

## Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria

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Dedicated to Prof. Dr. Dr. h.c. Eberhard Schnepf on the occasion of his retirement

**Summary.** We have investigated whether direct physical interactions occur between arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPRs), some of which are used as biocontrol agents. Attachment of rhizobia and pseudomonads to the spores and fungal mycelium of *Gigaspora margarita* has been assessed in vitro and visualized by a combination of electron and confocal microscopy. The results showed that both rhizobia and pseudomonads adhere to spores and hyphae of AM fungi germinated under sterile conditions, although the degree of attachment depended upon the strain. *Pseudomonas fluorescens* strain WCS 365 and *Rhizobium leguminosarum* strains B556 and 3841 were the most effective colonizers. Extracellular material of bacterial origin containing cellulose produced around the attached bacteria may mediate fungal/bacterial interactions. These results suggest that antagonistic and synergistic interactions between AM fungi and rhizosphere bacteria may be mediated by soluble factors or physical contact. They also support the view that AM fungi are a vehicle for the colonization of plant roots by soil rhizobacteria.

**Keywords:** *Pseudomonas fluorescens*; *Rhizobium leguminosarum*; *Gigaspora margarita*; Cell-to-cell adhesion; Cell surface.

**Abbreviations:** AM arbuscular mycorrhiza; PGPR plant growth promoting rhizobacteria; CBH cellobiohydrolase; DAPG 2,4-(di-acetyl-phloroglucinol; TY triptone-yeast; LB Lauria-Bertani.

### Introduction

The various microorganisms found routinely in the rhizosphere and known to contribute to soil fertility and crop yield include mycorrhizal fungi, free nitrogen-fixing bacteria and other plant growth promoting

rhizobacteria (PGPRs), such as rhizobia and pseudomonads (O'Gara et al. 1994). The arbuscular mycorrhizal (AM) fungi are one of the most important components of the soil microbial community, as they interact symbiotically with the roots of about 80% of all plant species (Bonfante and Perotto 1995). These mycorrhizal symbioses are present in most natural and agricultural ecosystems, where they are involved in many key processes including nutrient cycling, conservation of soil structure, plant health and enhancement of nitrogen fixation from rhizobia (Varma and Hock 1995). In the soil environment, external AM hyphae interact with other soil microorganisms, either directly or indirectly by modifying host physiology and the pattern of root exudation (Azcon-Aguilar and Barea 1992). On the other hand, the behaviour of mycorrhizal fungi can be influenced by other root symbionts or by PGPRs (Linderman 1992). Because of the importance of beneficial microorganisms in soil ecosystems, a better understanding of the interactions between AM fungi and rhizosphere bacteria, in particular PGPRs, is essential to ensure the safe release of microbial inoculants used as biopesticides. In particular, the release of microbial inoculants whose biocontrol potential involves the production of antifungal compounds (Fenton et al. 1992) may influence mycorrhizal associations.

There is very little information on the cellular interactions between AM fungi and PGPRs, as interest has

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been mostly focussed on the interactions of these fungi with their hosts. The close cell-to-cell interactions observed when AM fungi and rhizobia contact the host plant root are an important prerequisite for the formation of mycorrhizal and nodule endosymbioses, respectively (Bonfante and Perotto 1995, Brewin 1992). Other PGPRs, including pseudomonads, also adhere to the plant root surface, but do not form intracellular associations (Costacurta and Vanderleyden 1995).

The purpose of this paper was to investigate in vitro whether PGPRs attach to the hyphal structures of mycorrhizal fungi. This is the first step in determining whether direct cell-to-cell interactions are important factors in the interactions between these two substantial microbial populations. We have used PGPRs with and without antifungal activity in order to investigate whether such metabolic activities influence their interactions with AM fungi.

## Material and methods

### Fungal material

Sterilized seeds of *Trifolium repens* L. (clover) were sown in sterilized quartz sand. Mycorrhizal plants were obtained by injecting a sterilized suspension of spores of *Gigaspora margarita* Becker & Hall around the seedlings. Spores of *G. margarita* recovered from pot cultures of clover by wet sieving (Gerdemann 1963) were surface sterilized with 4% Chloramine T and 300 ppm streptomycin for 30 min, rinsed 5 times over 1 h with sterile distilled water and left to germinate in distilled water in petri dishes (30 spores per plate) at 30 °C in the dark. 70–80% germination was obtained within 10 days.

### Bacterial strains and culture conditions

The characteristics of the *Rhizobium* and *Pseudomonas* used in this study are listed in Table 1.

The bacteria were grown separately in TY (rhizobia) or LB (pseudomonads) liquid medium overnight at 28 °C with gentle shaking. After centrifugation of the bacterial cell suspension at 3000 g for 20 min, the supernatant was discarded and the pellet resuspended in 15 ml phosphate buffer (50 mM, pH 7.2). The concentration of bacterial cells in the suspension was between 10<sup>7</sup>–10<sup>8</sup> CFU/ml.

### Attachment assay

Germinated spores were incubated with a suspension of either *Rhizobium* or *Pseudomonas* bacteria overnight at 28 °C with gentle shaking. The fungal material was then washed 10 times in phosphate buffer over 1 h. Samples were prepared for observation by scanning and transmission electron microscopy and by confocal microscopy. Four classes of bacterial attachment were recognized: class 1, no attachment; class 2, a few bacteria attached; class 3, bacteria evenly spread on the fungal surface; class 4, thick coat of attached bacteria.

To check that no bacterial contamination occurred before preparation for microscopy, control samples were incubated without bacterial suspensions. The specificity of attachment was checked by incubating bacterial colonies in the presence of other organic and inorganic

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substances, such as cotton and quartz fibres, following the same protocol.

### Scanning electron microscopy

Samples were fixed with 2.5% glutaraldehyde in 100 mM Na-cacodylate buffer (pH 7.2) at room temperature for 2 h. After post-fixation in 1% OsO<sub>4</sub> for 1 h, the specimens were rinsed in buffer, dehydrated through a graded ethanol series followed by acetone, and critical point-dried on an Emitech K850. After drying, the specimens were coated with gold in a Hammer cool sputter coater and examined in a Cambridge Instrument (Stereoscan 120) SEM.

### Confocal microscopy

After incubation with the bacterial cell suspensions followed by extensive washing with phosphate buffer, unfixed spores and mycelia of *G. margarita* were stained with the Live/Dead BacLight Bacteria Viability kit (Molecular Probes Europe, Leiden, The Netherlands) according to the manufacturer's instructions. After incubating samples in the stain for 15 min in the dark (at room temperature), they were immediately observed with a ViewScan DVC-250 (Bio-Rad Microsciences, Ltd, Hemel Hempstead, Herts., U.K.). The BacLight kit contains a proprietary mixture of nucleic acid stains that distinguishes live bacteria with intact plasma membranes, which fluoresce green, from dead bacteria with impaired membranes, which fluoresce red.

### Transmission electron microscopy

Samples fixed and dehydrated as for SEM were embedded in LR White resin (SPI Supplies, West Chester, PA, U.S.A.). Thin sections were counterstained with uranyl acetate and lead citrate, or were treated with silver proteinate (PATAg reaction) to reveal sugar residues (Roland and Vian 1991). To detect cellulose molecules, a cellobiohydrolase (CBH)-colloidal gold complex was prepared and used as described by Balestrini et al. (1994).

## Results

All bacterial strains attached to AM fungal structures though to different extents: all *Rhizobium leguminosarum* strains adhered tightly to the fungal structures of *G. margarita*, whereas the *P. fluorescens* strains displayed a range of behaviours (Table 1).

*R. leguminosarum* strain B556 heavily colonized the fungal surfaces (Figs. 1, 2, and 10). When seen under SEM, many bacterial cells were attached to spores, hyphae and auxiliary cells of *G. margarita* grown in axenic culture (Figs. 1 and 2). They were particularly abundant on the spore wall. Strain 3841 formed a coat around the fungal hyphae, spores and auxiliary cells when observed with confocal microscopy (Figs. 16 and 17). Bacterial cells were sometimes irregularly distributed, producing patches around the hyphae.

Attachment of *P. fluorescens* strains varied considerably. Spores, clusters of auxiliary cells, and hyphae treated with WCS 365 cells were completely coated. The bacteria were often embedded in an amorphous

**Table 1.** Characteristics of bacterial strains

Bacteria	Hosts	Significant characteristics	WT/GMO	Class of attachment <sup>a</sup>
<i>P. fluorescens</i> strain CHA0	tobacco, wheat, others	DAPG/HCN/siderophore	WT	2
<i>P. fluorescens</i> strain WCS 365	potato	good root colonizer	WT	4
<i>P. fluorescens</i> strain F113	sugar beet	DAPG/HCN/siderophore	WT	2
<i>P. fluorescens</i> strain F113G22	sugar beet	DAPG-/HCN/siderophore	GMO	2
<i>Pseudomonas</i> sp. strain M114	sugar beet	HCN/ siderophore	WT	3
<i>R. leguminosarum</i> strain B556	pea	endosymbiont	WT	3
<i>R. leguminosarum</i> strain 3841	pea	endosymbiont	WT	4

<sup>a</sup> Four classes of bacterial attachment were defined: class 1, no attachment; class 2, a few bacteria attached; class 3, bacteria evenly spread on the fungal surface; class 4, thick coat of attachment bacteria

WT Wild type, GMO genetically-modified organism

extracellular matrix (Figs. 3, 4, 11, 12, and 14). By contrast, only a few cells of strain CHA0, which produces the antimicrobial compound DAPG (Table 1), were found on the surface of fungal structures. In Fig. 5, a few bacteria are attached to mature auxiliary cells. The same results were obtained with F113 (Figs. 6 and 15) and F113G22, namely a wild-type strain that produces DAPG and a transposon mutant that does not.

To rule out the possibility that fungal surfaces were already contaminated by other bacteria, spores of *G. margarita* were germinated but not incubated with bacteria. The fungal surfaces were always completely free of any bacterial cells (Figs. 7–9, 13, and 18).

When cotton and quartz fibres were used, comparable results were obtained, i.e., only strains which usually attached to fungal hyphae adhered to the non-living substrata (not shown).

After treatment of fungal and inorganic substrata with the *BacLight* kit, almost all adhering bacteria were shown to be alive (Figs. 10–12, 16, and 17).

TEM observations were performed chiefly on *Pseudomonas* WCS 365. When incubated in the presence of germinating hyphae, which possess thick and irregularly layered walls (Fig. 19), bacteria clustered on the hyphal surface. In some cases, direct contact between fungal and bacterial cells was observed (Fig. 20), whereas in other cases cellular interactions were mediated by extracellular material which reacted strongly with the PATAg reagent for poly-

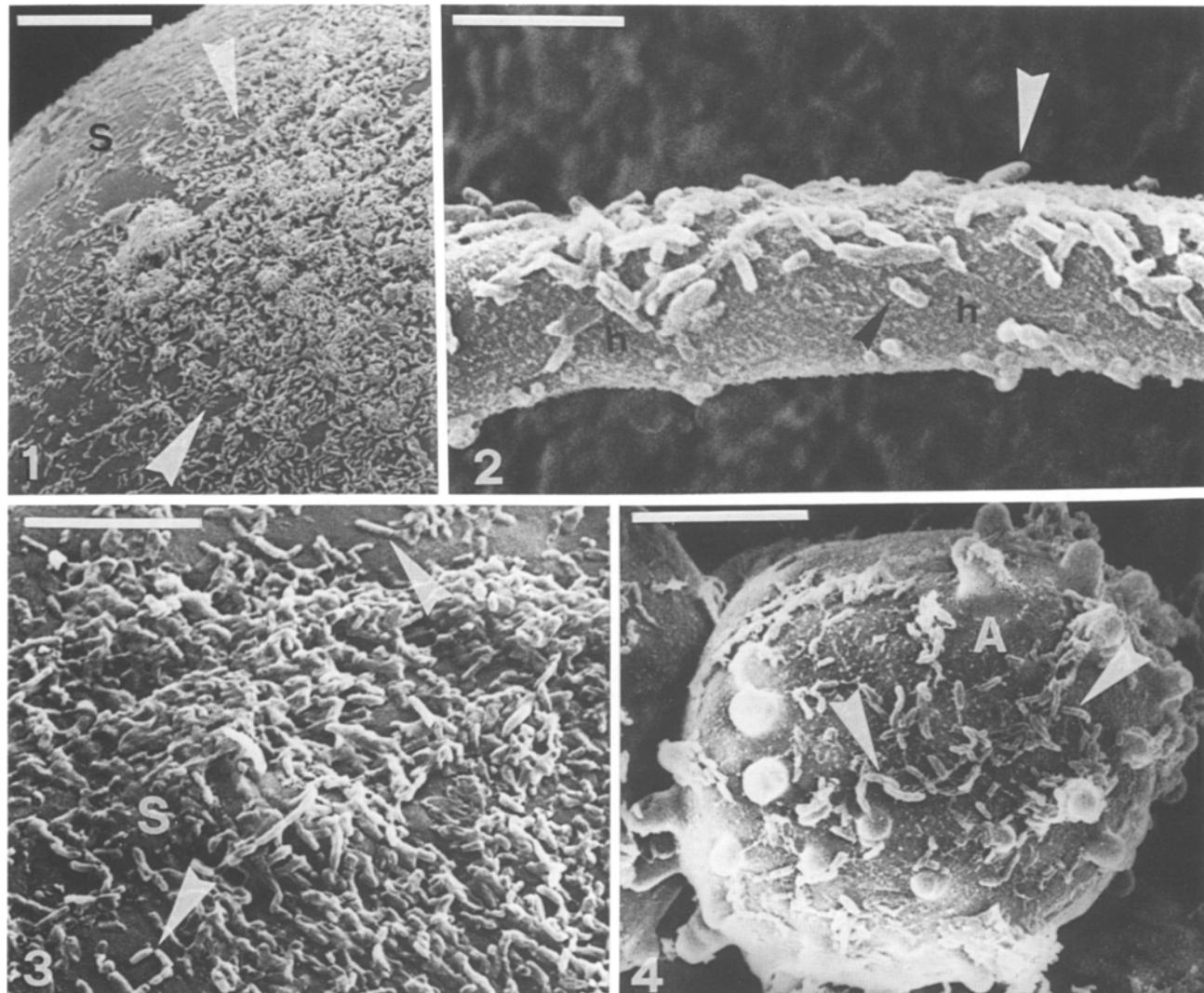
saccharides (Fig. 21). Similar material was also observed when WCS 365 was growing in liquid medium. Extracellular material was regularly labelled by the CBH-gold complex (Fig. 22), indicating the presence of β-1,4 glucans.

## Discussion

The rhizosphere is a narrow zone of soil surrounding the root but the scene of intense microbial activity. In the emerging field of molecular microbial ecology, the dynamics of rhizosphere communities and the interactions between their microbial components are crucial (O'Gara et al. 1994). Our experiments demonstrate that rhizobia and pseudomonads adhere to spores and hyphal structures formed by AM fungi, another important component of the rhizosphere. The density of attached bacteria is largely dependent on their strain. All *R. leguminosarum* strains attached to *G. margarita* spores, hyphae or auxiliary cells. *P. fluorescens* strains displayed greater variability. Strain WCS 365 formed a thick coat on the hyphae. Strain M114 was less efficient, while attachment of CHA0, F113, and F113G22 was very rare.

### *The fungal cell surface as a substratum for soil bacteria*

Soil microorganisms adhere to several organic and inorganic substrata (Bitton and Marshall 1980). They attach to plant surfaces to initiate symbiotic, patho-



**Figs. 1–4.** Scanning electron microscopy of PGPR strains in contact with *Gigaspora margarita*. S Spore, A auxiliary cell, h hypha

**Fig. 1.** The surface of a *G. margarita* spore is coated with bacterial cells of *R. leguminosarum* strain B556 (arrowheads). Bar: 25 µm

**Fig. 2.** Attachment of *R. leguminosarum* strain B556 (arrowheads) to the surface of a fungal hypha. Bar: 5 µm

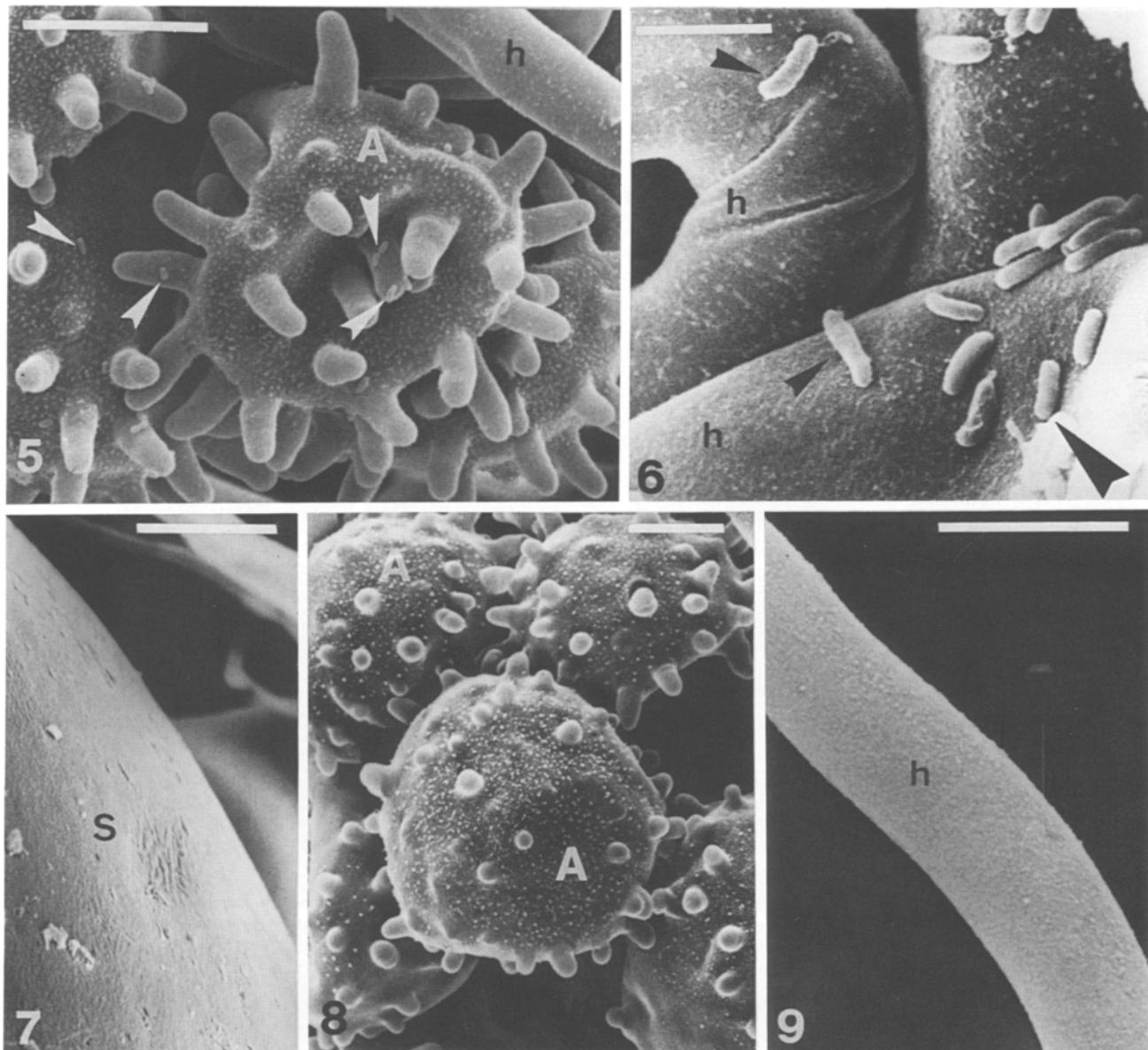
**Fig. 3.** The spore surface of *G. margarita* is completely coated with *P. fluorescens* strain WCS 365 (arrowheads). Bar: 10 µm

**Fig. 4.** An auxiliary cell formed by *G. margarita* and coated with strain WCS 365 bacteria (arrowheads). Bar: 10 µm

genic or saprotrophic interactions, but are also found as biofilms on inorganic surfaces. Adhesion to plants has been studied in detail for *Rhizobium* and *Agrobacterium*, where attachment to the cell surface is one of the earliest events in infection (Kijne et al. 1992, Citovsky et al. 1992). In both cases, a two-step mechanism has been suggested, namely weak binding followed by the strengthening of this attachment. During the first step, bacteria bind to specific receptors (Dazzo 1984) or specific bacterial adhesins are produced (Smit et al. 1987). During the second, firmer binding

is achieved by the production of cellulose fibrils or other bacterial extracellular polymers (Matthysse 1994).

A comparable two-step mechanism has also been demonstrated on inorganic surfaces, though here the first bond is generally electrostatic (van Loosdrecht et al. 1989). In our experiments, PGPRs bound to quartz fibres to the same extent as to AM fungi. Therefore it may be deduced that specific receptors are not involved, and that physicochemical parameters govern attachment to artificial or fungal surfaces. Elec-



**Figs. 5–9.** PGPR strains in contact with *Gigaspora margarita*. SEM. S Spore, A auxiliary cell, h hypha

**Fig. 5.** Mature auxiliary cells of *G. margarita* showing only a few bacteria (*P. fluorescens* strain CHA0) on their surfaces and on the echinulations (arrowheads). Bar: 10 µm

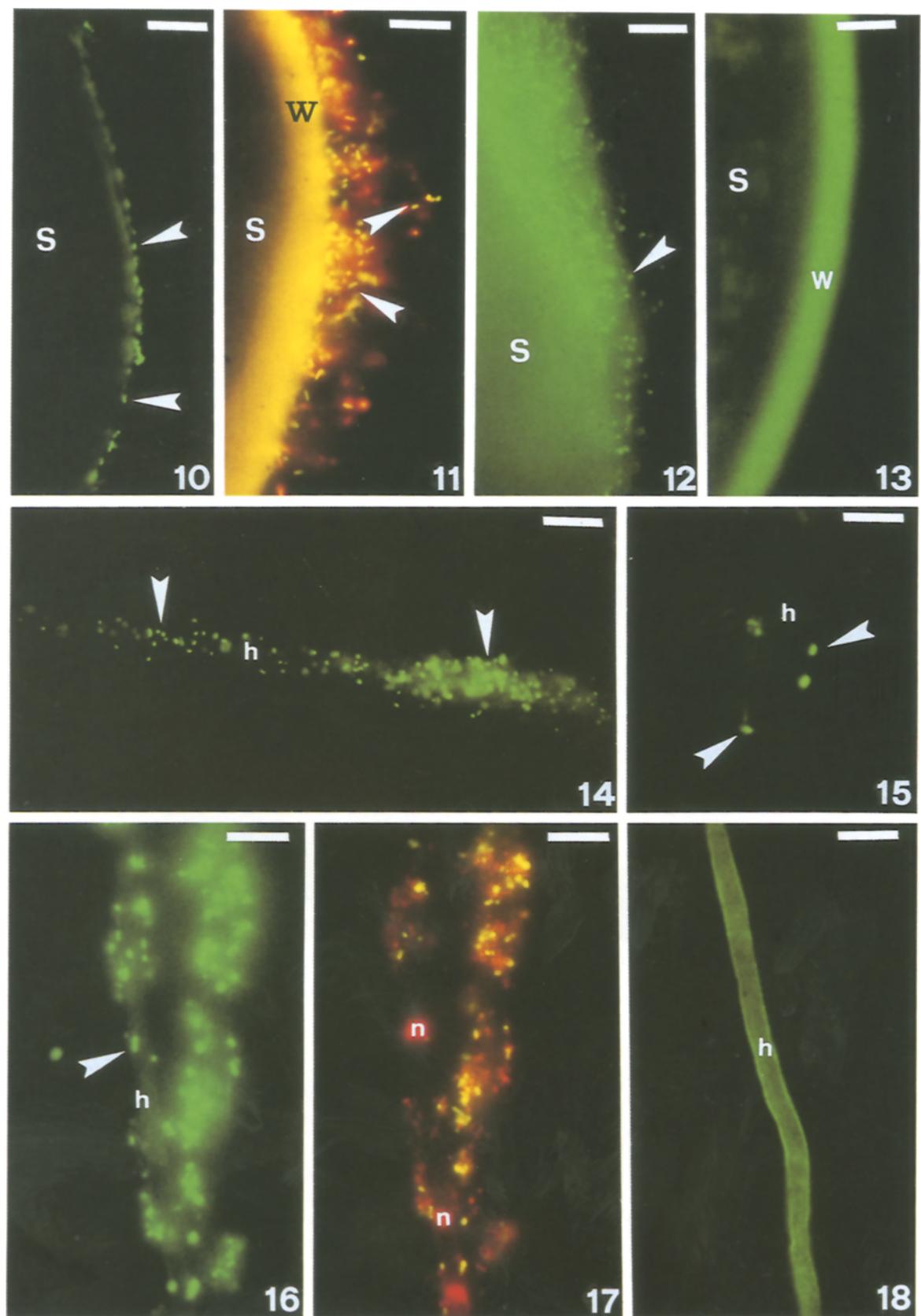
**Fig. 6.** Occasional bacteria (arrowheads) of *P. fluorescens* strain F113 remain attached to the hyphal surface of *G. margarita*. Bar: 2.5 µm

**Figs. 7–9.** As a control, spores were germinated under the same conditions but were not incubated with bacteria. No bacteria are visible on the surfaces of spores (Fig. 7), auxiliary cells (Fig. 8), or hyphae (Fig. 9). Bars: 10 µm

trostatic interactions may play a key role in the early stages of adhesion. It has been shown that electrostatic attraction between quartz grains and bacterial cells are crucial in adsorption (Mills et al. 1994), and that the hydrophobic and charge properties of the bacterial cell surface play an important role in attachment.

Differences in the ability of PGPR strains to bind to substrata may therefore be a consequence of their surface characteristics.

Following electrostatic attraction, specific bacterial cell surface structures may be used to secure more permanent attachment. Cellulose fibrils, which were



detected in the coat of PGPR bacteria surrounding fungal hyphae, may be part of this second step.

#### *The pattern of rhizobacterial adhesion: fungal versus plant surfaces*

The ability of the bacterial strains to adhere to fungal surfaces partly mirrored their attachment to plants. *P. fluorescens* strain WCS 365 attaches to potato roots, and has been isolated as a very good colonizer of their surface (Geels and Schippers 1983). The two strains of *R. leguminosarum* were isolated from nodules, which presupposes their effective attachment to root hairs. *Pseudomonas* strains CHA0 and F113 have been isolated from a soil which naturally suppresses black root rot of tobacco and from the rhizosphere of sugar beet plants, respectively (Défago et al. 1990, Shanahan et al. 1992). The morphological details of root surface colonization are not known.

Adhesion to fungal surfaces took two forms: free bacterial colonies around the hyphae or direct binding to the fungal wall. Distribution along the hypha was often irregular, suggesting that AM fungi are a non-uniform substratum. The reasons for this may be physiological (uneven release of nutrients and exudates along the hypha) or structural (thick cell walls with sloughing layers producing both interstices and warts). Comparable patterns have been described during attachment of PGPRs to the cell walls of ectomycorrhizal fungi and plants. Some *Pseudomonas* strains bind to *Laccaria laccata*, *Suillus bovinus*, and

*Paxillus involutus*, and a role for polar flagella has been suggested (Sen et al. 1994).

Root colonization by PGPRs also depends on their ability to stick to the surface. Ultrastructural observations reveal irregular attachment along the root, depending on the quantity of mucigel and exudates produced by the host. In addition, bacteria may be associated as free colonies in the rhizoplane or in direct contact with the root epidermal cells, thanks to polar attachment (Whiehe et al. 1994, Hoflich et al. 1995).

#### *Rhizobacteria and AM fungi: speculations on their interactions*

Two groups of physiologically distinct rhizobacteria were used in this study. The first includes pseudomonad strains (CHA0 and F113) that produce antifungal molecules, such as DAPG (Dowling and O'Gara 1994), and are used as biocontrol microorganisms. This metabolic feature, however, does not seem to affect attachment, since the adhesion of both the wild-type DAPG-producing strain and the non-DAPG-producing mutant strain F113G22 was equally weak. These data, together with observations by Barea et al. (1994), suggest that bacteria used as biocontrol agents do not affect AM fungi via long- or short-distance mechanisms.

The second group of strains included PGPRs that do not produce antifungal compounds. A synergistic effect on plant growth has often been observed when

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**Figs. 10–18.** The Live/Dead BacLight Viability kit (Molecular Probes) was used to stain bacteria attached to fungal structures of *G. margarita*. Living bacteria fluoresce bright yellow-green under the blue light, while dead bacteria fluoresce red under green light. Bars: Figs. 10 and 12, 25 µm; Figs. 11, 13, and 16–18, 10 µm; Fig. 15, 7 µm

**Fig. 10.** After incubation with *R. leguminosarum* strain B556, the surface of a *G. margarita* spore (S) shows a coating of living bacteria that fluoresce green (arrowheads)

**Fig. 11.** After treatment with a suspension of *P. fluorescens* strain WCS 365, the spore surface is coated with bacteria. Under blue and green light, the thick spore wall (w) shows strong yellow autofluorescence. Most bacteria are alive and fluoresce bright yellow-green (arrowheads). Only a few bacteria are dead and fluoresce red

**Fig. 12.** The same sample as Fig. 11 observed under blue light only. The living bacteria fluoresce green (arrowhead) and are embedded in an amorphous matrix

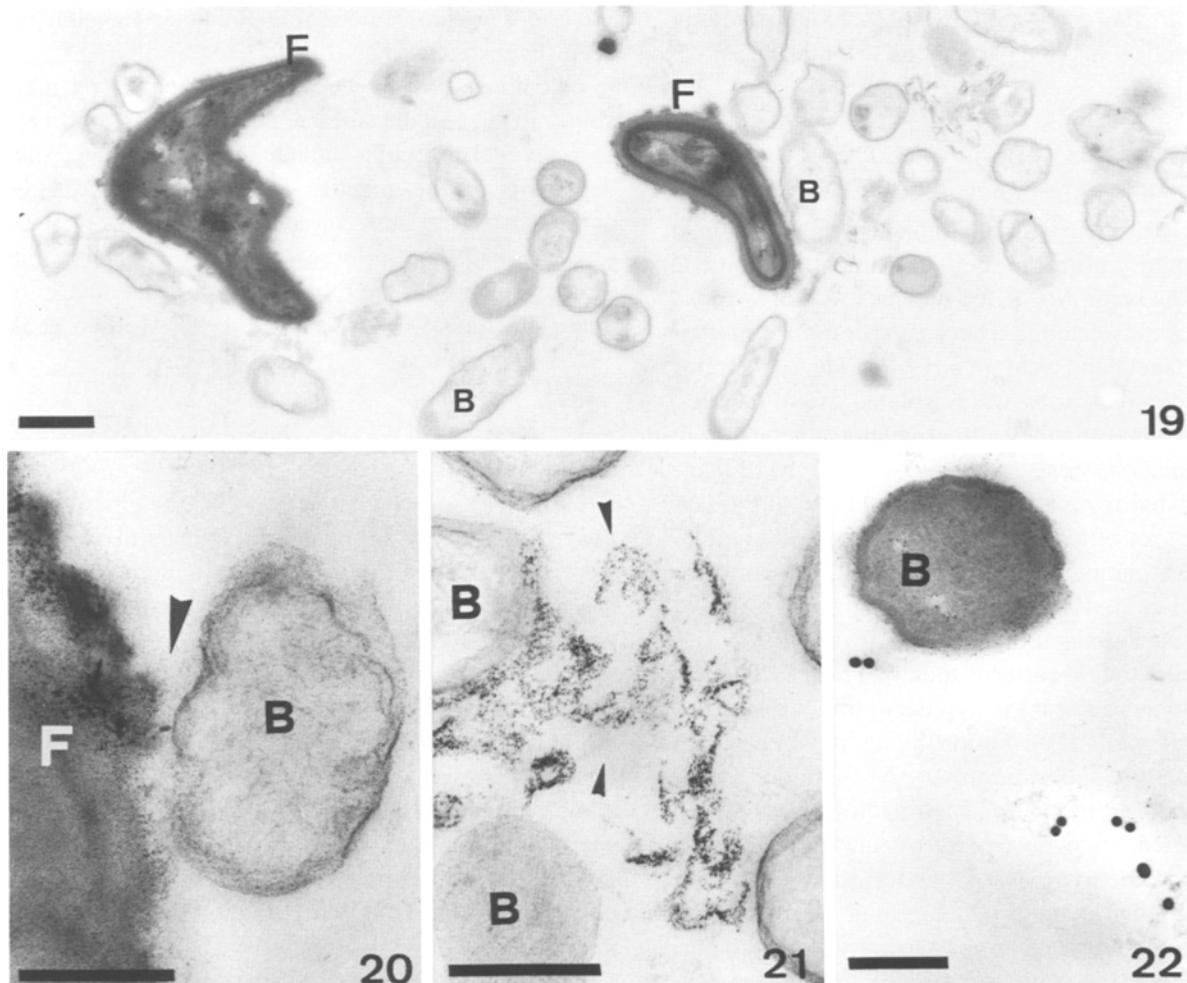
**Fig. 13.** Bacterial cells were never observed on the external surface of the wall (w) of sterilized spores

**Fig. 14.** A germinating hypha is unevenly covered by cells of *P. fluorescens* strain WCS 365

**Fig. 15.** A few living bacterial cells belonging to strain F113 have adhered to a fungal hypha (h)

**Figs. 16 and 17.** Several cells of *R. leguminosarum* strain 3841 cover the external surface of a fungal hypha. Under both blue and green light, living bacteria fluoresce yellow-green whereas dead bacteria fluoresce red. Fungal nuclei (n) distributed along the hyphae fluoresce red

**Fig. 18.** No bacteria are found on germinated mycelium which was not incubated with the bacterial suspensions



Figs. 19–22. Ultrastructural aspects of the attachment of *P. fluorescens* strain WCS 365 to AM fungal hyphae

**Fig. 19.** Hyphae of *G. margarita* (*F*) surrounded by numerous bacterial cells (*B*). Only some of the bacteria are directly in contact with the fungal surface. Bar: 1.5  $\mu\text{m}$

**Fig. 20.** Higher magnification of a bacterial cell which is in contact with the hyphal surface. The arrowhead indicates the irregular surface of the electron-dense fungal wall. Bar: 0.35  $\mu\text{m}$

**Fig. 21.** After staining with the PATAg reaction, which visualizes polysaccharides, some fibrillar material (arrowheads) can be observed around the bacterial cells surrounding the fungal hyphae. Bar: 0.4  $\mu\text{m}$

**Fig. 22.** The extracellular polysaccharides produced by strain WCS 365 are labelled with a CBH-colloidal gold complex, which is specific for  $\beta$ -1,4 glucans. Bar: 0.2  $\mu\text{m}$

PGPRs and AM fungi are inoculated at the same time. Boddey et al. (1991) have suggested that rhizobacteria with colonizing capacities interact with AM fungi by using hyphae as the vehicle for their distribution and/or to enhance colonization. Our results provide a cellular basis for this suggestion, showing that some of these PGPR bacteria adhere en masse to the extra-radical hyphae, probably increasing their mobility along the growing root. This is a further example of

cell-to-cell interactions in the rhizosphere of mycorrhizal plants.

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