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Ultrastructural Spatial Distribution of Bacteria Associated with Sporocarps of *Glomus mosseae*

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Abstract

Transmission electron microscopy studies showed many bacteria associated with the sporocarps of *Glomus mosseae*, occurring not only on the hyphal surface, but also on the surface of spores within the sporocarps and in microniches formed by peridial hyphae. Sequential cuts of the sporocarps showed the spatial distribution of the bacteria, and evidenced bacterial cells embedded in spore walls by means of tunnels mined within the electron-dense spore wall layers. Quantitative determinations of microorganisms associated with *G. mosseae* sporocarps showed the occurrence of large numbers of bacteria, fungi and actinomycetes on the sporocarp surface and in the sporocarp homogenate. Moreover, chitinolytic microbes were detected in the sporocarps.

Keywords: *Glomus mosseae*, sporocarps, ultrastructure, associated bacteria

1. Introduction

Mycorrhizal symbioses have been shown to provoke such striking changes in the composition and size of rhizospheric microbial populations (Ames et al., 1984; Meyer and Linderman, 1986; Garbaye et al., 1990; Azcon-Aguilar and

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Barea, 1992; Bansal and Mukerji, 1994), that the new name "mycorrhizosphere" is now widely used (Lindermann, 1988). Mycorrhizal fungi have been shown to provide a physical and nutritional substrate for many soil microbes (Foster and Nicolson, 1981; Tisdall, 1991). In recent years different types of microbes have been reported to occur in the mycorrhizosphere and have been described as plant-growth promoting rhizobacteria (PGPR) (Meyer and Linderman, 1986; Paulitz and Linderman, 1989; Garbaye, 1991; Azcon-Aguilar and Barea, 1992), nitrogen fixing bacteria (Meyer and Linderman, 1986; Ho, 1988; Tilak et al., 1989), and antagonists towards plant pathogens (Secilia and Bagyaraj, 1987; Catskà, 1994; Azcon-Aguilar and Barea, 1996; Citernesi et al., 1997).

Different taxa of microbes in the mycorrhizosphere have been reported to be associated either with the hyphae, or with the sporocarps of different mycorrhizal fungi (Mayo et al., 1986; Vancura et al. 1989; Garbaye, et al., 1990; Varese et al., 1996). The occurrence of bacteria within mantle hyphae of ectomycorrhizae, arbutoid mycorrhizae and spores of AM fungi, has been reported in ultrastructural studies (Buscot, 1994; Filippi et al., 1995; MacDonald and Chandler, 1981; Walley and Germida, 1996).

Nevertheless, little is known about the resident microbial populations of AM fungal hyphae and spores, which are raised in pot-cultures and used world-wide in mycorrhizal experiments. Accordingly, this work was performed with the aims of: i) determining the quantity of microorganisms occurring in the sporocarps of *Glomus mosseae*; ii) studying ultrastructural spatial distribution of mycorrhizospheric microbes inside the sporocarps.

2. Materials and Methods

Plant and fungal material

The AM fungal species used, *Glomus mosseae* (Nicol. and Gerd.) Gerdeman and Trappe (Kent isolate), kindly provided by Dr. Barbara Mosse in 1980, has been produced and maintained for 17 years in the pot-culture collection of the Istituto di Microbiologia Agraria, University of Pisa, Italy. Pot-cultures of *G. mosseae* were produced and maintained in association with *Fragaria vesca* L. Voucher specimen of the isolate was deposited in the herbarium of the Dipartimento di Scienze Botaniche, University of Pisa, *Herbarium Horti Botanici Pisani* (PI) as PI-HMZ 4. The soil used for pot-cultures was a sandy soil containing 16.4 ppm available P_2O_5 (Olsen), 19.1 ppm available K_2O (ammonium acetate), 0.2% total N (Kjeldahl), 0.56% organic matter and pH (H_2O) was 7.3. The soil was sterilized by autoclaving twice at 121°C for 25 min. to eliminate indigenous AM fungi. The pot-cultures were grown in a glasshouse

with a day-time temperature of 21°C, a night-time temperature of 10°C, and a maximum light intensity of 30.7 klx. They were watered daily by an automatic device.

Transmission electron microscopy (TEM)

Mycorrhizal roots and sporocarps of *G. mosseae* were recovered from pot-cultures by the wet-sieving and decanting method (Gerdemann and Nicolson, 1963) down to a mesh size of 50 μm; 50 sporocarps were collected from the wet-sieved material and utilised for TEM studies. The sporocarps were washed twice in 0.05 M phosphate buffer, pH 6.8, and fixed in 3% glutaraldehyde in the same buffer, for 4 h at 4°C. They were rinsed several times in the same buffer and postfixed in 1% Osmium tetroxide overnight at room temperature. The sporocarps were then dehydrated and embedded as described in Filippi et al. (1995). Ultrathin sections were cut by using a diamond knife, mounted on a Reichert UK 12 ultramicrotome, stained with uranyl acetate followed by lead citrate, and observed under a Siemens Elmiskop IA electron microscope. Semithin sections were cut as described, and stained according to Glavert (1965).

Mycorrhizospheric microbes associated with Glomus mosseae sporocarps

Quantitative analyses were carried out on microorganisms occurring on the surface and in the homogenate of *G. mosseae* sporocarps.

Sporocarp surface

Sporocarps of *G. mosseae* (100) were collected from the wet-sieved material, suspended in 100 ml of sterile distilled water (SDW) and shaken for 15 min in a 150 rpm mechanical shaker. Serial dilutions were prepared from the rinsing water, and analyses of total number of bacteria and fungi were carried out. Bacteria, fungi and actinomycetes populations were assayed by standard microbial counts, on the following media: total heterotrophic bacteria on Nutrient agar (Difco, Detroit, USA), total fungi on PDA agar (Difco), amended with 100 μg ml^{-1} rifampicin and total actinomycetes on Waksman agar (Casitone 5 g/l, NaCl 5 g/l, glucose 10 g/l, beef extract 3 g/l, agar 20 g/l). Chitinolytic microorganisms were assayed by using chitin agar (Lingappa and Lockwood, 1962; Ames et al., 1984).

Sporocarp homogenate

The same sporocarps used for the previous analyses were washed in SDW (13 times), then collected in 1 ml SDW, and homogenized using sterile pestle and

mortar. The homogenate was then recovered, suspended in SDW and processed as previously described. Sample dry weights were determined by calculating percentage of moisture from the fresh weight material, recovered by filtration on tared weighing dishes, after drying (48 h, 80°C) until its weight was constant.

3. Results

Ultrastructural studies

Transmission electron microscopy revealed multiform bacterial cells inside the sporocarps. Detailed ultrastructural studies evidenced bacteria in the interhyphal spaces of the sporocarps, occupying the microniches formed by the peridial hyphae interwoven around spores (Figs. 1, 2). Sequential cuts of the sporocarps showed that bacteria were attached to the outer hyaline wall layer of the spores, when this layer was present (Fig. 1, C, D). Many morphologically similar bacteria were observed adhering to hyphal walls (Fig. 2, A, C, D). It is interesting to note the finding of some germinating bacterial spores inside the sporocarps (Fig. 1, E).

Sections of the fungal spores showed bacterial forms within the outer layer of the multilayer electron-dense wall, which were found in holes probably caused by their own lytic activity (Fig. 3). In fact, small vesicles were observed in many spore walls, suggesting an enzymatic activity by the bacteria. The wall-degrading bacteria did never penetrate into the inner part of the electron-dense wall layer. Similar bacterial cells were detected also on hyphae, actively degrading the walls (Fig. 4).

Mycorrhizospheric microbes associated with the sporocarps of Glomus mosseae

The numbers of microorganisms isolated from sporocarps are reported in Table 1. The quantities of fungi, bacteria and actinomycetes in sporocarp homogenate were comparable with those obtained from sporocarp surface.

Chitinolytic microorganisms ranged from 3.7×10^7 cfu/g dry matter on sporocarp surface to 5.4×10^7 cfu/g dry matter in sporocarp homogenate. Chitinolytic bacteria were highly represented in sporocarp homogenate, where their number increased up to 57% of the total chitinolytic microorganisms (Table 2).

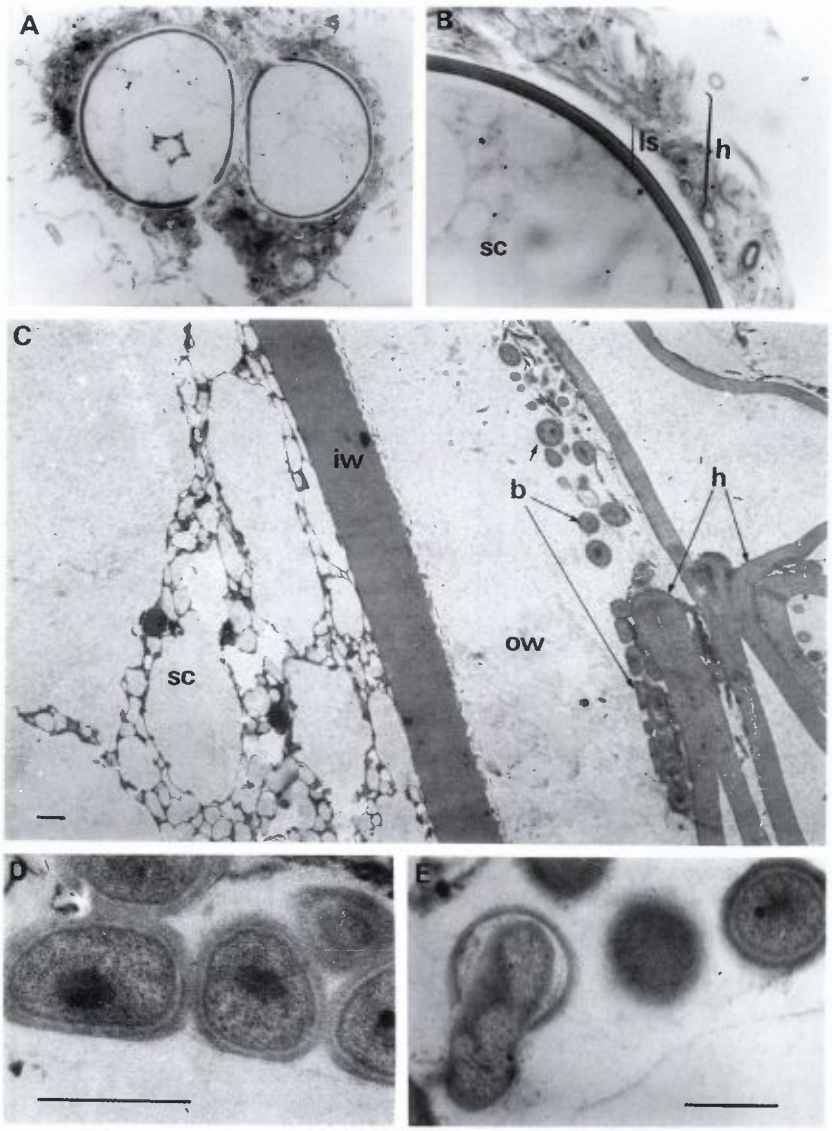

Figure 1. Light micrographs and transmission electron micrographs of *Glomus mosseae* sporocarps. (A) semi-thin section showing the network of interwoven hyphae around two spores; (B) semi-thin section showing spore cytoplasm (sc), different spore wall layers (ls), hyphae surrounding a spore (h); TEM micrographs showing: (C) spore cytoplasm (sc), the inner electron-dense spore wall layer (iw), the outer electron-transparent spore wall layer (ow), bacteria (b) occurring between the spore wall and the peridial hyphae (h); (D, E) high magnifications showing bacteria adhering to the outer spore wall layer (D) and the typical germination of a bacterial spore (E). Bars = 1 μm.

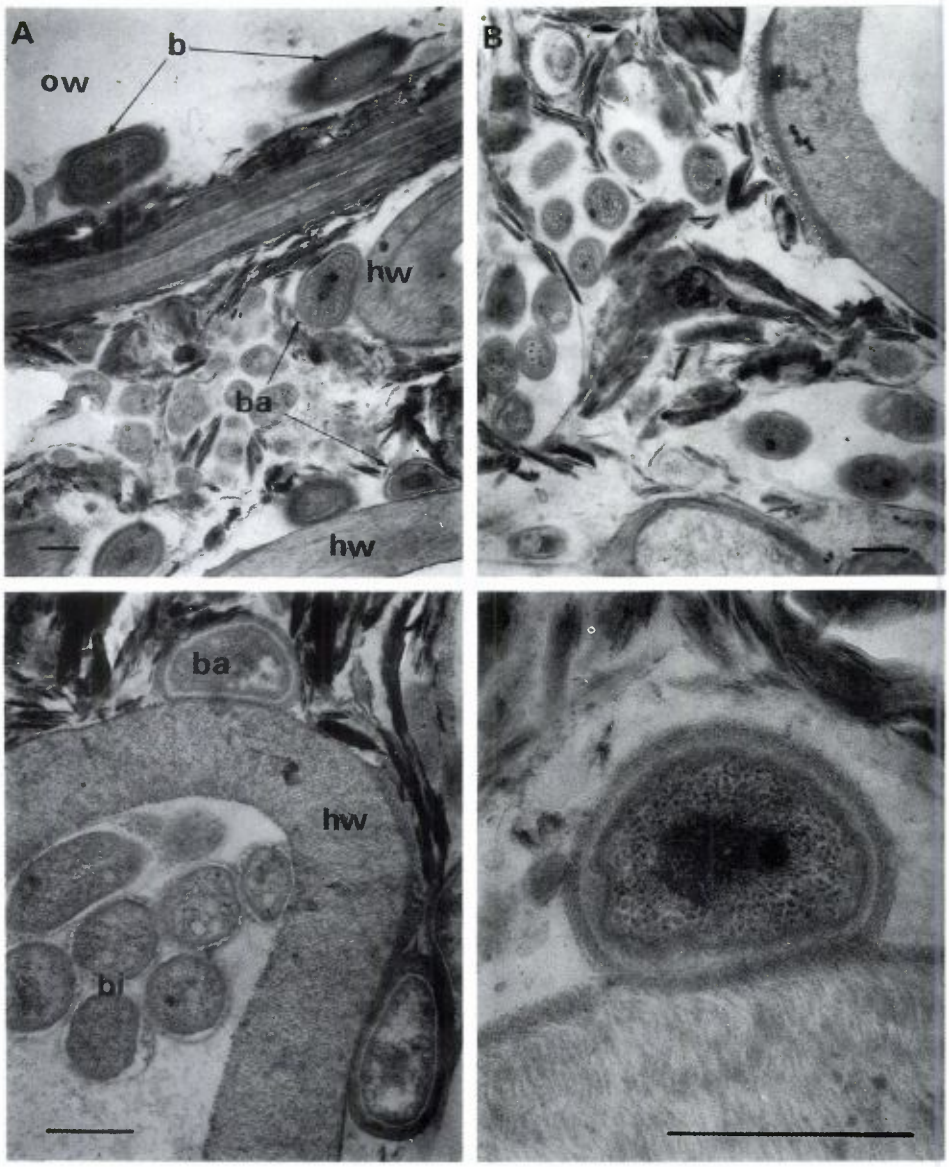

Figure 2. Transmission electron micrographs of *Glomus mosseae* sporocarps showing: (A) bacteria (ba) adhering to the hyphal wall (hw) and bacteria (b) adhering to the outer electron-transparent spore wall layer (ow); (B) morphologically similar bacterial cells in microniches formed within peridial spaces; (C) bacteria (bi) colonizing a dead hypha; (D) a bacterial cell adhering to an hyphal wall. Bars = 1 μm.

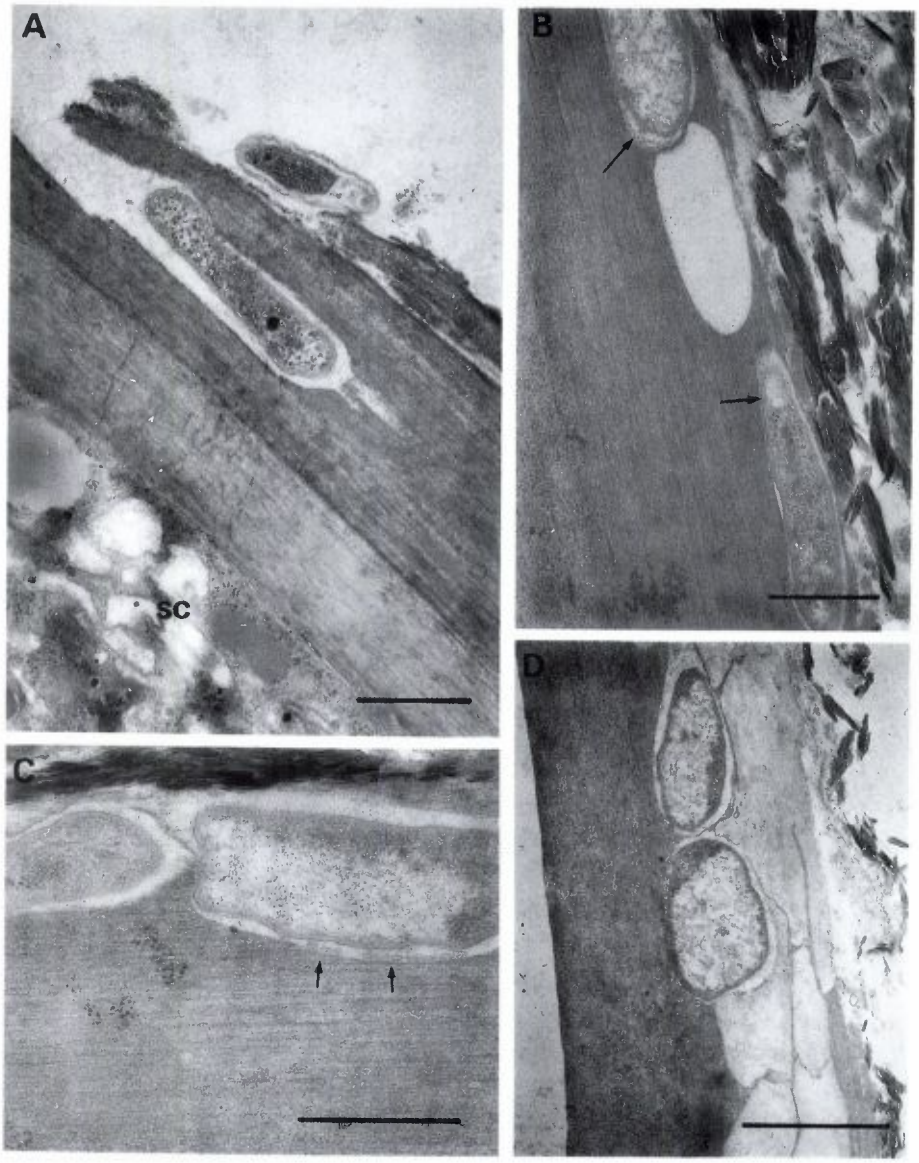

Figure 3. Transmission electron micrographs showing bacteria embedded in the outer electron-dense spore wall layer of *Glomus mosseae*; arrows (B, C) indicate putative digestive vesicles. Bars = 1 μm.

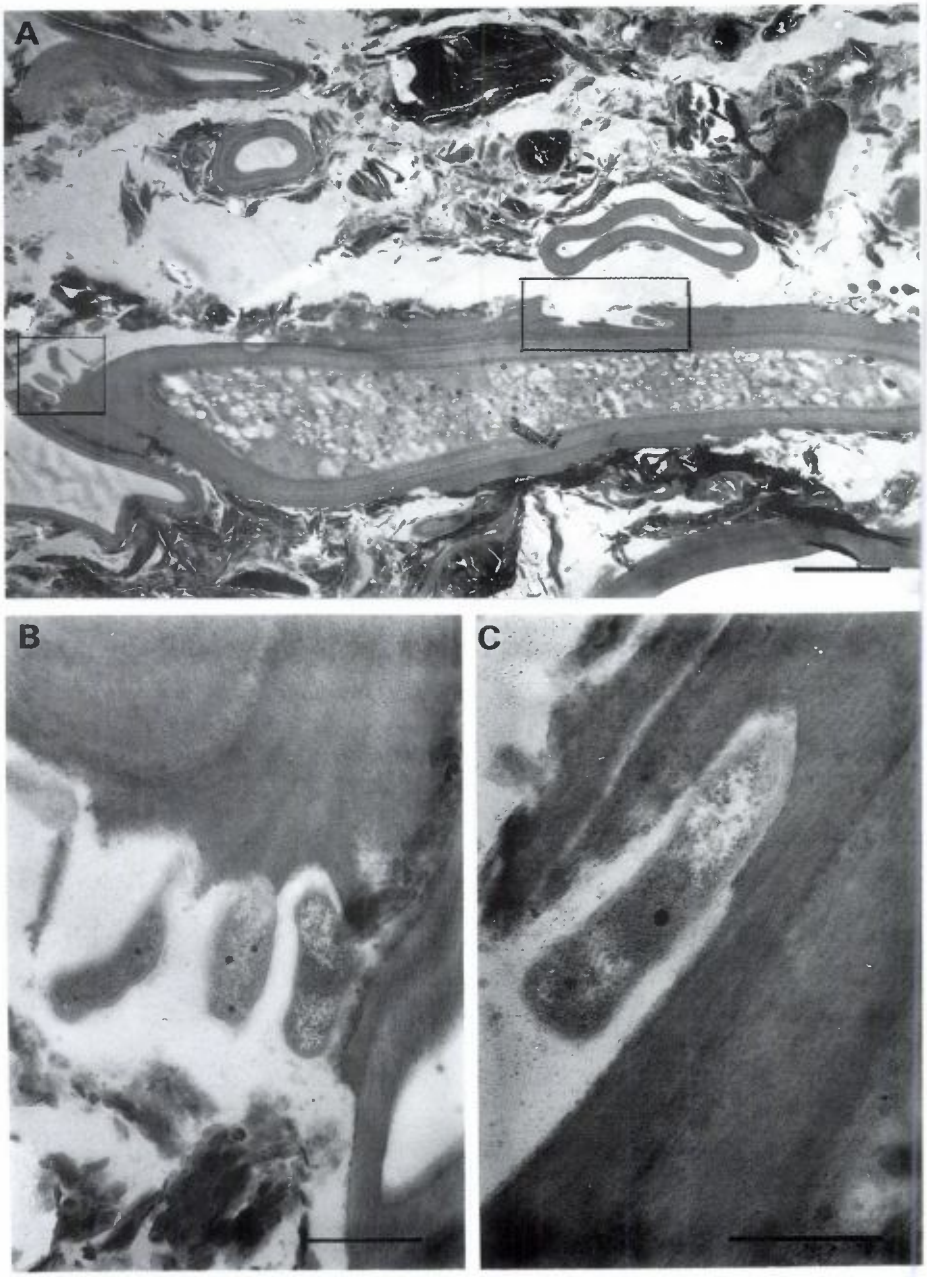

Figure 4. Transmission electron micrographs of peridial hyphae of *Glomus mosseae* sporocarps showing: (A) a living hypha surrounded by many bacteria degrading the outer electron-dense wall layer (bar = 10 µm); (B, C) enlargements of parts of (A) (bars = 1 µm).

Table 1. Numbers of microorganisms (bacteria, actinomycetes and fungi) on sporocarp surface (SS) and in sporocarp homogenate (SH) of *Glomus mosseae* grown in strawberry pot-cultures

(cfu/g dry matter)	Sporocarps surface	Sporocarp homogenate
Bacteria	$1.2 \times 10^8 \pm 1.9 \times 10^7$	$6.1 \times 10^8 \pm 1.1 \times 10^8$
Actinomycetes	$1.6 \times 10^6 \pm 1.2 \times 10^6$	$4.4 \times 10^6 \pm 1.8 \times 10^6$
Fungi	$4.1 \times 10^5 \pm 8.3 \times 10^4$	$1.0 \times 10^6 \pm 4.7 \times 10^5$

Means are followed by the standard errors.

Table 2. Chitinolytic microorganisms occurring on the surface and homogenate of *Glomus mosseae* sporocarps

	Sporocarp surface	Sporocarp homogenate
Total number (cfu/g dry matter)	3.7×10^7	5.4×10^7
% Bacteria	7.88	57.13 *
% Actinomycetes	80.49	15.79 *
% Fungi	4.56	28.07 *

Means were calculated after angular transformation. *Indicate values in the rows significantly different at $P<0.01$.

4. Discussion

The results of this work show that the sporocarps of *G. mosseae* harbor a high number of microorganisms, which occur in microniches formed by peridial hyphae and spores, and within the electron-dense spore wall layers.

Our TEM studies showed the occurrence of many bacteria on the spore surface and in the interhyphal spaces of the sporocarps, which probably provide a rich environment for their establishment and metabolic maintenance. Bacterial cells, which we found intimately associated with *G. mosseae* spore walls, have been reported to occur also in ecto- and arbutoid mycorrhizae (Buscot, 1994; Filippi et al., 1995; Varese et al., 1996). The occurrence of bacterial cells, in the electron-dense wall of *G. mosseae* spores and hyphae, probably responsible of the lytic activity observed in the wall, confirms recent results obtained by Walley and Germida (1996). They observed bacteria and microbial activity on the outer spore wall of *Glomus clarum* spores: such bacteria were so intimately associated with the wall that they could not be removed even by using 10% concentration of the sterilizing agent for up to 120 min decontamination time.

Our TEM results are in agreement with such observations and show that the bacterial cells occurring within the sporocarps of *G. mosseae* are able to make tunnels while embedded in the wall. These results may explain the phenomenon, often reported in taxonomical descriptions, of the "disappearance" of the outer hyaline spore wall layer in *G. mosseae* (Walker, 1983; Morton, 1988). This layer is composed mainly of chitin (Sbrana et al., 1995), which is degraded by common mycorrhizospheric microbes.

Recently Bianciotto et al. (1996) showed that the cytoplasm of the AM fungus *Gigaspora margarita* harbors a further bacterial symbiont belonging to the genus *Burkholderia*, though its functional significance remains unknown. On the other hand, the occurrence of different taxa of microbes, positively interacting with the plants, was reported by other authors, who isolated nitrogen fixing bacteria from sporocarps of ectomycorrhizal fungi, from spores of *Glomus* species and from arbutoid mycorrhizas (Li and Castellano, 1987; Tilak et al., 1989; Filippi et al., 1995).

Many authors reported that microorganisms can both stimulate and inhibit or delay AM fungal spore germination (Mosse 1959; Daniels and Trappe, 1980; Paulitz and Linderman, 1989; Azcon-Aguilar and Barea, 1992). Accordingly, microbes living in intimate contact with spores and degrading their outer wall layer, such as those described in this paper, could strongly aid spore germination of AM fungi. Indeed, the occurrence of undetected microbes associated with AM fungal spores might account for their erratic germination (Giovannetti, Avio and Salutini, 1991).

Concerning microbes associated with *G. mosseae* sporocarps, our hypothesis is that the quantity and quality of hyphal exudates and/or metabolic products may play a fundamental role in the selection of the resident microbial populations. These microorganisms should be considered a stable component of the mycorrhizosphere and should be taken into account when studying the extent of biodiversity in AM fungi, including infectivity and effectiveness of different isolates.

In conclusion, a better understanding of the relationships between mycorrhizal symbionts and mycorrhizosphere microorganisms could help to unravel the peculiar role of each group of organisms on the growth, survival and fitness of crop plants, and to optimize the management of the delivery of mycorrhizospheric microbial populations into agroecosystems.

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REFERENCES

Ames, R.N., Reid, C.P.P., and Ingham, E.R. 1984. Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. *The New Phytologist* **96**: 555–563.

Azcon-Aguilar, C. and Barea, J.M. 1992. Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: *Mycorrhizal Functioning. An Integrative Plant-Fungal Process.* M.F. Allen, ed. Chapman & Hall, New York, London, pp. 163–198.

Azcon-Aguilar, C. and Barea, J.M. 1996. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens – an overview of the mechanisms involved. *Mycorrhiza* **6**: 457–464.

Bansal, M. and Mukerji, K.G. 1994. Positive correlation between VAM-induced changes in root exudation and mycorrhizosphere mycoflora. *Mycorrhiza* **5**: 39–44.

Bianciotto, V., Bandi, C., Minerdi, D., Sironi, M., Volker Tichi, H., and Bonfante, P. 1996. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. *Applied and Environmental Microbiology* **62**: 3005–3010.

Buscot, F. 1994. Ectomycorrhizal types and endobacteria associated with ectomycorrhizas of *Morchella elata* (Fr.) Boudier with *Picea abies* (L.) Korst. *Mycorrhiza* **4**: 223–232.

Catskà, V. 1994. Interrelationships between vesicular-arbuscular mycorrhiza and rhizosphere microflora in apple replant disease. *Biologia Plantarum* **36**: 99–104.

Citernesi, A.S., Fortuna, P., Filippi, C., Bagnoli, G., and Giovannetti, M. 1996. The occurrence of antagonistic bacteria in *Glomus mosseae* pot-cultures. *Agronomie* **16**: 563–571.

Daniels, B.A. and Trappe, J.M. 1980. Factors affecting spore germination of the vesicular-arbuscular mycorrhizal fungus *Glomus epigaeus*. *Mycologia* **72**: 457–471.

Filippi, C., Bagnoli, G., and Giovannetti, M. 1995. Bacteria associated to arbutoid mycorrhizae in *Arbutus unedo* L. *Symbiosis* **18**: 57–68.

Forster, S.M. and Nicolson, T.H. 1981. Microbial aggregation of sand in a maritime dune succession. *Soil Biology and Biochemistry* **13**: 205–208.

Garbaye, J. 1991. Biological interactions in the mycorrhizosphere. *Experentia* **47**: 370–375.

Garbaye, J., Duponnois, R., and Wahl, J.L. 1990. The bacteria associated with *Laccaria laccata* ectomycorrhizas and sporocarps: the effect on symbiosis establishment on Douglas fir. *Symbiosis* **9**: 267–273.

Gerdemann, J.W. and Nicolson, T.H. 1963. Spores of mycorrhizal Endogone species extracted from soil by wet-sieving and decanting. *Transactions of the British Mycological Society* **46**: 235–244.

Giovannetti, M., Avio, L., and Salutini, L. 1991. Morphological, cytochemical, and ontogenetic characteristics of a new species of a vesicular-arbuscular mycorrhizal fungus. *Canadian Journal of Botany* **69**:161–167.

Glavert, A.M. 1965. Section staining, cytology, autoradiography and immunochemistry for biological specimens. In: *Techniques for Electron Microscopy*. D. Kay, ed., BSP, Oxford, pp. 254–310.

Li, C.Y. and Castellano, M.A. 1987. *Azospirillum* isolated from within sporocarps of the mycorrhizal fungi *Hebeloma crustuliniforme, Laccaria laccata* and *Rhizopogon vinicolor*. *Transactions of the British Mycological Society* **88**: 563–565.

Linderman, R.G. 1988. Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizosphere effect. *Phytopathology* **78**: 366–371.

Lingappa, Y. and Lockwood, J.L. 1962. Chitin media for selective isolation and culture of actinomycetes. *Phytopathology* **52**: 317–323.

Ho, I. 1988. Interaction between VA-mycorrhizal fungus and *Azotobacter* and their combined effects on growth of tall fescue. *Plant and Soil* **105**: 291–293.

MacDonald, R.M. and Chandler, M.R. 1981. Bacterium-like organelles in the vesicular-arbuscular mycorrhizal fungus *Glomus caledonium*. *The New Phytologist* **89**: 241–246.

Mayo, K., Davis, R.E., and Motta, J. 1986. Stimulation of germination of spores of *Glomus versiforme* by spore associated bacteria. *Mycologia* **78**: 426–431.

Meyer, J.R. and Linderman, R.G. 1986. Selective influence on population of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. *Soil Biology and Biochemistry* **18**: 191–196.

Morton, J.B. 1988. Taxonomy of vesicular-arbuscular mycorrhizal fungi: classification nomenclature, and identification. *Mycotaxon* **32**: 267–324.

Mosse, B. 1959. The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. *Transactions of the British Mycological Society* **42**: 273–286.

Paulitz, T.C. and Linderman, R.G. 1989. Interactions between fluorescent *Pseudomonas* and VA mycorrhizal fungi. *The New Phytologist* **113**: 37–45.

Sbrana, C., Avio, L., and Giovannetti, M. 1995. The occurrence of calcofluor and lectin binding polysaccharides in the outer wall of arbuscular mycorrhizal fungal spores. *Mycological Research* **99**: 1249–1252.

Secilia, J. and Bagyaraj, D.J. 1987. Bacteria and actinomycetes associated with pot-cultures of vesicular-arbuscular mycorrhizas. *Canadian Journal of Microbiology* **33**: 1069–1073.

Tilak, K.V.B.R., Li, C.I., and Ho, I. 1989. Occurrence of nitrogen fixing *Azospirillum* in vesicular-arbuscular mycorrhizal fungi. *Plant and Soil* **116**: 286–288.

Tisdall, J.M. 1991. Fungal hyphae and structural stability of soil. *Australian Journal of Soil Research* **29**: 729–743.

Vancura, V., Orozco, M.O., Grauova, O., and Prikryl, Z. 1989. Properties of bacteria in the hyphosphere of a vesicular-arbuscular mycorrhizal fungus. *Agricultural Ecosystem Environment* **29**: 421–427.

Varese, G.C., Portinaro, S., Trotta, A., Scannerini, S., Luppi-Mosca, A.M., and Martinotti, M.G. 1996. Bacteria associated with *Suillus grevillei* sporocarps and ectomycorrhizae and their effects on in vitro growth of the mycobiont. *Symbiosis* **21**: 129–147.

Walley, F.L. and Germida, J.J. 1996. Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria. *Mycorrhiza* **6**: 43–49.

Walker, C. 1983. Taxonomic concepts in *Endogonaceae*: spore wall characteristics in species descriptions. *Mycotaxon* **18**: 443–455.