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Bacteria Associated to Fruit Bodies of the Ecto-Mycorrhizal Fungus *Tuber borchii* Vittad.

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Abstract

A microbial population was isolated from young sporocarps of *Tuber borchii* Vittad. to study its possible role in ascus opening. The bacteria in the sporocarps had a mean value of 10^6 CFU/g and were generally found in quantities higher than that found in the bulk soil (10^3). In the sporocarps examined the predominant bacteria were *Pseudomonas fluorescens* (30% of the total population) and spore-forming, gram-positive, bacteria (15% of total). These bacterial species were tested to evaluate their capacity to degrade cellulose and chitin, the most important components of the hyphal walls. Ultrastructural examination of the tested sporocarps revealed the presence of bacteria in the interhyphal space, a portion of which were embedded in the ascus wall. It is suggested that the presence of *Pseudomonas* strains and Bacillaceae in *T. borchii* Vittad. sporocarps could be related to their chitinolytic and cellulolytic activities, which could in turn be involved in ascus opening and, perhaps, in spore germination.

Keywords: *Tuber borchii* Vittad., bacteria, ultrastructure, ascus

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1. Introduction

Mycorrhizae are mutualistic symbioses which are common in natural soils and form between single mycobionts and fine rootlets. They play an important role in increasing the uptake of phosphorus and other poorly mobile nutrients in the plant (Danduran et al., 1997). The part of soil where these associations develop is termed the rhizosphere, which is composed of the interacting trinity of the plant, the soil immediately adjacent to the root and the organisms associated with the roots (Lynch, 1987).

The microbial populations within the rhizosphere differ quantitatively and qualitatively from those in the bulk soil (Danduran et al., 1997). In particular, some bacteria associated with the rhizosphere of ectomycorrhizae stimulate fungus growth and mycorrhizal infection (Duponnois and Garbaye, 1991; Duponnois and Garbaye, 1990; Frey-Klett et al., 1997; Ali and Jackson, 1989); these bacteria have been named mycorrhiza helper bacteria (MHB) (Duponnois and Garbaye, 1991; Garbaye and Duponnois, 1992). Moreover, the development of the symbiosis alters the carbohydrates exuded by the roots both quantitatively and qualitatively, thereby modifying the microbial equilibrium in the rhizosphere. This particular condition has been described as a "mycorrhizosphere effect" and apparently promotes the occurrence of some MHB (Garbaye, 1994; Linderman, 1988; Bowen and Theodorou, 1979). This selective activity in the mycorrhizosphere has also been found in the fruit bodies of various ectomycorrhizal fungi (Bowen and Theodorou, 1979; Linderman, 1988; Filippi et al., 1995; Li and Castellano, 1987).

Many edible ectomycorrhizal Ascomycetes of the genus *Tuber* are of great economic importance due to the organoleptic properties of their fruit bodies (truffles). The geographic area of the Comunità Montana dell'Alto e Medio Metauro (a consortium of communes in the mountain areas of the upper and mid-Metauro River Valley, which is located in central Italy) is characterised by the presence of several species of white truffle, a valuable variety. Consequently, a program of reforestation using seedlings inoculated in nurseries with these ectomycorrhizal fungi would be of noteworthy economic interest for the region and, at the same time, would allow the recovery of several unused marginal areas. However, at the present time, the white truffle mycelium that is available for *in vitro* studies comes from sporocarps and not from spore germination, making large-scale plant mycorrhization programs difficult.

The aim of this research was to study, by means of a microbiological and ultrastructural approach, the MHB inside the sporocarps of *Tuber borchii* Vittad., a white truffle species, in order to investigate their possible role in the processes affecting the ascospore germination.

2. Materials and Methods

Collection of bacterial strains

Fruit bodies of *T. borchii* Vittad. were harvested at early stages of maturation from January to March in the territory of the Comunità Montana dell'Alto e Medio Metauro (central Italy). All together, 70 fruit bodies of *T. borchii*, collected in seven mountain areas (ten from each area) characterised by different botanical species, were examined.

Bacterial strains were collected from these fruit bodies and the adjacent bulk soils. The sporocarps were washed under running tap water and dried with cold air. The outer layer of the sporocarps was then blazed with alcohol and removed with a sterile scalpel. Only the inner part of the sporocarps (1–5 g f.w.) was aseptically placed in a sterile physiological solution (8.5 g NaCl l^{-1}) and homogenised with a potter (5 min).

Samples of bulk soil (1 g f.w.) were shaken in a sterile physiological solution (9 ml) for 3 min. Homogenates and bulk soil suspensions were used to prepare appropriate dilutions which were then placed on the following selective growth media: Tryptone Soy Agar (TSA Oxoid), Nutrient Agar (NA Difco) and Pseudomonas Agar Base with C-N supplement (PSA Oxoid). After being placed on TSA and PSA media, the appropriate dilutions were maintained for 10 min at 80°C to discriminate for the Bacillaceae and then placed on the NA medium. The bacterial colonies grown on PSA medium were subsequently inoculated on King's B agar (KAB) and observed under UV light at 350 nm to distinguish fluorescent *Pseudomonas* from non-fluorescent species (King et al., 1954). All agar plates were incubated for 36–48 h at 28°C and microbial growth was evaluated by counting and converted into CFU/g.

The bacterial colonies grown on selective media were purified, identified using API systems (Biomerieux-France) and biochemical analysis, according to Bergey's Manual, and finally cryopreserved at –20°C in glycerol.

Testing for cellulolysis by bacteria

Sixty-five bacterial strains belonging to the species *Pseudomonas fluorescens* and 40 belonging to the genus *Bacillus* were suspended in 5 ml of sterile physiological solution. The number of cells was determined by measuring absorbency at 520 nm and adjusted to a McFarland range value of 0.2. Aliquots of the suspensions were then placed on Petri dishes containing CMC Agar [1 g $(NH_4)_2SO_4$; 2 g KH_2PO_4; 3 g Na_2HPO_4; 0.1g $FeSO_4.7H_2O$; 0.5 g $CaCl_2$; 1 ml trace elements (10 mg H_3BO_5; 10 mg $MnSO_4$; 70 mg $ZnSO_4$; 50 mg $CuSO_4$; 10 mg

MoO_3; 1 l distilled water); 1 g yeast extract; 5 g carboxymethylcellulose, 10 g agar; 1 l distilled water], pH 7. The agar plates were incubated for 4–9 days at 28°C, fixed with NaCl 5M for 20 min and then treated with Congo Red (0.1% in H_2O) for 20 min.

Testing for chitinolysis by bacteria

Sixty-five bacterial strains belonging to the species *Pseudomonas fluorescens* and 40 belonging to the genus *Bacillus* were tested for the capacity to reduce chitin. The strains were treated as for the cellulolysis test, then placed on Chitin Agar (Lingappa and Lockwood, 1962) and incubated for 7–10 days at 28°C.

Morphological analysis

Small fragments of the inner part of the ascocarps were fixed with a mixture containing 2.5% glutaraldehyde and 2% paraformaldehyde diluted in 0.5 M cacodylate buffer (pH 6.8) for 4 h at 4°C. The samples were then washed in the cacodylate buffer and post-fixed with 1% OsO4 and 1.5% $K_4Fe(CN)_6$ for 2 h at room temperature. After further washing in the cacodylate buffer, the specimens were dehydrated through graded concentrations of acetone and embedded in Epon-Araldite. Semithin sections were cut, conventionally stained with 1% toluidine blue and observed with a Leitz Orthoplan light microscope. Ultrathin sections were placed on copper grids coated with a Formvar-carbon layer, stained with lead citrate and observed with a Zeiss EM 902 transmission electron microscope. Morphometrical analyses on the sporocarps were carried out with a Zeiss Axioskop light microscope connected to a Kontron KS300 image analysis system, which allowed the measurement of the perimeters of sectioned asci. In order to obtain information on possible modifications in ascus size during maturation, we considered only those asci containing immature spores showing the primary wall alone and asci containing mature spores showing the secondary wall with well-developed ornamentations.

Statistical analyses

Mean values of microbial populations were calculated in triplicate in all 70 fruit bodies and in the respective bulk soil samples. The statistical analyses were effectuated using the Chi-square test.

3. Results

Bacteria in sporocarps

The total cultivable bacterial population inside *T. borchii* sporocarps in early stages of maturation was characterised by a significant homogeneity despite the different harvesting areas (Table 1).

Six hundred and fifty bacterial strains were isolated from 70 sporocarps of *T. borchii* collected in mountain areas characterised by the presence of different botanical species (i.e. brooms, pines, grasses). Among the isolated strains, 400 belonged to the genus *Pseudomonas*, 200 were spore-forming gram-positive bacilli and the remaining ones were bacteria belonging to the genera *Staphylococcus* and *Micrococcus*.

Table 1. Bacterial genera isolated from *T. borchii* sporocarps collected in areas characterised by different botanical species.

Collection areas	Botanical species	Bacterial genera
A	*Cytisus scoparius*	*Pseudomonas, Bacillus*
B	*Pinus pinea*	*Pseudomonas, Bacillus, Micrococcus*
C	*Poa pratensis* *Dactylis glomerata* *Lolium rigidum* *Festuca pratensis* *Bromus erectus* *Trifolium pratensis*	*Pseudomonas, Bacillus, Staphylococcus*
D	*Poa pratensis* *Dactylis glomerata* *Lolium rigidum* *Festuca pratensis* *Bromus erectus* *Trifolium pratensis*	*Pseudomonas, Bacillus*
E	*Cytisus scoparius*	*Pseudomonas, Bacillus, Micrococcus*
F	*Pinus pinea*	*Pseudomonas, Bacillus*
G	*Pinus pinea*	*Pseudomonas, Bacillus, Micrococcus*

Table 2. Mean values (CFU/g) of the microbial population in *T. borchii* sporocarps and in bulk soil.

Collection areas	Microbial population	
	Sporocarps	Bulk soil
A	2.3×10^7	2.2×10^2
B	1.2×10^5	1.1×10^3
C	1.3×10^4	1.7×10^3
D	7.9×10^1	6.3×10^2
E	2.9×10^2	1.2×10^3
F	1.7×10^5	2.1×10^3
G	3.0×10^3	2.7×10^3

$t = 1.015$, $df = 12$, $P = 0.330$.

Table 3. Mean values (CFU/g) of *Pseudomonas fluorescens* and Bacillaceae in sporocarps and bulk soil samples from the different collection areas.

Collection areas	*Pseudomonas fluorescens*		Bacillaceae	
	Sporocarps	Bulk soil	Sporocarps	Bulk soil
A	9.6×10^6	2.2×10^1	3.4×10^6	1.7×10^1
B	3.6×10^4	1.1×10^2	1.8×10^4	8.8×10^1
C	3.9×10^3	1.7×10^2	1.9×10^3	1.3×10^2
D	2.3×10^1	6.3×10^1	1.1×10^1	5.0×10^1
E	8.7×10^1	1.2×10^2	4.3×10^1	9.6×10^1
F	5.1×10^4	2.1×10^2	2.5×10^4	1.6×10^2
G	9.0×10^2	2.7×10^2	4.5×10^2	2.1×10^2

Pseudomonas fluorescens: $t = 1.011$, $df = 12$, $P = 0.332$; Bacillaceae: $t = 1.015$, $df = 12$, $P = 0.330$.

Forty percent of the 400 *Pseudomonas* strains tested in this study belonged to the fluorescent group; these fluorescent bacteria were identified as *Pseudomonas fluorescens* (30%) and *Pseudomonas putida* (10%).

Quantitatively, the values for the microbial population in the sporocarps (mean value of 10^6) were nearly always higher than that found in the bulk soil (10^3) (Table 2). In detail, the population of *Pseudomonas fluorescens* represented 30% of the total sporocarp bacteria, while it made up only 10% of

the bacteria present in the bulk soil. Similarly, the Bacillaceae population represented 15% of the whole sporocarp microbial population, in comparison to a value of 8% in the bulk soil (Table 3).

Bacterial cellulolysis

Forty-eight *Pseudomonas fluorescens* strains isolated from sporocarps and 48 spore-forming Bacillaceae strains, randomly chosen from the 200 isolated in fruit bodies, were analysed for cellulolytic activity. The results obtained showed that only 30 and 25 strains, respectively, of the 48 *Pseudomonas fluorescens* and 48 spore-forming Bacillaceae presented the light orange halo around bacterial colonies grown on the selective medium, which was indicative of cellulose degradation.

Bacterial chitinolysis

The bacterial strains tested for cellulose utilisation were further assayed for their capacity to digest chitin. The presence of a clear halo around the bacterial colonies demonstrated the chitinolytic activity of the bacterial strains. Twenty-five of the 30 *Pseudomonas fluorescens* strains and all the 25 spore-forming Bacillaceae strains tested were found to be positive for chitinolytic activity.

Morphological analysis

The observations made using light and electron microscopy confirmed that the sporocarps were in early stages of maturation; in fact, many spores showed only the primary wall (Fig. 1A) and, moreover, no free spores were found inside the sporocarps and all ascus walls were intact (Figs. 1A–1B).

Ultrastructural investigations revealed many bacterial cells inside the sporocarps and in particular, in the interhyphal space, sometimes directly in contact with the hyphal wall but never inside living hyphal cells (Fig. 1B). These bacteria generally occurred as aggregates of morphologically similar individuals, likely belonging to different species, embedded in a fine fibrillar matrix (Figs. 1B–2A). They were rod-shaped and were generally surrounded by a homogeneous electron-transparent area, probably due to their lytic activity, whereas no evidence of a coating mucus layer was found (Fig. 2A). Many bacteria were observed adhering to the hyphal walls, and some appeared to be partially embedded in the ascus walls. These bacteria seemed to have partially penetrated into the ascus wall (Fig. 2B); however, they were found only in the outer layer of the wall.

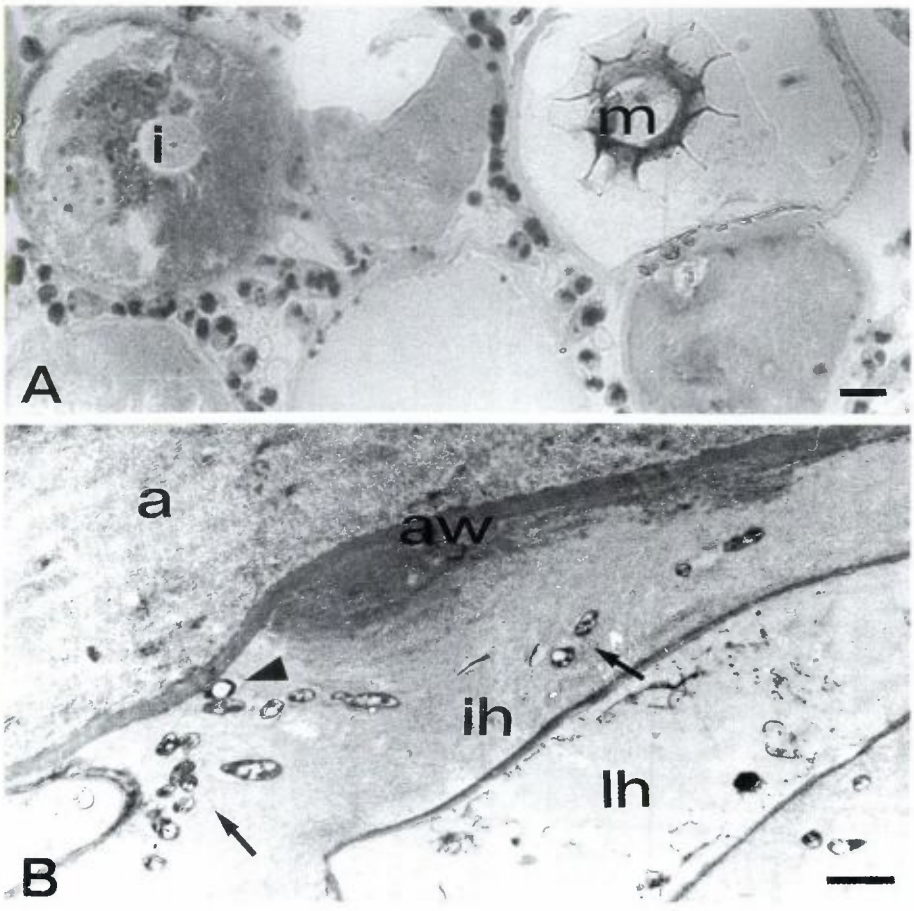

Figure 1. Light and transmission electron micrographs of *Tuber borchii* sporocarps. (A) Semithin section showing asci of different size and content; note the ascus containing a mature spore displaying well-developed ornamentations (m) and the ascus containing an immature spore surrounded by a primary wall alone (i). All ascus walls appear to be intact. Bar = 10 μm. (B) Electron micrograph showing many bacteria (arrows) present in the interhyphal space (ih) and also adhering (arrowhead) to the ascus wall (aw). (a) = ascus; (lh) = living hypha. Bar = 1 μm.

Morphometrical analyses revealed differences in the perimeters of the asci containing mature spores (mean value ± SD = 280.4 ± 27.6 μm, n=25) and the perimeters of asci with immature spores (168.7 ± 17.8 μm). Bacteria were found to adhere only to the larger asci.

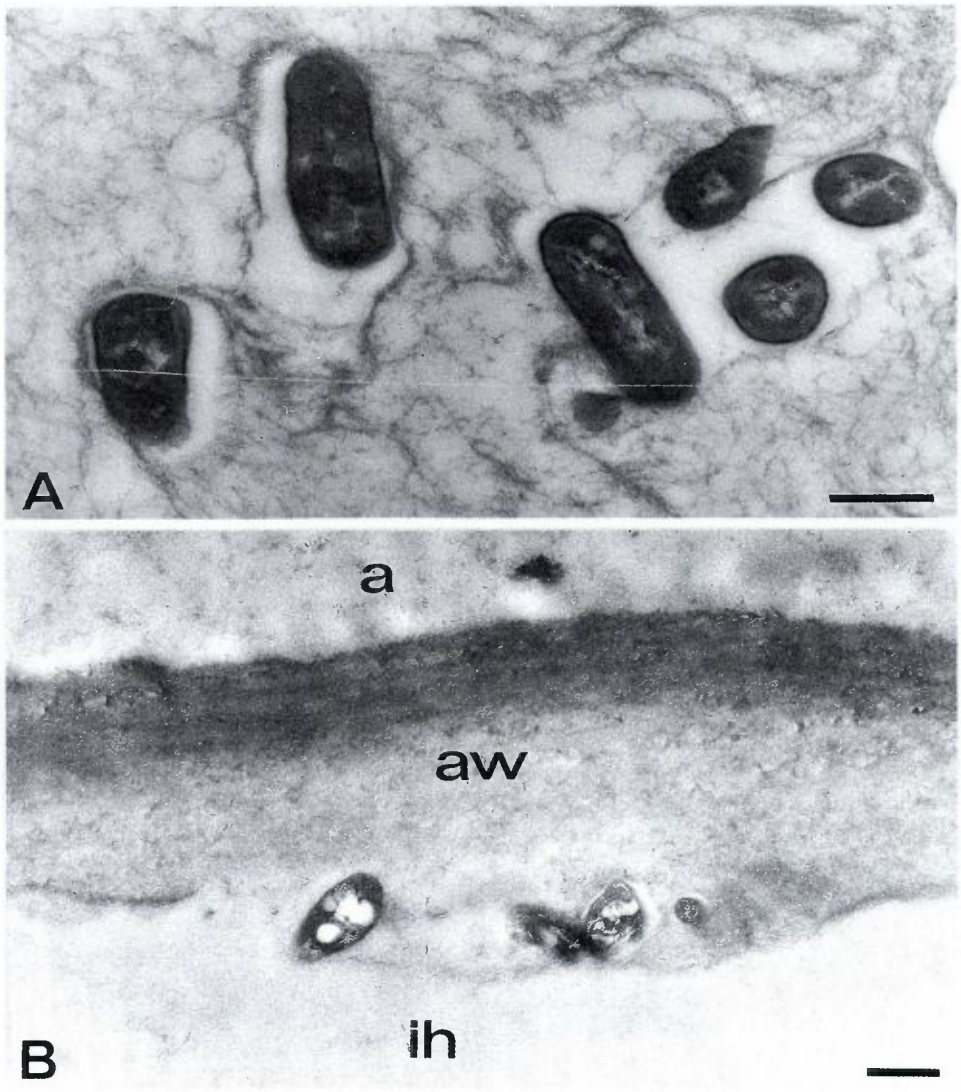

Figure 2. Transmission electron micrographs showing: (A) bacteria dispersed in the fine fibrillar matrix of the interhyphal space, generally surrounded by homogeneous electron-transparent areas; and (B) bacteria partially embedded in the outer layer of the ascus wall (aw). (a) = ascus; (ih) = interhyphal space. Bars = 0.5 µm.

4. Discussion

Our investigations carried out on the microbial population in *T. borchii* sporocarps at early stages of maturation demonstrated the following points: 1)

the microbial population of all the sporocarps analysed was nearly always higher than that present in the bulk soil; 2) fluorescent *Pseudomonas* species and Bacillaceae were the most frequent bacterial groups in the sporocarps; 3) most of these bacteria were able to degrade cellulose and chitin; 4) the bacteria inside the sporocarps were distributed in the interhyphal space, at times partially embedded in the ascus walls.

The total population of bacteria found inside the *T. borchii* fruit bodies showed a great degree of homogeneity, despite the fact that the different harvesting areas were characterised by the presence of a variety of botanical species. From a quantitative point of view, the total population of bacteria inside the fruit bodies was generally greater than that found in the bulk soil. In particular, the amount of *Pseudomonas fluorescens* in the sporocarps was three times that found in the bulk soil. Similarly, twice the amount of spore-forming bacteria were found in the sporocarps vs the bulk soil. It is possible that the internal environment of the sporocarps of *T. borchii* exerts a selective and stimulating effect on the growth of bacterial strains commonly present in bulk soil. A similar effect was also observed in fruit bodies of the ectomycorrhizal fungus *Cantharellus cibarius* (Danell et al., 1993), of the endomycorrhizal fungus *Glomus mosseae* (Filippi et al., 1998) and in the mycorrhyizosphere (Mamaun and Oliver, 1989; Frey-Klett et al., 1997).

The location of the bacteria in the interhyphal space – and not in living hyphae – of young fruit bodies suggests a random incorporation of the bacteria surrounding the hyphae during development and growth of sporocarps. These bacteria were at times found to have partially penetrated into the ascus walls, probably because of their own lytic activity. The hyphal walls are composed mainly of cellulose and chitin, which *Pseudomonas fluorescens* and spore-forming bacteria isolated from our sporocarps are able to degrade. However, the bacteria were found to be only partially embedded in the outer layer of the ascus wall. The early stage of maturation of the sporocarps tested probably did not allow the observation of the latest phases of bacterial lytic activity in the ascus wall. Such wall degradation could play a role in the process of ascus opening and, consequently, of spore scattering.

Interestingly, the microbial lytic degradation took place only in the walls of the largest asci containing mature spores, which seemed to have become thinner than those of smaller asci. Moreover, it is possible that this lytic activity could be extended, in older sporocarps, also to spore walls, as previously observed in some endo-mycorrhizal fungi (Varese et al., 1996; Filippi et al., 1998; Walley and Germida, 1996), thus affecting spore germination processes (Paulitz and Linderman, 1989; Azcon-Aguillar and Barea, 1992). Studies on *T. borchii* sporocarps in late stages of maturation are in progress.

In conclusion, the presence of a substantial number of *Pseudomonas* and Bacillaceae strains in *T. borchii* Vittad. sporocarps could be related to their chitinolytic and cellulolytic activities, which could facilitate ascus opening and, perhaps, spore germination.

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