Adhesion of ectomycorrhizal bacteria to plant cells: an *in vitro* evidence

B. Citterio, V. Patrone, B. Baldelli,* M. Malatesta*
Istituto di Scienze Tossicologiche, Igienistiche e Ambientali and *Istituto di Istologia e Analisi di Laboratorio, University of Urbino "Carlo Bo", Urbino, Italy

In this study we have investigated, by combining microbial and microscopical techniques, the adhesion ability of bacteria present in *Tuber borchii* ectomycorrhizosphere. Our data demonstrate that a common pool of bacteria – *Pseudomonas*, *Bacillus*, *Micrococcus* and *Moraxella* – occurs in all ectomycorrhizal homogenates and that most of these bacteria are able to attach *in vitro* to plant cells.

Key words: bacteria, adhesion, ectomycorrhiza, *Tuber*.

Correspondence: Manuela Malatesta, Istituto di Istologia e Analisi di Laboratorio, via Zeppi s.n., University of Urbino "Carlo Bo", 61029 Urbino, Italy.
Phone: international +39.0722.320168.
Fax: international +39.0722.322370.
E-mail: malatesta@uniurb.it

Paper accepted on December 19, 2003

European Journal of Histochemistry
2004; vol. 48 issue 2 [Apr-Jun]: 191-194

Mycorrhizal symbioses occur in the "mycorrhizosphere", defined as the rhizosphere of a root infected by a mycorrhizal fungus. As the fungus uses some of the root exudates and modifies root functions, microbial communities in the mycorrhizosphere differ from those in the rhizosphere and in the soil (Ames et al., 1984; Linderman, 1988; Bianciotto, 2001). Bacteria are often associated with ectomycorrhizas and are probably involved in the dynamics of mycorrhiza formation (Garbaye and Duponnois, 1992; Varese et al., 1996, Gamalero 2003). In this study we investigated, by combining microbial and microscopical techniques, some bacterial strains of *Tuber borchii* ectomycorrhizosphere and we tested them *in vitro* for adhesion to plant cells.

Twenty-one samples of *T. borchii* ectomycorrhizas of different botanical species were harvested in mountain areas of the Comunità Montana dell'Alto e Medio Metauro (Central Italy) and identified by morphological analysis of the mantle. Mycorrhized apices (1 g f.w.) were aseptically collected, submitted to 4 washings in sterile physiological solution (8.5 g NaCl/L) to remove external bacteria and homogenised with a potter (10 min). Homogenates suspensions were diluted, placed on TSA (Triptone Soy Agar, Difco) and incubated for 36-48 h at 28°C to evaluate the microbial growth. After a preliminary screening (Gram staining, oxidase and catalase tests, sugar reduction), bacteria occurring in all ectomycorrhizal homogenates were identified. After being placed on TSA medium, the appropriate dilutions were maintained for 10 min at 80°C to discriminate for the *Bacillus*. The bacterial colonies were purified, identified using API systems (Biomerieux-France) and biochemical analysis according to Bergey's manual, and finally cryopreserved at -20°C in glycerol. We found that all ectomycorrhizal homogenates contained bacterial strains belonging to the genera *Pseudomonas*, *Bacillus*, *Micrococcus* and *Moraxella*. A total of 25

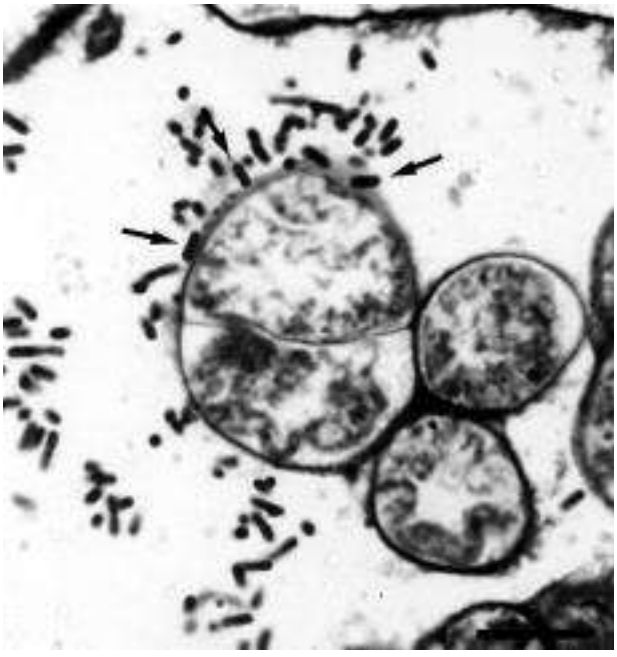

Figure 1. Bacteria occur in association with *Rubus fruticosus* cell surface (arrows). Scale bar: 10 µm.

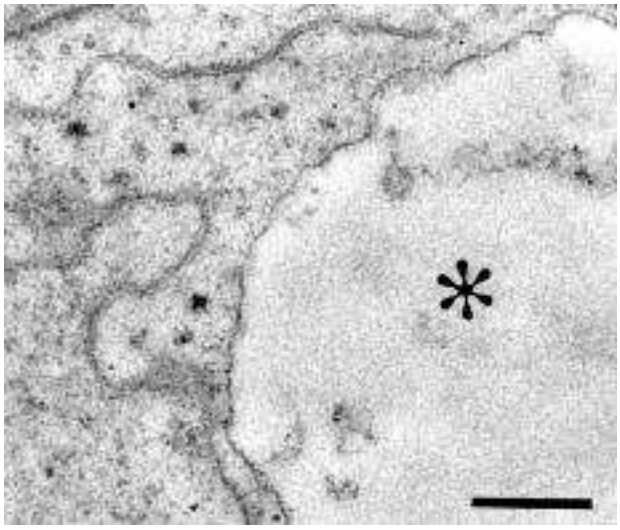

Figure 3. In the cytoplasm of *Rubus fruticosus* cells various membranous structures and vacuoles (asterisk) are present. Scale bar: 0.5 µm.

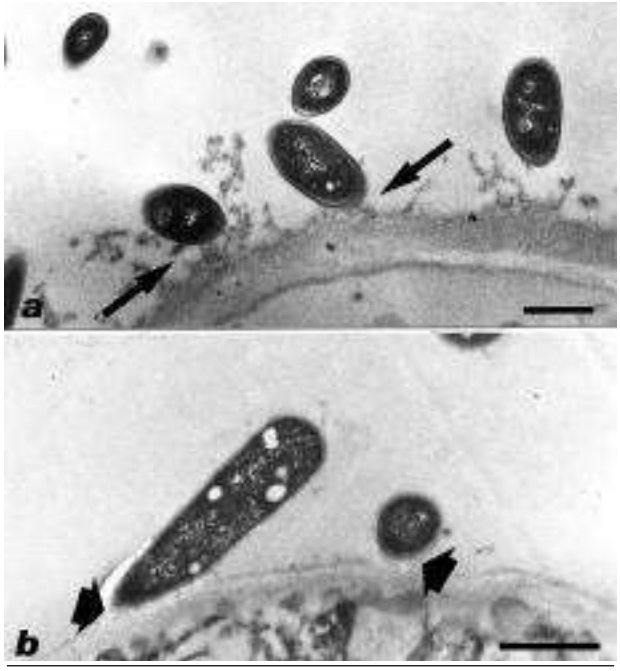

Figure 2. Bacteria belonging to the genus *Pseudomonas* attached to *Rubus fruticosus* cells: some of them (a) are embedded in the fine fibrillar material coating the cell surface (arrows), others make direct contact with the cell wall (arrowheads). Scale bars: 1 µm.

bacterial strains from these genera were used to test their possible attachment to plant cells. *Rubus fruticosus* cells, a widespread and reliable *in vitro* model, were grown in KM (Kao and Michayluk) medium (Mezzetti et al., 1997). Ten mL plant cell culture were inoculated in 100 ml KM medium and incubated at 24°C in an orbital shaker at 115 rpm in the dark. The different bacterial strains were grown for 24 h in order to obtain a synchronous culture and the viability was tested using 5-Cyano-2,3-ditolyl tetrazolium chloride (Polysciences Europe GmbH). A loop of these bacteria (10^3 CFU/mL) was then added to 100 mL KM medium containing 10^5 plant cells and kept at 24°C in the orbital shaker for 2 days. The samples were then washed twice with sterile physiological solution, filtered and resuspended in the culture medium. In order to preserve a good ultrastructural morphology of both bacterial and plant cells as well as the cell-bacteria relationships (Figures 2 and 3), the cell suspensions were fixed by adding 2.5% glutaraldehyde directly to the culture medium. After 1 h fixation at 4°C, the cells were washed in 0.2 M cacodylate buffer pH 5.6, post-fixed with 1% OsO_4 for 1 h, washed again, dehydrated and embedded in LRWhite resin. Semithin sections, stained with 1% toluidine blue, were observed with a Leitz Orthoplan light microscope and revealed the presence of bacteria and their distribution among the plant cells. As expected, the bacterial population present in each sample was constituted by morpho-

logically homogeneous individuals, showing either rod-like or ovoid shapes. In 23 of the 25 samples analysed numerous bacteria appeared to be associated to the plant cell surface (Figure 1); in detail 10 samples showed a weak (8-10 bacteria/cell), 10 a moderate (10-20 bacteria/cell) and 3 a strong (more than 30 bacteria/cell) attachment. The adhering bacteria belonged to all the tested genera, but two *Pseudomonas* strains were non-adhering. Ultrathin sections, contrasted with lead citrate, were observed with a Zeiss EM 902 electron microscope, thus revealing the fine spatial relationships between bacterial and plant cells. Bacteria were observed adhering by means of the fine fibrillar material coating the plant cell surface (Figure 2a) as well as by making direct contact with the plant cell wall (Figure 2b).

In conclusion, our data demonstrate that, in spite of the various host plants, a common pool of bacterial strains - *Pseudomonas*, *Bacillus*, *Micrococcus* and *Moraxella* - occurs in *T. borchii* ectomycorrhizosphere, and most of these bacteria are able to attach *in vitro* to plant cells. It is likely that such bacteria would be related with *T. borchii* life cycle, as suggested by their presence in the sporocarps of the same fungus (Citterio et al., 1995), where they seem to be involved in ascus opening by producing cellulolytic and chitinolytic enzymes (Gazzanelli et al., 1999; Citterio et al., 2001). Some of these bacteria are known to attach to root cells (Hawes and Brigham, 1992); moreover, *Pseudomonas* species isolated from *T. borchii* ascocarps have been demonstrated to adhere to *Tuber* hyphae (Sbrana et al., 2000; Sbrana et al., 2002). As our *in vitro* model showed, the microbial attachment can take place by means of the mucilaginous material coating plant cells, but the bacteria can also establish a direct contact with the plant cell wall.

Acknowledgments

We are particularly grateful to Prof. Bruno Mezzetti for providing us with *Rubus fruticosus* cells.

References

Ames RN, Reid CPP, Ingham ER. Rhizosphere bacterial population response to root colonization by a vesicular-arbuscular mycorrhizal fungus. New Phytol 1984;96:555-63.

Bianciotto V, Andreotti S, Balestrini R, Bonfante P, Perotto S. Extracellular polysaccharides are involved in the attachment of *Azospirillum brasilense* and *Rhizobium leguminosarum* to arbuscolar mycorrhizal structures. Eur J Histochem 2001;45:39-49.

Citterio B, Cardoni P, Potenza L, Amicucci A, Stocchi V, Gola G, et al. Isolation of bacteria from sporocarps of *Tuber magnatum Pico, Tuber borchii Vitt.* and *Tuber maculatum Vitt.* Identification and biochemical characterization. In: Stocchi V, Bonfante P, Nuti MP, editors. Biotechnology of ectomycorrhizae. Plenum Press: New York; 1995. p. 241-8.

Citterio B, Malatesta M, Battistelli S, Marcheggiani F, Baffone W, Saltarelli R, et al. Possibile involvement of *Pseudomonas fluorescens* and *Bacillaceae* in structural modifications of *Tuber borchii* fruit bodies. Can J Microbiol 2001;47:264-8.

Gamalero E, Fracchia L, Cavaletto M, Garbaye J, Frey-Klett P, Varese G.C, Martinotti M.G. Characterization of functional traits of two fluorescent pseudomonas isolated from basidiomes of ectomycorrhizal fungi. Soil Biol Biochem 2003;35: 55-6.

Garbaye J, Duponnois R. Specificity and function of mycorrhization helper bacteria (MHB) associated with the *Pseudostuga menziesii-Laccaria laccata* symbiosis. Symbiosis 1992;14:335-44.

Gazzanelli G, Malatesta M, Pianetti A, Baffone W, Stocchi V, Citterio B. Bacteria associated to fruit bodies of the ecto-mycorrhizal fungus *Tuber borchii Vittad.* Symbiosis 1999;26:211-22.

Hawes MC, Brigham LA. Impact of root border cells on microbial populations in the rhizosphere. Adv Plant Pathol 1992;8:119-48.

Linderman RG. Mycorrhizal interaction with the rhizosphere microflora: the mycorrhizosphere effect. Phytopatology 1988;78:366-71.

Mezzetti B, Savini G, Carnevali F, Motti D. Plant genotype and growth regulator interaction affecting in vitro morphogenesis of blackberry (*Rubus fruticosus*) and raspberry (*R. idaeus*). Biol Plantarum 1997; 39:139-50.

Sbrana C, Bagnoli G, Bedini S, Filippi C, Giovannetti M, Nuti MP. Adhesion to hyphal matrix and antifungal activity of *Pseudomonas* strains isolated from *Tuber borchii* ascocarps. Can J Bot 2000; 46: 259-68.

Sbrana C, Agnolucci M, Bedini S, Filippi C, Lepera A, Toffanin A, Giovannetti M, Nuti MP. Diversity of culturable bacteria populations associated to Tuber borchii ectomycorrhizas and their activity on *T. borchii* mycelial growth. FEMS Microbiol Lett 2002;2:195-201.

Varese GC, Portinaro S, Trotta A, Scanerini S, Luppi-Mosca AM, Martinotti MG. Bacteria associated with *Suillus grevillei* sporocarps and ectomycorrhizas and their effects on *in vitro* growth of the mycobiont. Symbiosis 1996;21:129-47.