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P. Bonfante · R. Balestrini · E. Martino · S. Perotto
C. Plassard · D. Mousain

Morphological analysis of early contacts between pine roots and two ectomycorrhizal *Suillus* strains

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Abstract Selection of ectomycorrhizal strains for application in forestry is mostly based on the evaluation of symbiotic performance in small-scale experiments. Two *Suillus collinitus* strains isolated from a Mediterranean and an alpine area were inoculated onto two pine tree species (*Pinus pinea* and *P. nigra* ssp. *laricio* var. *corsicana*) typical of these two environments. The early events during contact between the cell surfaces of plant and fungal partners were analysed morphologically using ultrastructural and immunocytochemical techniques. All four plant-fungus combinations led to a similar degree of mycorrhizal infection and to a similar colonization pattern. The first contact of fungal hyphae with root cap cells usually involved breakdown of the outermost electron-opaque layer of the plant cell walls. Hyphae further developed between this layer and the underlying wall strata. Ultrastructural observations revealed that *S. collinitus* strain J3-15-24, isolated from a Mediterranean area, induced a defence reaction in the roots of *P. nigra*, which grows typically in alpine areas. These observations suggest functional differences between the two fungal strains in their mycorrhizal capabilities.

Key words *Suillus* · Ectomycorrhizas · Cell surfaces · *Pinus* · Affinity techniques

Introduction

Ectomycorrhizas are an excellent example of plant-fungus interactions in which both partners develop in a controlled fashion, culminating in the formation of a deeply modified root. Morphological aspects of ectomycorrhizal roots have been studied in a number of plant-fungus combinations and recently reviewed by Smith and Read (1997). Major morphogenetic events for the fungal symbiont are (1) the aggregation of hyphae to produce a tissue-like structure (the mantle) covering the root surface and (2) a labyrinthic growth between the epidermal and cortical cells to produce the Hartig net. In the host plant there are changes in the structure of the root meristem (Clowes 1981) and in the shape of the root epidermal cells, which enlarge considerably along the radial axis [see Smith and Read (1997) for a review of ectomycorrhiza development]. Most morphological data have been obtained from the analysis of fully developed ectomycorrhizas and early stages in the establishment of the interaction have rarely been considered (Piché et al. 1983; Lei et al. 1990; Kottke 1997). However, molecular studies have already shown that plant and fungal development is finely regulated during establishment of ectomycorrhizal associations. The expression of mRNA populations and the amounts of some proteins (ectomycorrhizins) change with time after contact, some fungal genes being strongly expressed early in the development of the association [see Martin et al. (1995) for a review]. In particular, fungal cell wall proteins and cell surface molecules have been identified as key molecules in the establishment of symbiosis between *Pisolithus tinctorius* Coker & Couch and *Eucalyptus globulus* subsp. *bicostata* Kirkp (Tagu and Martin 1996). It is, therefore, important to gather more precise morphological information on the first events of fungal contact with the host root. Differences in the behaviour of fungal isolates at these early stages may have consequences for the symbiotic performance at later stages and could be important parameters in the selection of ectomycorrhizal strains with high symbiotic performance for forestry.

We have investigated the first cell-to-cell interactions between two isolates of the ectomycorrhizal species *Suillus collinitus* (Fr.) Kuntze and the roots of pine

P. Bonfante (✉) · R. Balestrini · E. Martino · S. Perotto
Dipartimento di Biologia Vegetale dell'Università and
CSMT-CNR, Viale Mattioli 25, I-10125 Turin, Italy
Fax: +39-011-6707459; e-mail: Bonfante@csmt.to.cnr.it

C. Plassard · D. Mousain
Laboratoire de Recherches sur les Symbiotes des Racines,
INRA ENSA, 21 Place Viala,
F-34060 Montpellier Cedex 1, France

trees. One of the isolates was obtained from a Mediterranean ecosystem and the other was from an alpine area. We studied their early interactions with *Pinus pinea* L. and *P. nigra* Arnold ssp. *laricio* Poiret var. *corsicana*, plant species typical of Mediterranean and mountain regions, respectively. The different possible combinations of plant and inoculant also allowed us to investigate whether strains originating from a specific site show better symbiotic capabilities in association with host plants growing in the same type of environment. Previous studies already demonstrated that the fungal strains used in this study have high symbiotic capabilities (Mousain et al. 1994) but are genetically distinct (Bonfante et al. 1997).

Material and methods

Fungal cultures and mycorrhizal synthesis

Both strains of *S. collinitus* (Fr.) O. Kuntze were isolated from basidiocarps. Strain J3-15-24 originates from a basidiocarp collected under *P. pinea* in a Mediterranean area (La Grande Motte, Hérault, France), whereas J3-15-31 was collected under *P. sylvestris* in an alpine forest (Les Jaussaud, Hautes-Alpes, France). Fungal cultures were maintained on agar nutritive medium, as described in El Karkouri et al. (1996).

The two strains were inoculated on seedlings of *P. pinea* and *P. nigra* ssp. *laricio* var. *corsicana* to check their mycorrhizal phenotype. Mycorrhizal plants of *P. nigra* were obtained in large Petri dishes according to the method described by Wong and Fortin (1989). The same inoculation technique could not be used for *P. pinea* due to the large seedling size and the difficulty in obtaining axenic germination from seeds. Therefore, 3-month-old seedlings were inoculated and grown in 400-ml anti-cooling containers (Thermoflan, Le Vigan, France) as described in Conjeaud et al. (1996). For both plant species, non-inoculated control seedlings were grown in the same conditions. Inoculation experiments with 20 plates of *P. nigra* and 12 containers of*P. pinea*, were replicated in triplicate for each fungus/plant combination.

One month after inoculation, plates with *P. nigra* plants were scanned under the stereomicroscope. Since primary roots were never found to be mycorrhizal, observations were limited to the secondary rootlets. These were scored as (1) non-mycorrhizal, (2) with superficial hyphae or (3) ectomycorrhizal at different developmental stages. Samples from each group of secondary roots were prepared for morphological observation. Mycorrhizal tips as a percentage of total rootlets were determined 2 months after inoculation. The mycorrhizal infection of *P. pinea* seedlings was estimated 4.5 months after inoculation by observing the whole root system under a stereomicroscope and estimating the percentage mycorrhizal apices.

Microscopy and affinity techniques

Fungal cultures, mycorrhizal and non-mycorrhizal rootlets were prepared for light, fluorescence and transmission electron microscopy according to the protocols described in Bonfante and Spanu (1992). All experiments were replicated in triplicate for each material. Thin sections were subjected to the periodic acid, thiocarbohydrazide, Ag proteinate (PATAg) reaction for polysaccharide location. Chitin residues were revealed by fluorescence and electron microscopy using wheat germ agglutinin (WGA) bound to fluorescein isothiocyanate (FITC) and to colloidal gold, respectively (Bonfante and Spanu 1992). The bound lectins were purchased from Sigma Co., St.Louis. The presence of β-1,3-glucans was revealed using a poly-glucopyranose polyclonal antibody binding specifically to these polysaccharides. The antibody was developed by Northcote et al. (1989) and is obtainable from Euromedex, Schiltigheim, France. To demonstrate the presence of chitin and glucans in the same section, double-labelling experiments were performed by successively treating the sections with the anti-polyglucopyranose polyclonal antibody and the WGA-gold complex, as described by Peterson et al. (1996), colloidal gold granules of 15 and 40 nm, respectively.

Fig. 1–6 Ultrastructural features of *Suillus collinitus* growing in pure culture; *bars* 0.4 μm ▶

Fig. 1 Both longitudinal and transverse hyphal cell walls (*w*) of *S. collinitus* J3-15-31 are thin and electron transparent (*d* dolipore septum, *v* vacuole)

Fig. 2 After PATAg reaction, the plasma-membrane (*pl*) and dispersed extracellular material (*arrows*) are stained

Fig. 3 The hyphal walls are labelled after treatment with a WGA-gold complex to detect chitin (*arrows*). Weakly osmiophilic masses (*asterisk*) cover the wall surface

Fig. 4 Immunolocalization of β-1,3-glucans. Labelling is found regularly distributed over the fungal walls (*arrows*) as well as associated with the dolipore septum

Fig. 5 In the double-labelling experiments, gold granules locating chitin (*arrows*) are colocalized with the smaller β-1,3-glucan granules

Fig. 6 The hyphal surface of *S. collinitus* strain J3-15-24 is decorated by weakly osmiophilic masses that covering the whole surface (*asterisk*)

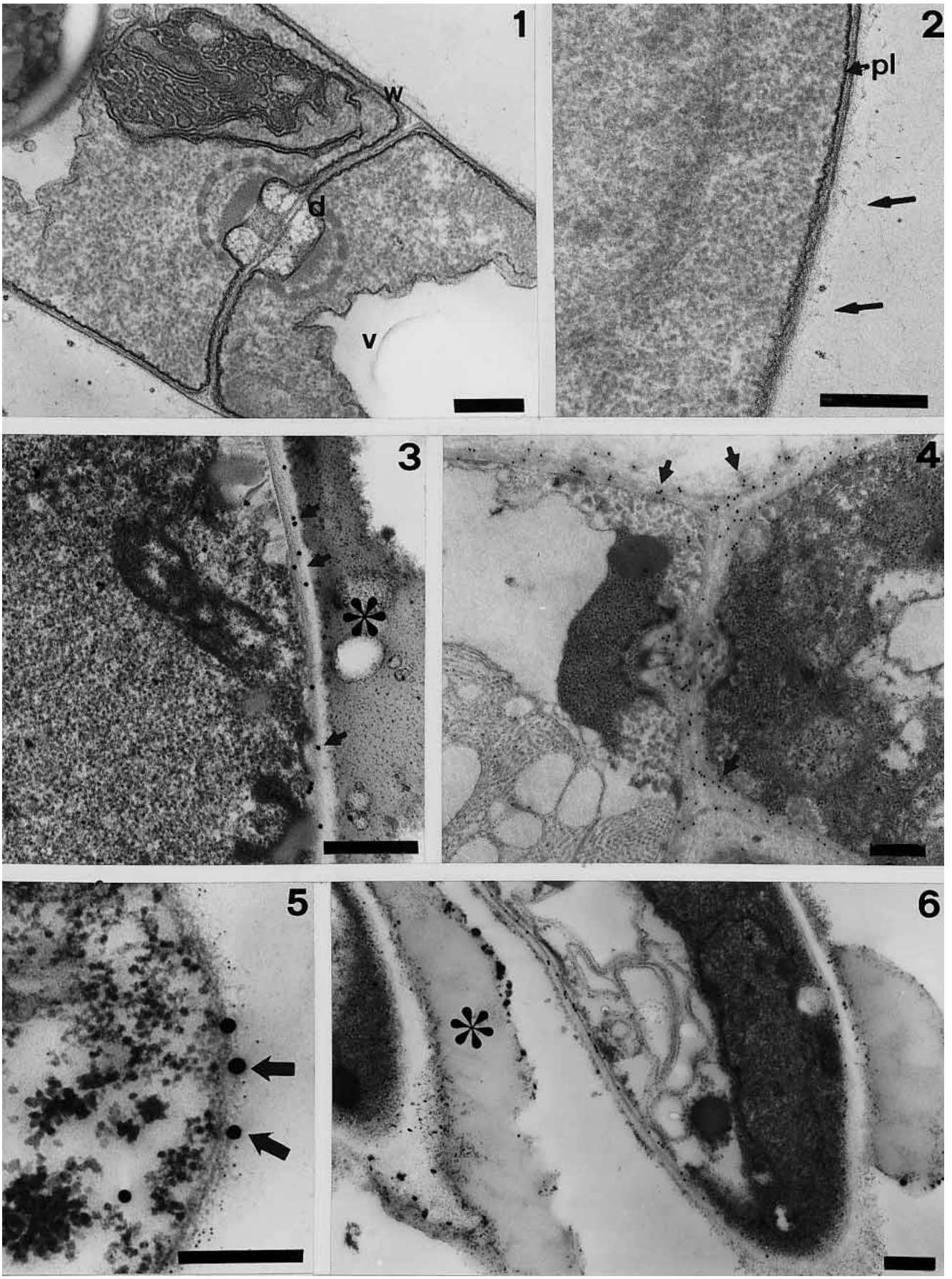
Results

Assessment of mycorrhizal infection

Both strains of *S. collinitus* formed ectomycorrhizal roots with the two pine tree species. Fully developed mycorrhizal roots grown either in vitro or in pot conditions were sampled and evaluated for mycorrhizal infection. No differences were found for the two *Suillus* strains inoculated on *P. pinea* and grown in pots: a mean of 45% mycorrhizal apices was found. Lower values were obtained for roots of *P. nigra* grown on plates. In this growth condition, 25% of the tips were mycorrhizal when the pine was inoculated with *S. collinitus* strain J3-15-31, while only 10% of the tips were mycorrhizal after inoculation with *S. collinitus* strain J3-15-24.

General features of *Suillus collinitus* strains

Ultrastructural analysis of the two *Suillus* strains showed a dense cytoplasm rich in organelles limited to the apical part of the growing hyphae. Both longitudinal and transverse cell walls were usually thin and electron transparent (Figs. 1, 2). The PATAg reaction labelled the plasma membrane and revealed a loose extracellular material (Fig. 2). The hyphae were labelled by WGA-FITC especially on the apex and on the septa, whereas the basal hyphal regions were usually unla-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belled (not shown). In contrast, with the WGA-colloidal gold complex gold granules were distributed along the walls of resin-embedded materials irrespective of the distance from the apex (Fig. 3). Labelling with an antibody which binds β-1,3-glucans was found to be regularly distributed over the fungal walls (Fig. 4) and associated with the dolipore septa. When chitin and β-1,3-glucans were revealed in the same sections by double-labelling experiments, the large gold granules locating chitin co-localized with the smaller granules bound to β-1,3-glucans (Fig. 5).

The most basal part of the hyphae was vacuolated and organelles were located in the peripheral cytoplasm. With aging, the hyphal surface changed its features: the loose extracellular material (Fig. 2) was replaced by weakly electron-opaque masses (Figs. 3, 6), first seen as small droplets, probably lipids, increasing in size until they covered the whole wall surface in the older hyphae. No differences were found between the two fungal strains either in wall architecture or in the presence or distribution of chitin and glucans.

Morphological features of short roots and ectomycorrhizal tips

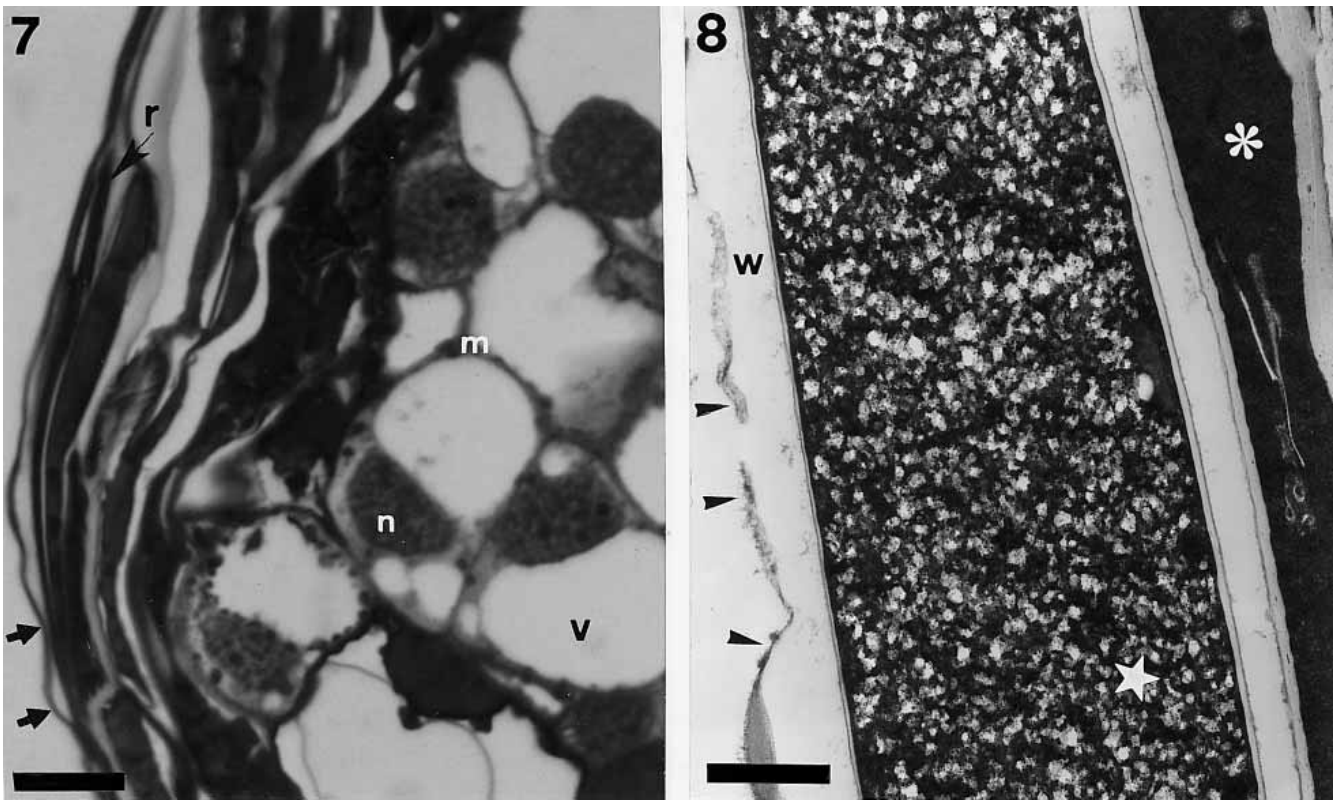
Non-inoculated plants of *P. pinea* and *P. nigra* had short roots with rounder tips, small apical meristems, a differentiated region with 3–4 layers of cells and a poorly developed stele [see Wilcox (1968) for a full description]. The meristematic cells were often vacuolated and covered by root cap cells which were collapsed or filled with black deposits (Fig. 7). At the ultrastructural level, these root cap cells showed either a granular or an homogenous content, and possessed an electron-transparent cell wall lined by a thin electron-dense outer layer (Fig. 8). The latter covered the whole surface of the tip (cap and epidermal cells) and was mostly observed in the roots developing on plates.

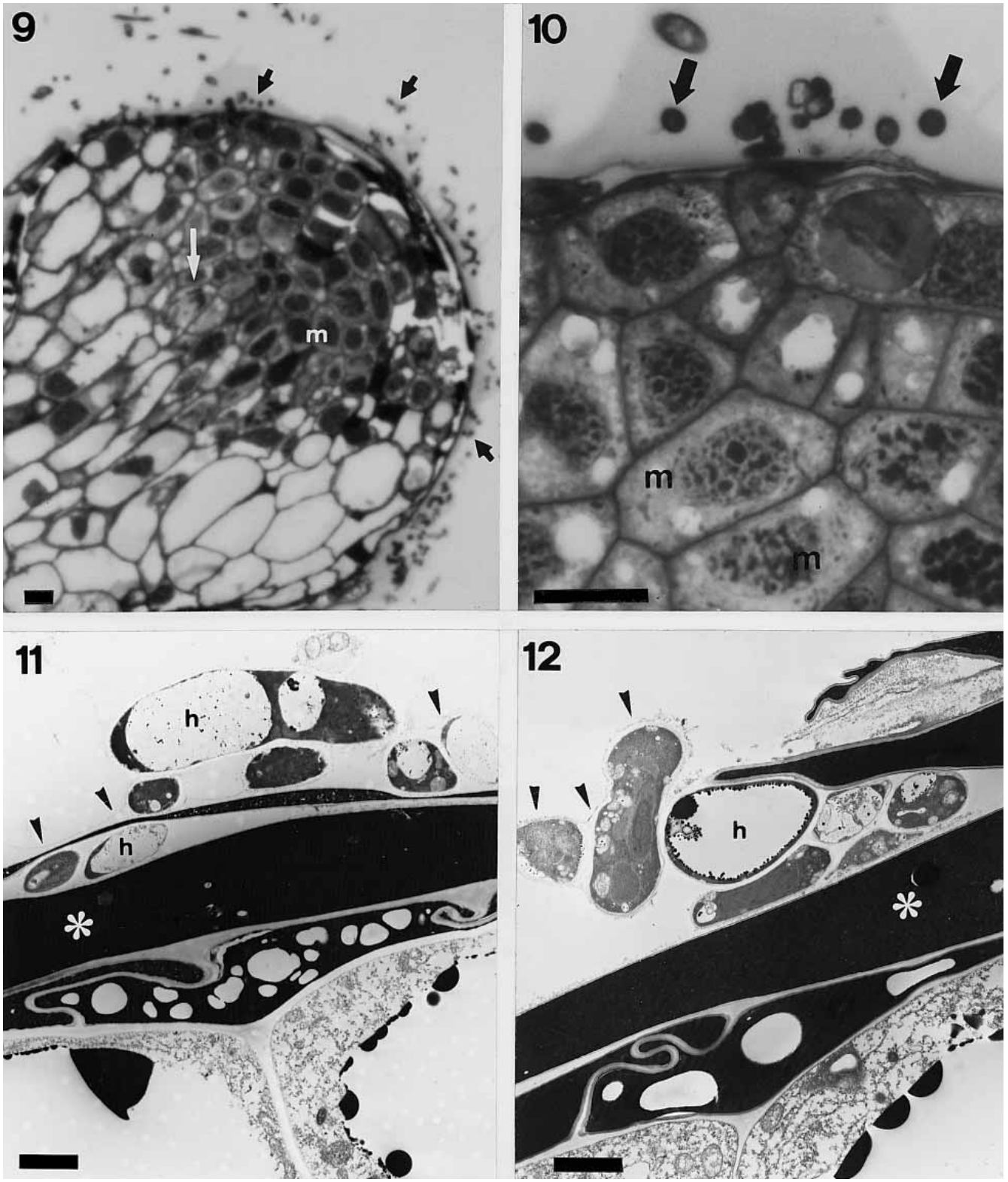
In plants inoculated with *S. collinitus* strains, the presence of the fungus led to the morphological changes (tip swelling, dichotomous branching) well described in the literature (Smith and Read 1997). One month after inoculation, many rootlets showed these features even though hyphae were not detected on the surface. The root surface was similar to that of non-inoculated controls, although the apical meristematic cells were less vacuolated (not shown).

Figs. 7–8 Short roots from non-inoculated plants of *Pinus nigra*

Fig. 7 Detail of a short root with a rounded apex and a small apical meristem containing cells with large vacuoles. The root apex is covered by collapsed cap cells with opaque contents (*r*) and limited by a thin electron-dense layer (*arrows*); *bar* 10 μm (*m* meristematic cells, *n* nucleus, *v* vacuole)

Fig. 8 An electron micrograph of collapsed root cap cells shows either a granular (*star*) or an homogenous (*asterisk*) content and an electron-transparent wall (*w*) lined by a thin electron-dense layer (*arrows*); *bar* 0.4 μm





Figs. 9–12 Roots of *Pinus nigra* inoculated with *S. collinitus* strain J3-15-31

Figs. 9, 10 The rounded tip has a meristem with living and actively dividing cells (*white arrow*). *Black arrows* indicate the external hyphae of *S. collinitus; bars* 20 μm (*m* meristematic cells)

Figs. 11, 12 External hyphae (*h*) of *S. collinitus* contact the sloughing cap cells (*asterisk*) and develop among them. A thin electron-opaque layer (*arrowheads*) covers some hyphae; *bars* 2 μm

The early stages of interaction and the plant-fungal contact area

Hyphae in contact with the root surface were firstly observed at the very tip of the rounded rootlets (Figs. 9, 10). On both pine tree species, *S. collinitus* J3-15-31 and *S. collinitus* J3-15-24 made contact with the sloughing root cap cells and grew among them (Figs. 11, 12). The first contact was established between the thin electron-dense layer lining the outer part of the cap cell walls and the loose extracellular material covering the fungal wall (Fig. 13). Following this first contact, the electron-dense layer appeared broken, with small fragments adhering to the fungal surface (Fig. 14), while the fungal wall came into close contact with the inner part of the host wall. At this stage, loosening in the texture of the fungal wall was observed (Figs. 15, 16), although labelling with WGA allowed the fungal surface to be identified. The thin electron-dense layer became detached from the host surface, and the fungus which developed underneath (Fig. 17) was ultimately seen to occupy the space defined by the electron-dense layer and the inner part of the host cell wall (Fig. 18). Labelling with the polyclonal antibody against β-1,3 glucans allowed the fungal wall to be clearly identified also at this stage.

Following these events, hyphae proliferated, increased in diameter and started to organize into the mantle. When the mycorrhiza became fully organized, the most external hyphae of the mantle were highly vacuolated and often showed extracellular masses similar to those found when the fungus was grown in pure culture (Fig. 6). These morphological features were found in all plant-fungal combinations and in all experimental conditions tested. However, this colonization pattern was observed more rarely in plates in which *P. nigra* was challenged by *S. collinitus* strain J3-15-24; only 10% of the roots were mycorrhizal. Here, hyphae were often seen as isolated patches at the surface of rounded roots, without further development. At the ultrastructural level, the host root cap cells laid down wall deposits as thickenings in the middle of the wall (Figs. 19, 20). These were limited to the contact area with the fungus, appeared amorphous in structure, and were regularly labelled by the antibody raised against β-1,3 glucans (Fig. 20). Thickenings and labelling were not found on the cap cell walls (Fig. 21) in adjacent regions from which the fungus was absent. Likewise, no labelling was found on root cell walls in samples inoculated with *S. collinitus* strain J3-15-31 (Fig. 22).

Figs. 13–18 Rootlets of *P. nigra* inoculated with *S. collinitus* strain J3-15-31. Electron micrographs of the tip region shown in Fig. 9; *bars* 0.2 μm ▶

Fig. 13 First contact between the outer loose layer of the hyphal wall and the electron dense layer lining the wall of a cap cell (*arrowhead*) (*h* fungus)

Fig. 14 Arrows indicate the breakdown of the electron-dense layer and the small pieces still adhering to the fungal surface. The fungal wall is labelled by the WGA-gold complex to detect chitin (*h* fungus)

Fig. 15 Fungal wall in close contact with the host wall (*w*). WGA gold-granules label the loose fungal wall (*arrowheads*)

Fig. 16 Higher magnification of the plant-fungal contact area. WGA gold-granules label the loose fungal wall (*arrowheads*) (*w* host wall)

Fig. 17 The electron-dense layer (*arrow*) is detached from the host surface and the fungus (*h*) is developing underneath

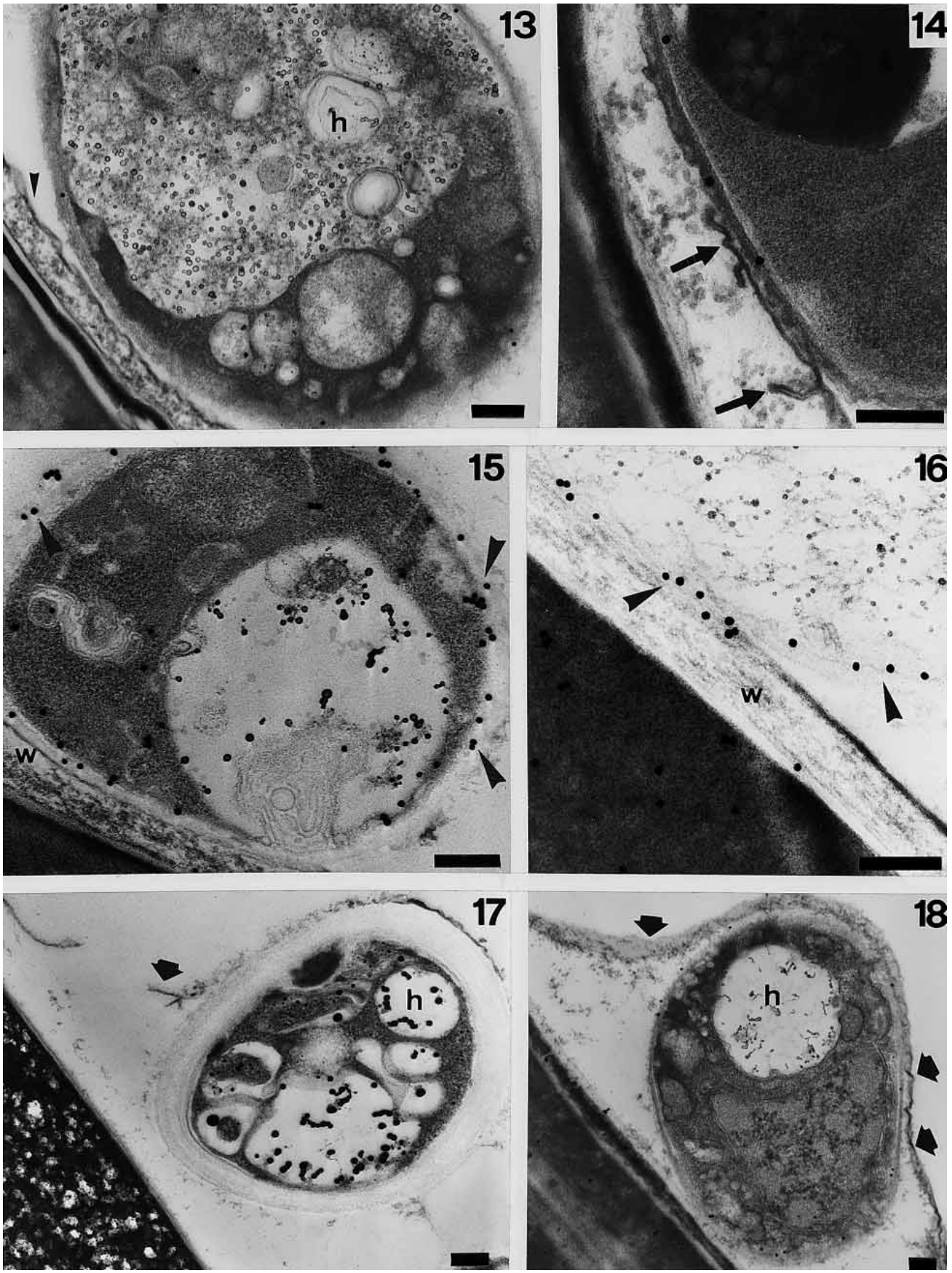
Fig. 18 The fungus (*h*) develops in the space between the electron-dense layer (*arrows*) and the host cell wall. The fungal wall is labelled by the polyclonal antibody to locate glucans

Discussion

Morphological analysis of pine roots developed in the presence of *S. collinitus* strains revealed a colonization pattern very similar to that described by Piché et al. (1983) for *Pinus strobus* and *Pisolithus tinctorius*. Hyphae first colonize the surface of secondary roots and then proliferate under the root cap cells, where vacuolar phenolics are stored, as widely reported for gymnosperm mycorrhizas (Smith and Read 1997). Our results provide, however, some novel information on factors that may influence the degree of compatibility between the symbionts and eventually lead to the different symbiotic capabilities of ectomycorrhizal strains.

A clear event in the establishment of early contacts between the partners is the breakdown of a thin electron-dense layer lining the cell wall. As a specialized cell wall domain, it covers the whole surface of the secondary rootlets, represented by cap and epidermal cells. This layer has so far received little attention: it has been reported by Kottke (1997), who interpreted it as a kind of cuticle. The importance of such a cuticle-like layer in the interaction between *Laccaria amethystea* and *Picea abies* was suggested by the observation that hyphae attach to this layer on the root cap cells and dissolve it. However, cytochemical and biochemical characterization of this structure is lacking, and the possible similarities with the cuticle layer described on conifer leaf epidermis (Tenberge 1992; Kerstiens 1996) await further investigation. Our observations show that the fungus breaks this layer and makes firm contact with the underlying part of the host wall. At this stage, the fungal wall seems to loosen in texture, even though chitin and glucans – the major skeletal molecules in many higher fungi – are still found. Interestingly, a very similar pattern has been described during early interactions between *Pisolithus tinctorius* and *Eucalyptus globulus* (Laurent P, Tagu D, De Carvalho D, Nehis U, De Bellis R, Balestrini R, Bauw G, Inz D, Bonfante P, Martin F in preparation).

Ectomycorrhizal fungi have been suggested to produce hydrolytic enzymes, including polygalacturonases, which would help loosen the host wall (Cairney and Burke 1994). However, in our experimental conditions, strains J3-15-24 and J3-15-31 did not produce significant levels of polygalacturonases, in contrast to some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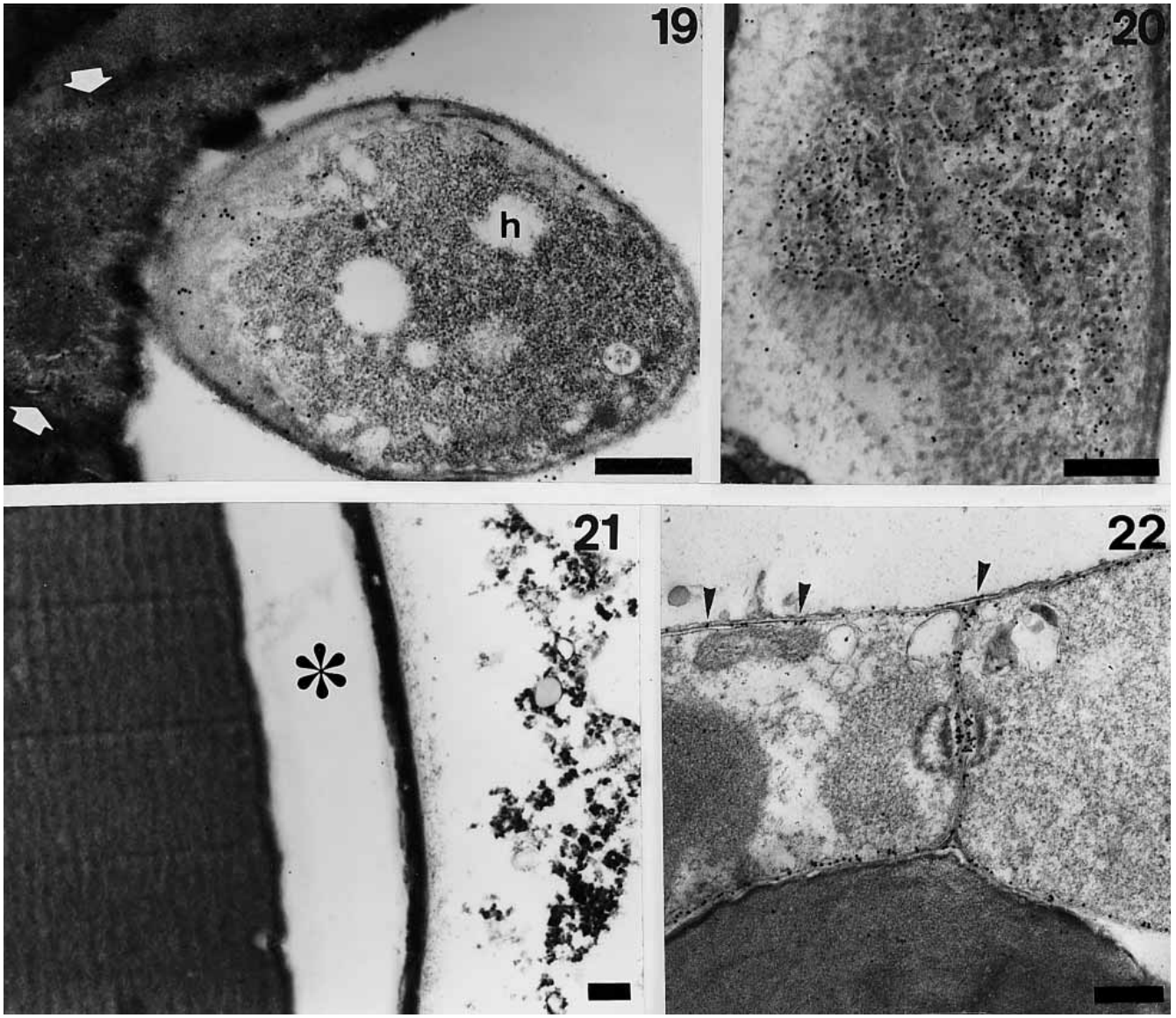

Figs. 19–22 Rootlets of *P. nigra* inoculated with *S. collinitus* strains J3-15-24 (Figs. 19–21) and J3-15-31 (Fig. 22). Electron micrographs of the tip region, covered by collapsed cap cells; *bars* 0.4 μm

Fig. 19 A new cell wall material is laid down by the host cap cell in contact with the fungus. The deposit appears as a thickening limited in size (*arrows*) and amorphous in structure. It is labelled by an antibody against β-1,3-glucans. The fungal (*h*) wall is also labelled, as shown in Fig. 18

Fig. 20 Detail of the amorphous material regularly labelled by the antibody against β-1,3-glucans. The other cell wall domains do not show gold granules

Fig. 21 In the regions where the fungus is not present, no gold granules are present on the cell wall (*asterisk*)

Fig. 22 In the samples inoculates with *S. collinitus* strain J3-15-31, labelling is present only on the hyphal cell walls (*arrowheads*)

endomycorrhizal fungi (Perotto et al. 1998). On the basis of the present morphological observations, other lytic enzymes should be investigated.

The two *Suillus* strains can form mycorrhizas in combinations with both *P. pinea* and *P. nigra*. This indicates that *S. collinitus* J3-15-24 and J3-15-31, which originate from a Mediterranean and an alpine region, respectively, and are genetically distinct (Bonfante et al. 1997), are compatible with these plants irrespective of the environment of origin. However, inoculation on plates revealed some differences in the behaviour of the two *S. collinitus* strains: only a small number of mycorrhizal tips were found when the Mediterranean fungal isolate was inoculated on *P. nigra*, a species adapted to alpine areas. Differences in mycorrhizal efficiency when hosts are challenged by different isolates of ectomycorrhizal fungi have been reported (Lei et al. 1990; Burgess et al. 1994). Several mechanisms have been suggested to reduce mycorrhizal efficiency, such as in-

creased deposition of phenolic materials at the contact zone (Malajczuk et al. 1984). In our experiments, hyphae of the *S. collinitus* J3-15-24 strain were found to elicit a defence reaction in the host plant with the formation of wall thickenings containing β-1,3-glucans. In addition to several plant-pathogen interactions where β-1,3-glucans (callose) have been detected (Xu and Mendgen 1994), callose has also been described in the interaction of arbuscular mycorrhizal (AM) fungi with symbiosis-defective mutant plants. In fact, pea mutants with a myc$^-$ phenotype show localized callose depositions on the epidermal walls adjacent to the fungal hyphae (Gollotte et al. 1993), demonstrating that AM fungi can evoke defence reactions. Ectomycorrhizal fungi can also release elicitors which trigger defence reactions in cell cultures (Salzer et al. 1996), even though the eliciting activity is reduced in planta, as also described for AM symbiosis (Gianinazzi-Pearson et al. 1996).

Our results thus provide morphological evidence for an eliciting activity *in planta* by one of the *S. collinitus* strains. The defence response we found was limited to the experiments in which *P. nigra* was challenged on plates by a fungal strain originating from an environment different from that of the host plant. In contrast, no reactions were observed when seedlings were inoculated with a fungal strain from the same area. This different behaviour may be due to the different symbiotic capabilities of the two strains used, which are also genetically distinct. However, it may also indicate a higher symbiotic compatibility between partners living in the same geographic region and/or adapted to the same environment. Of course, more isolates must be examined to test this hypothesis. The extent to which in vitro conditions like the plate system may influence the plant defence reaction, must also be clarified since this can be modulated by factors such as a nutrient gradient (Salzer et al. 1996).

In conclusion, morphological analysis of the early contacts between *S. collinitus* strains and pine tree species showed that the breakdown of an electron-opaque layer at the surface of the cap cells is an important step in the establishment of symbiosis. This raises questions on the molecular mechanisms involved and whether the electron-dense layer, described at the pine root surface as the outermost wall layer, is common to other woody plants. Further, genetically different fungal strains may show subtle differences in their mycorrhizal capabilities. When selecting strains for reforestation, it may be relevant to consider carefully the use of strains capable of evoking a defence reaction on hosts which are not fully compatible.

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