacteria were seldom isolated. Microbial presence, expressed as cfu per gram of dry matter, was 10^5 to 10^8 for *Pseudomonas* spp. and 10^2 to 10^4 for spore-forming bacillaceae.

From sporocarps of *T. borchii* 600 bacterial strains were randomly isolated on the basis of morphological characteristics of colonies grown on the different media used. The pseudomonads (300 isolates) were selected for further analysis. Tests performed for the preliminary identification suggested that about 90% pseudomonads could be referred to *Pseudomonas* sp. and that 38% of these isolates could be assigned to the fluorescent group.

Two hundred sixtysix pseudomonads isolates were further processed by the Biolog system to ascertain their taxonomic identification, and 68% were identified at species level, 24% at genus level and 8% were not identified. Two hundred sixty (98%) strains were assigned to the genus *Pseudomonas*, and among these *P. fluorescens*, *P. tolaasii* and *P. corrugata* were the most frequent, with 91, 42 and 33 isolates respectively (Table 1).

Preliminary assays of IAA in *Tuber* spp. fruit bodies had shown a content of 232.9±11.8 μg g^{-1} fresh weight in *T. borchii*. To test whether IAA presence could be due also to bacterial production 90 isolates of *Pseudomonas* spp. were analysed for IAA production *ex-planta* and results showed quantitatively variable IAA production, ranging between 7.8±0.7 and 725.1±87.5 μg l^{-1} of culture medium.

Table 1. Taxonomical identification carried out using the Biolog system of 266 pseudomonads isolated from within *Tuber borchii* sporocarps

Species and biovar	Number of isolates (%)	
Ewingella americana	2	(0.7)
Pseudomonas spp.	65	(24.4)
P. fluorescens biotype G*	40	(15.0)
P. fluorescens biotype A*	24	(9.0)
P. fluorescens biotype B*	11	(4.1)
P. fluorescens biotype F*	2	(0.7)
P. fluorescens biotype C*	15	(5.6)
P. corrugata	33	(12.4)
P. tolaasii	42	(15.8)
P. putida	1	(0.3)
P. synxantha	8	(3.0)
Xanthomonas maltophilia	4	(1.5)
Not identified	20	(7.5)

*Biotype classification proposed by Stainer et al., 1966.

Table 2. Biochemical characterization of thirthy *Pseudomonas* strains isolated from within *Tuber borchii* sporocarps

Strain number	Species	IAA production (μg/l culture medium±S.E.)	PHL production	HCN production	Protease activity	Collagen. activity	Carboxy methyl cellulase activity
19/6	*P. corrugata*	8.82±1.0	±	–	+		
6/3	*P. corrugata*	13.92±0.4	–	–	+	+	+
15/3	*P. corrugata*	18.51±3.0	–	+	+	+	–
14/2	*P. corrugata*	19.33±4.8	–	–	±	±	+
11/4	*P. corrugata*	33.44±6.9	–	–	–	–	–
4/3	*P. corrugata*	41.42±14.4	–	–	–	–	–
20/13	*P. corrugata*	307.12±67.5	–	±	+		
9/4	*P. fluorescens* A	15.60±2.9	–	–	+	+	+
6/1	*P. fluorescens* A	19.42±9.1	–	–	+	+	–
9/3	*P. fluorescens* A	76.03±3.1	–	–	–	–	±
17/2	*P. fluorescens* B	71.67±30.2	–	–	+	+	+
15/11	*P. fluorescens* C	15.12±3.6	–	–	+		
4/1	*P. fluorescens* G	50.74±17.9	–	–	–	–	+
18/2	*P. fluorescens* G	52.30±15.5	–	+	+		
3/7	*P. fluorescens* G	67.48±14.2	–	–	–	–	–
3/3	*P. fluorescens* G	83.83±43.9	–	–	–	–	–
14/1	*P. tolaasii*	10.68±3.4	–	–	+	+	–
14/9	*P. tolaasii*	11.23±2.33	–	–	+		
14/4	*P. tolaasii*	15.72±1.5	–	–	+	+	–
1/13	*Pseudomonas* sp.	7.86±0.7	–	–	+		
20/16	*Pseudomonas* sp.	9.35±1.6	–	–	+		
18/1	*Pseudomonas* sp.	10.90±1.7	±	–	+		
7/1	*Pseudomonas* sp.	11.86±3.2	–	–	+	+	–
7/11	*Pseudomonas* sp.	13.54±3.8	–	–	+	+	+
7/6	*Pseudomonas* sp.	16.68±5.1	–	+	+		
16/4	*Pseudomonas* sp.	17.29±9.2	–	–	–	–	+
7/2	*Pseudomonas* sp.	21.10±6.1	–	–	+	+	+
11/2	*Pseudomonas* sp.	97.06±4.7	–	+	–	–	+
3/5	*Pseudomonas* sp.	131.71±35.0	–	–	–	–	–
20/15	unidentified	113.57±4.9	–	±	+		

Blank cells indicate unassayed strains.

Biological characterization of 30 *Pseudomonas* spp. isolates was carried out through their biocontrol traits. All isolates tested released fluorescent siderophores in SA medium, whereas chitinolytic activity remained below detection level. Other traits were differentially expressed: PHL and HCN

Table 3. Bacterial strains isolated from within *Tuber borchii* sporocarps showing antagonistic activity against some phytopathogenic fungi. Antagonistic test carried out on 30 *Pseudomonas* using *in vitro* dual cultures.

Fungal species	Number of isolates with antagonistic activity
Cryphonectria parasitica	28
Diplodia mutila	15
Phytophthora cinnamomi	20
Phytophthora cactorum	18
Botrytis cinerea	21
Fusarium oxysporum f.sp. *dianthii*	0

production was limited to a few strains, while proteases, belonging either to the serine- or in particular to the chymotrypsin-like group, were commonly released. Some isolates were also found to be positive to tests for collagenase and carboxy-methyl-cellulase activity in culture (Table 2).

The same strains were tested for biological control of some plant root phytopathogenic fungi in dual cultures, and variable antagonistic activity of strains challenging *Botrytis cinerea*, *Cryphonectria parasitica*, *Diplodia mutila*, *Phytophthora cactorum* or *Phytophthora cinnamomi* was observed, with inhibition radii ranging between 2 and 5 mm (Table 3). Mycelial growth of *Fusarium oxysporum* f.sp. *dianthii* remained unaffected by the *Pseudomonas* tested. The growth of all phytopathogenic fungi used was reduced by the presence of the biocontrol agent *Pseudomonas fluorescens* F113 (inhibition radii 5–10 mm) used as reference strain.

4. Discussion

The presence of dense bacterial populations in the inner part of mature sporocarps of *Tuber borchii* can be deduced from the viable cell counts and the electron microscopy investigations reported in this study. Although the presence of bacteria in fruit bodies of ectomycorrhizal fungi has been described (Swartz, 1929; Li and Castellano, 1987; Garbaye et al., 1990; Danell et al., 1993; Varese et al., 1996), including non-sterilized *Tuber* sporocarps (Pacioni, 1990), here the characterization of bacterial populations isolated from the inner core of surface-sterilized *T. borchii* is reported for the first time.

All but two samples examined contained 10^5–10^8 cfu of pseudomonads, or spore-forming bacillaceae, or both. The two sporocarps of *T. borchii* giving levels of bacterial microbiota below detection limit (i.e. 10^{-1}–10^2) were very

small, and it was therefore difficult to exclude direct effects of the sterilization procedure.

Owing to the variety of geographical areas, of climate and soils from which the truffles originated, we suggest that the presence of bacterial populations inside the sporocarps of *T. borchii* is consistent, widespread and apparently specialized. The latter aspect is supported by the fairly reduced variety of bacterial groups isolated and identified, among which fluorescent *Pseudomonas* predominate, representing 38% of total pseudomonads isolated. In other *Tuber* sporocarps the percentage of fluorescent *Pseudomonas* with respect to total bacterial populations ranged between 20 and 42% (Basaglia and Nuti, 1996). A higher fraction of fluorescent *Pseudomonas* has also been reported to occur in *Cantharellus cibarius* sporocarps with respect to the surrounding soil (Danell et al., 1993). These findings suggest that an enrichment by the fungal sporocarp for specific bacterial groups may be more likely than an incidental incorporation of surrounding microbiota.

Seasonal fluctuations have been reported (Mamoun et al., 1986; Olivier and Mamoun, 1988) for *Pseudomonas* spp. in the rhizoplane and rhizosphere of *Tuber* mycorrhizae. Additional data are needed to ascertain whether the reduced biodiversity, at least for culturable populations, with respect to overall surrounding microbiota is dependent upon these fluctuations or on a direct effect by the fungus and/or the plant.

Among isolates identified at species level, *P. fluorescens, P tolaasii* and *P. corrugata* were the most frequent, representing 34, 16 and 12% of total pseudomonads, respectively. Biotypes A, B, C, F and G of *Pseudomonas fluorescens* are represented. *P. tolaasii* was also identified, and this bacterium has been repeatedly reported to cause alterations, i.e. bacterial blotch disease, to sporocarps and stems of cultivated mushrooms such as *Agaricus* and *Pleurotus* (Fermor and Lynch, 1988; Bedini et al., 1998).

In order to gain an insight into the possible physiological role of *Pseudomonas* spp., a number of metabolites, described as positive or negative effectors on different organisms, was investigated.

Siderophores and proteases were produced by all strains studied, while 2,4-diacetylphloroglucinol and HCN were released by a minority of strains. The above four metabolites have been described as biocontrol substances and found to be produced by *Pseudomonas* species (Fenton et al., 1992; Cronin et al., 1997; Dunne et al., 1997; Dowling et al., 1998). Many strains showed positive responses to the assay for cellulolytic activity carried out with carboxy methyl cellulose, and it is well-known that cellulolytic microorganisms are involved in control of phytopathogenic fungi like *Pythium* spp. (Thrane et al., 1997). The absence of fungal contaminants from the core samples of sporocarps examined, though consistent populations of fungal saprophytes in truffle orchard soil have been enumerated (Mamoun et al., 1986; Olivier and Mamoun, 1988), could

be interpreted as a consequence, either direct or indirect, of the presence *in situ* of bacteria producing antimycotic compounds. All isolates tested were not expressing chitinolytic activity in our culture and assay conditions, contrary to findings of Gazzanelli et al. (1998). The biocontrol activity of *Pseudomonas* was confirmed also via in vitro biological tests, which indicates that 50–90% of strains tested were able to control the growth of *Botrytis, Cryphonectria, Diplodia* and *Phytophthora*. No antagonistic activity was detected towards *Fusarium oxysporum*, which was actively controlled by the reference biocontrol strain *P. fluorescens* F113 (a siderophore, PHL and HCN producer) indicating that antifungal activity of isolates from *T. borchii* was weak. Some strains also showed collagenase activity, which is known to be implicated in plant root nematode control by rhizospheric bacteria (Cronin et al., 1997). Overall, it appears that *Pseudomonas* from within sporocarps are equipped to express some biocontrol activity towards some common plant root pathogens.

It is noteworthy that all 90 strains examined produced the phytostimulatory substance indole-3-acetic acid in culture. The sensitive assay with monoclonal antibody has revealed unusual ability to synthesize this phytohormone, at concentrations to be considered higher than other bacteria, owing the higher sensitivity and specificity of the method we adopted, compared to the Salkowski reaction (Costacurta and Vanderleyden, 1995; Costacurta et al., 1998).

The quantification of IAA amounts in *Tuber* sporocarps indicated the level of 232.9±11.8 μg IAA g^{-1} f.w. for *T. borchii* and further analyses are needed to understand whether bacterial production contribute to total IAA concentration; however this substance could have positive or negative effects on the ectomycorrhizal fungus depending on the concentration and localization within the sporocarp. Our finding suggests that for either effects, the number of viable cells found in the core of truffles is adequate since, in the case of producer bacteria, these cells are physically close to the target cells of the fungus.

Results obtained from the present in vitro characterization assays represent an indication of some bacterial activities which could be of benefit for the fungus *in vivo*, explaining such a close interaction between these organisms, though bacterial functionality should be ascertained with experiments to be carried out in a nutrient-poor environment, more relevant to natural conditions.

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