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# Extramatrical structures of hydrophobic and hydrophilic ectomycorrhizal fungi

**Abstract** The extramatrical mycelia of *Suillus bovinus, Rhizopogon luteolus* and *R. vinicolor,* all examples of hydrophobic (*ho*), mat-forming mycorrhizal fungi, were examined while associated with their hosts in the unsterilized rhizoscope, and efforts were made to produce and examine similar structures in vitro. Comparisons were made with four hydrophilic (*hi*) mycorrhizal fungi, *Thelephora terrestris, Cenococcum geophilum, Laccaria laccata* and *Hebeloma crustuliniforme.* The *ho* fungi formed linear structures (coarse, rhizomorph-like cords, with vessels in the center) and fans, both in the rhizoscope and in vitro. The same was seen in mycorrhizal mycelia in forest soils. These cords did not themselves give rise to the fans peripherally, and were not proper rhizomorphs, but were created continuously from single *exploring* air hyphae in the preexisting fan. Thus the *ho* exploring hyphae aggregated into strands, which grew in thickness only when no suitable, exploitable substrate was found. The assembly of hyphae creating *ho* cords was seen in the air as well as on inert hydrophilic (glass) or hydrophobic (plastic) surfaces, but never in water. It is hypothesized that the *ho* cell wall surface glues hyphae together while cords are formed. Water disturbed strands and mantles already formed. The *ho* exploring hyphae could also create *ho* mycelial patches (as in a mat) at the water-air interface of a number of substrates. The periphery of these patches seemed to be composed of shorter exploiting hyphae penetrating different water-soaked substrates. Exploring, aerial hyphal tips of the *ho* fungi were shown to "excrete" water droplets from openings in the *ho* cell wall surface, both in vitro and in the rhizoscope. In the rhizoscope, droplet excretion was apparently directly governed by photosynthesis in the shoot of the seedling. It is proposed that the drop exudation represents a kidney-like function of the extramatrical hyphae and a bridge to drier soil particles to initiate nutrient uptake by the hyphae. The ecological function of the different extramatrical structures of *ho* fungi are discussed. The *ho* cords or hyphae may translocate water only in the vessels or symplastically and not in the cell walls. The *ho* property may be essential among the S-selected (stress-tolerant) factors in these forest fungi. The transfer from water-repelling exploring structures into more *hi* exploiting structures in water contact with surrounding soil debris is, therefore, of great importance. The *hi* fungi did not form rhizomorph-like strands, in most cases, but an extending hyphal mycelium, representing foraging, exploring and exploiting structures at the same time. In the field, short strands may be found. On the *hi* fungi droplets were also produced but readily fused into a water sheath around the hypha. The hyphae thus tended to wick water via the cell wall.

**Key words** Cords · Exploring hyphae · Hydrophobia · Hydrophilia · Air · Water exudation

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## Introduction

Basidiomycetes vary their modes of growth, e.g. as cords, hyphae and sclerotia, depending on their ecological needs (the environment and the connection with food sources etc.). In the saprotrophs, exploiting and exploring mycelia are often two morphologically distinct modes; one switches over to the other in culture, after chemical or physical induction, e.g. by water, oxygen, carbon dioxide, or nutrients (Jennings and Bravery 1991; Rayner 1991; Wessels 1993).

In mycorrhizal fungi, the situation has been examined in some detail (Brownlee et al. 1983; Finley and Read 1986; Straatsma and Bruisma 1986; Unestam 1991) and appears to be similar to that of the saprobes (Cairney 1992). Unestam (1991) pointed out the variation between mycorrhizal fungi in the water repellency

of the mycelium, from very hydrophobic (*ho*) to extremely hydrophilic (*hi*) and claimed that the surface property must vary, depending on the function of each mycelial structure. Since many forest tree mycobionts are water repelling, i.e. hydrophobic, from the mantle out through the extramatrical structures, it would at least be necessary for them to have water-accepting, if not completely *hi* tips in order to exploit the mostly hydrophilic substrates in the soil (Unestam 1991) and take up soluble inorganic and organic nutrients. It would be expected that a switch or gradual conversion of the hydrophobic hyphae into more hydrophilic exploiting hyphae would be found after more careful examination of the extramatrical structures, in vitro as well as in vivo. More ruderal mycorrhizal fungi, often hydrophilic by nature, could better resist flooding than the hydrophobic forest fungi (Stenström 1991), and were thus assumed to be inherently equipped to penetrate water-soaked soil substrates.

Unestam (1991) claimed that the *ho* property of many forest mycorrhizae was an ecological adaptation to the periodically rather dry conditions that are often typical of the upper soil layers of the boreal forest (Read 1991). He could not explain, however, why water condensed so readily on the hydrophobic structures, or if the drops were produced by the fungus for any physiological reason. Many ruderal *hi* fungi would be more adapted to evenly humid soils where water loss is less of a problem.

The aim of the work presented here was to study continually how the different extramatrical structures, strands, mycelia, hyphae etc., developed in relation to water and were transformed and connected to one another under undisturbed conditions in vitro and in the unsterile rhizoscope, as compared to natural conditions. A number of hydrophobic, mycorrhizal forest fungi were used in the study and were compared with more ruderal, hydrophilic ones.

## Materials and methods

### Fungal strains

Three mycobionts found to be hydrophobic and four hydrophilic ones were cultured in rhizoscopes and aseptically in vitro. The differences in water repellency between fungi were tested according to Unestam (1991), before and after cultivation.

*Ho* fungi: two pine mycorrhizal fungi, *Suillus bovinus* (L. ex Fr.) O. Kuntze and *Rhizopogon luteolus* (Fr. & North), were cultured as mycorrhiza in rhizoscopes after inoculation with basidiospores (Unestam 1991) collected from fruit bodies in forests near Uppsala. They were transferred from plant to plant as mycorrhiza (Unestam and Stenström 1989). From such mycorrhizal roots, a pure culture of *R. luteolus* (strain R1) was isolated on MMN agar after sterilization in 30% $H_2O_2$ and repeated washing in distilled water. *S. bovinus* (strain L1) was, however, isolated in pure culture from internal fruit body tissue.

A pure culture as well as spores of *R. vinicolor* (Smith) (specific on *Pseudotsuga menziesii*) were provided by Dr. Michael A. Castillano at Oregon State University, Corvallis, Ore. The spores were used to inoculate Douglas fir seedlings in the rhizoscope.

*Hi* fungi: pure cultures of *Laccaria laccata* (Scop. ex Fr.) Bk. & Br., strain Nancy 238A and *Hebeloma crustiliniforme* (Bull. ex Fr.) Quèlet, strain Nancy SIV, were obtained from Drs. Elna Stenström and J. E. Nylund, respectively, at our department and *Thelephora terrestris* (Ehrb.) Fr., strain 886, from Dr. N. Fries, Uppsala. Mycorrhization on *Pinus sylvestris* seedlings in the rhizoscope was obtained after placing washed and blended mycelia on the roots. *Cenococcum geophilum* (Fr.), strain Nancy SIV, was only in pure culture.

### Culture media

MMN with glucose instead of sucrose was used as nutrient medium for all fungi in the form of MMN agar (1.5% agar) in plastic petri plates (9 cm). Olive oil (0.2%) was dispersed in the agar medium when indicated, by adding it dissolved in a small amount of 96% alcohol before autoclaving. Also, 0.2% Tween 80 in MMN as well as 1–10% glucose agar were used in some experiments. In other cases, pure water agar was employed. Petri dishes were always sealed with a double layer of Parafilm to maintain the humid atmosphere during fungal growth.

Rhizoscopes were made from 14-cm plastic petri dishes (Unestam and Stenström 1989). The substrate in the rhizoscopes was peat mixed with LECA pellets (1:3, v:v; see Unestam 1991).

### Plants

Seeds of *Pinus sylvestris* L. (central Swedish provenance) or *Pseudotsuga menziesii* (Mirb.) Franco (Gift from Dr. J. Trappe, Oregon State University, Corvallis, Ore.) were sterilized with $H_2O_2$ and the roots allowed to develop in a moist, nonsterilized LECA/peat (3:1, v:v) mixture. Seedlings were pregrown in miniature greenhouses under light (mercury-halogen lamps, 200 $\mu Em^{-2}S^{-1}$), and the substrate was watered regularly with Ingestad's conifer nutrient solution (Unestam and Stenström 1989) at very low concentration.

Seedlings 5- to 8-weeks old were carefully lifted and immediately placed in rhizoscopes (3–5 per scope) and the roots inoculated with a spore suspension ($10^5$ spores/ml) or with blended mycelial agar. The rhizoscopes were also watered sparsely with Ingestad's solution.

### Recorded processes

Growth and development of mycelia were studied in vitro. Growth and development of the extramatrical mycorrhizal mycelia were studied in rhizoscopes. Hydrophobia and hydrophilia as well as hyphal water exudation were recorded in both systems. Hyphal reactions to the wet substrate were followed in both types of cultures.

### Differentiation and hydrophobia

Petri dish agar plates were inoculated with vigorous (5 × 5 mm) agar mycelium pieces in the center. Hyphal growth, adaptation, and differentiation, when growing on the MMN, glucose, or water agar, or onto glass or polyethylene plastic replacing part of the agar, were observed regularly in the microscope without opening the dishes. Also, hyphae growing through oil drops on the agar surface were studied. Water exudation and retraction by the hyphae were studied by microscopy through the lid in the culture petri dish.

After extensive mycorrhiza formation in the rhizoscopes, the cultures were manipulated and studied with a stereomicroscope. Water agar blocks, autoclaved litter leaves, or water-soaked peat particles (pH 3.4) were placed on the substrate surface, and fungal growth and differentiation from the mycorrhizal roots were followed regularly: growth in the air (linear organs and exploring structures), on air-water interfaces and into agar (exploiting hyphae), patch formation, water drop and crystal formation on the fungal structures etc. were noted. By studying the formed droplets or adding extremely small water droplets to the fungal struc-

tures, the degree of hydrophobia could be established (Unestam 1991) under varied circumstances.

## Results

### Fungal structures in the rhizoscope

Studies in the rhizoscope revealed six distinct extramatrical fungal structures of the hydrophobic fungi (Figs. 1, 2) from the mantle to the exploited substrate: (1) Hyphae extending out from the hydrophobic mantle (not shown in Fig. 1) readily gave rise to (2) hydrophobic (*ho*) linear, rhizomorph-like cords, the thicker of which had wide, empty, vessel-hyphae in the center with dissolved septa, as seen in interference contrast microscopy (cf. in vitro below). These running cords were sometimes 5 cm or more long (Finley and Read 1986) and were always formed freely in the air. The cords extended into thinner strands and finally (3) *ho* hyphae, which explored the soil enviroment. Both cords and hyphae often carried oxalate crystals on their surface (see in vitro below). (4) Knot-shaped clusters of hydrophobic hyphae were often formed on wet, solid rhizoscope particles (as well as on agar cultures, see below) and were readily seen in the microscope on the surface of the added water agar blocks. These knots looked like sclerotia initials. (5) Hyphae, penetrating wet soil particles, moist and partly degraded leaves, water agar blocks etc. could be called exploiting hyphae, and must be water accepting if not fully hydrophilic. (6) White, hydrophobic fungal patches (interwoven strands, cords, hyphae, knots, and sometimes mycorrhizal roots) were preferentially formed on substrates such as alder leaves (Unestam 1991), but also occasionally in air pockets of peat particles and even on the surface of added water agar blocks (Fig. 3a). Patches always formed in the air on top of the air-water interface, but never inside the water.

*Ho* mycorrhizal roots of the rhizoscope being in contact with water agar soon turned brown where submerged (Fig. 3 a), thereby showing a strong defensive, phenolic reaction to being submersed (Stenström 1991). The mantle of the nonsubmersed parts often spread, on top of the agar surface, into a small white patch (Fig. 3a). Cords crossing over and dipping into an agar surface often turned black or brown (Fig. 3b).

Using the *hi* fungi *T. terrestris, L. laccata* and *H. crustuliniforme* in the rhizoscope, hyphae or strands extending out from the mycorrhizal mantel often dipped into wet areas and particles and were very seldom seen in the air. The hyphae readily penetrated agar blocks and showed very little differentiation, no cords (*T. terrestris* had thin, short strands), no knots, and no patches. All structures seemed essentially hydrophilic. In disturbed humid forest soil, hyphae and short bunches of hyphae of *L. laccata* were, however, often seen in air spaces.

Submersed mycorrhizal roots of *hi* fungi never turned brown (Fig. 15).

### Hyphal growth in vitro

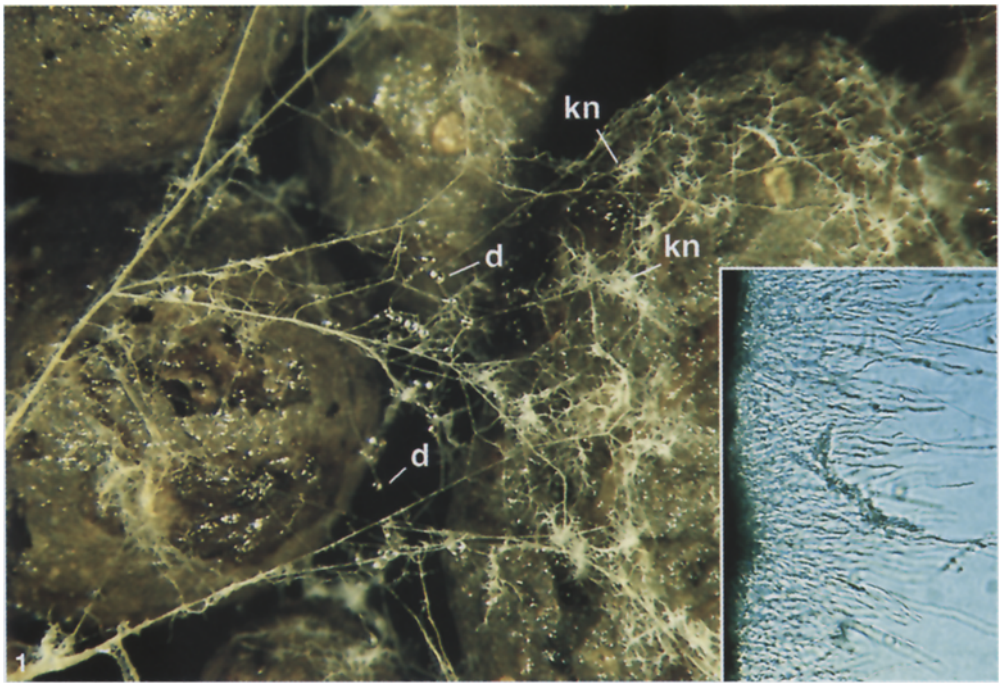
Table 1 and Fig. 2 summarize the characteristics of the *ho* hyphae of the three examined forest fungi and make comparisons with four *hi* fungi. The *ho* fungi readily formed aerial hyphae on MMN agar. Groups of submerged agar hyphae were also formed but this kind of growth normally lagged far behind the air mycelial front in *S. bovinus* and *R. luteolus* (Figs. 2, 4). In *R. vinicolor,* however, the agar hyphae were seen immediately behind the front of the air hyphae. Studies with *ho* fungi, however, showed that when 0.2% olive oil was included in some growth media, patchy submersed growth was supported, overtaking aerial growth. Even high glucose concentrations, 5–10%, favored growth inside the agar, particularly together with 0.2% oil. When a detergent, 0.2% Tween 80, was included in MMN or 1% glucose agar, this tendency was even more pronounced and the entire mycelium was often submersed (Fig. 2). Using the three *ho* fungi, knot-like structures were frequent on top of the agar surface of all media and formed agar-penetrating hyphae (Figs. 2, 5; cf. Fig. 1) that spread into shallow and narrow groups of exploiting hyphae into the agar. The knots appeared very clearly on oil-MMN agar, where the extensive air mycelium was suppressed (Fig. 5).

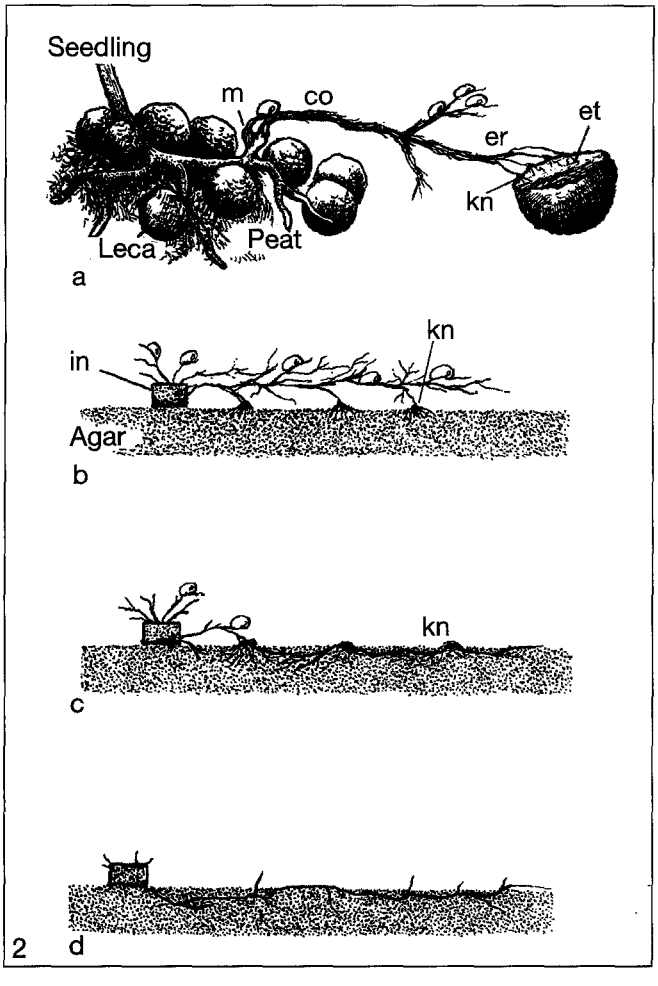
Figure 2 illustrates that for the *hi* fungi, the submersed mycelium extended far out into the agar before any aerial hyphae were seen growing from the inoculum (placed upside down) at the center of the colony and air hyphae were very sparsely seen over any agar surface. *L. laccata* was more extreme than *H. crustuliniforme, C. geophilum* and *T. terrestris* in this respect (Table 1). No knots were formed by these fungi.

### Cord formation in vitro and in rhizoscopes

In vitro, cords were seen on top of MMN agar only in old cultures of *ho* fungi but they formed more readily on 1% glucose agar or on the plastic (Fig. 6) or glass surfaces. Cords were also sometimes formed in the mycelium on top of water agar, but were never formed inside the agar.

At first glance, Fig. 1 might give the impression that the exploring mycelium fan was produced from the cords. Clearly that was not the case; cords formed only in and from an extensive mycelium (Finley and Read 1986). Thinner strands formed when two or more hyphae (e.g. branches from a hypha) grew in parallel and were glued to each other. Anastomoses were commonly seen between parallel hyphae (Fig. 6). When more hyphae (branches) joined, a thick cord was finally created, often having central, empty, vessel-hyphae (Fig. 6; Agerer 1988). Such differentiated cords were described in many species by Agerer (1987–1991). Even in vitro, crystals identified in transmission electron microscopy-Xray as calcium oxalate (Sten Hellqvist, Department of Ecology and Environmental Research),



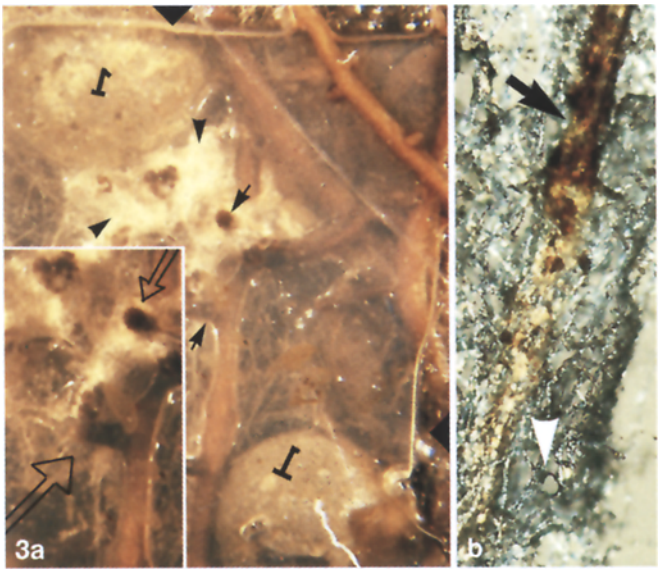




**Fig. 1** Extramatrical structures of *Rhizopogon luteolus* (*ho*) in the rhizoscope. Cords extending from the mycelium of a mycorrhizal short root (see Fig. 2) branch into thinner strands and exploring hyphae that often form knot-like clusters of hyphae (*kn*) on the LECA surface, from which exploiting hyphae (not seen) penetrate the LECA pellet to the right. Water drops (*d*) are attached to many exploring hyphae. ×18. Insert: water agar block placed in the rhizoscope penetrated by exploiting hyphae. ×30

**Fig. 2** Schematic diagram of mycelial behavior in the rhizoscope (**a**) and in vitro on nutrient agar (**b–d**). **a–c** Represent hydrophobic, **d** hydrophilic fungi. **a** Mycorrhizal root tip (*m*) and its hyphae which give rise to a hydrophobic cord (*co*), exploring hyphae (*er*), hyphal knots (*kn*), and exploiting hyphae (*et*). **b** Hydrophobic hyphae grow out from the inverted inoculum (*in*) over the agar, primarily in the air. Water drops attach to air hyphae. Behind the front, agar hyphae appear, often developing from hyphal knots (*kn*) on the agar surface. **c** In Tween 80 nutrient agar submersed growth is favored. **d** Hydrophilic fungi such as *Laccaria laccata* grow almost entirely in the agar or on the surface, with occasional single hyphal tips in the air

**Table 1** Hydrophobic and hydrophilic properties of fungal hyphae grown in vitro on 1% glucose agar and further onto glass or plastic. Drops on surface of agar, glass, or plastic were produced by and attached to hypha. Drops either formed a string of beads (Fig. 10), or a water mantle wicked along the hypha (Figs. 10, 11). (*Ll* Observations as for *Laccaria laccata*)

| | Air hyphae | | Water attachment to hyphae on | | |
|---|---|---|---|---|---|
| | Hyphae | Drops on | Agar surface | Glass | Plastic |
| *Rhizopogon luteolus* | Frequent | Frequent | Short mantle or bead string on tip | Short mantle on tip. Drops locally on hypha | Short mantle on tip plus distinct drops on hypha |
| *Rhizopogon vinicolor* | Frequent | Frequent | Often long mantle or bead string on tip | Long water mantle on tip. Drops locally on hypha | Long mantle on tip plus distinct drops on hypha |
| *Suillus bovinus* | Frequent | Frequent | Short mantle or bead string on tip | Short mantle on tip. Drops locally on hypha | Short mantle on tip plus distinct drops on hypha |
| *Laccaria laccata* | Very sparse | No | Mantle on all hypha. No drops or beads | Mantle on all hypha. No distinct drops | Mantle on all hypha. No distinct drops |
| *Hebeloma crustuliniforme* | Sparse | Sparse | Mantle on all hypha. No drops or beads | Mantle on all hypha. No distinct drops | Mantle on all hypha. No distinct drops |
| *Thelephora terrestris* | Sparse | Sparse | Mantle on all hypha. No drops or beads | Mantle on all hypha. No distinct drops | Mantle on all hypha. No distinct drops |
| *Cenococcum geophilum* | Sparse | Sparse to common | Mantle on all hypha. No drops or beads | Mantle on all hypha. No distinct drops | Mantle on all hypha. No distinct drops |

were readily formed on *ho* cords (Fig. 7) and hyphae. We have seen similar crystals on air-exposed hyphae of *L. laccata* in vivo but never in vitro or in the rhizoscope.

None of the *hi* fungi produced strands or cords in vitro. Instead they formed a sparse network of hyphae on the agar or other surfaces frequently interconnected by anastomoses, (most evident in *C. geophilum,* Fig. 8) representing the same pooling of resources (Rayner 1991) as the anastomoses in the hydrophobic cords. Occasionally, two or three hyphae were glued together by water (Fig. 9).

In forest soil, spider-web-like patches of *L. laccata* were seen to have hydrophilic bunches of hyphae reminiscent of strands. According to Agerer (1987–1991, 1988) such strands remain undifferentiated in *Laccaria ametystina* and *T. terrestris* and are not described in *Hebeloma edurum.*

## Hyphal interaction with water

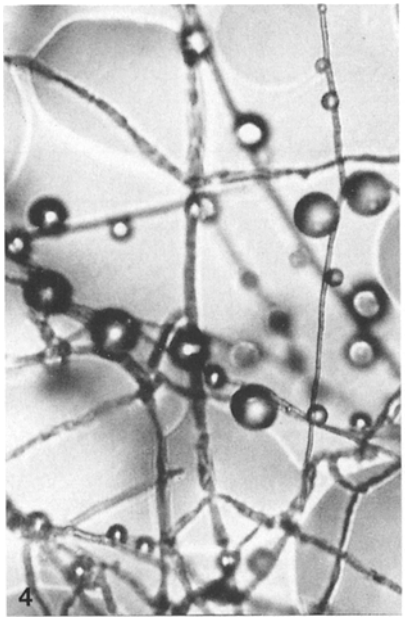
In the three *ho* fungi, when grown in the rhizoscope together with compatible hosts, spherical water drops were frequently formed on aerial hyphae (Fig. 1; Table 1) and strands, indicating their mainly hydrophobic nature (Unestam and Stenström 1989; Unestam 1991). In vitro at 20° C, all three *ho* fungi had tips carrying water drops when grown in the air, on the agar, on the glass surface or on the hydrophobic plastic surface (Fig. 10; Table 1). When frequent on glass or water agar, the drops fused into a string of beads, still showing their origin from sites on the mainly *ho* hyphal surface (Fig. 11), or into a short water mantle (Fig. 11; Table 1). In vitro, some difference could be seen among the three *ho* fungi (Table 1). A longer smooth mantle or string or of beads was seen surrounding the peripheral hypha of *R. vinicolor,* when growing on the agar surface, than for the two others. This may indicate a lesser degree of hydrophobia near the tip.

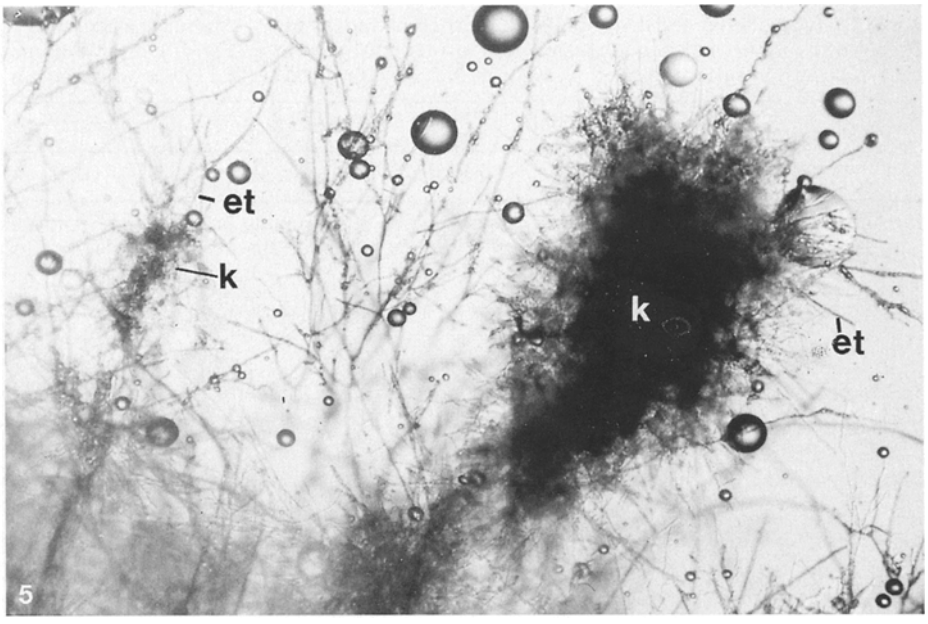
In young in vitro cultures of the *hi* fungi, only sparse hyphae were sticking up from the agar (Fig. 2) and water drops were sometimes seen on air hyphae of *H. crustuliniforme, T. terrestris* and *C. geophilum* but never on *L. laccata.* On plastic, glass, or agar, the four *hi* fungi formed an even, broad water mantle, without tendencies toward bead formation (Fig. 9; Table 1).

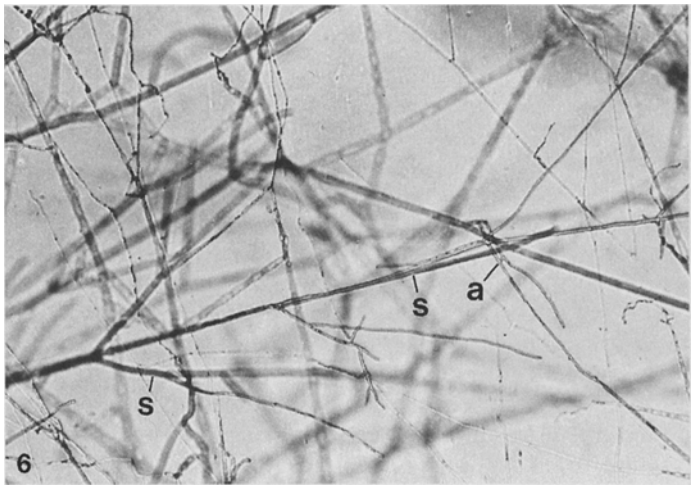
## Drop exudation

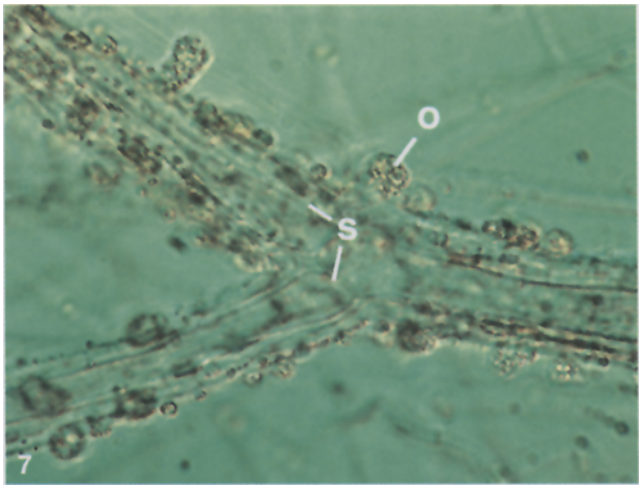
Drop formation on the fungal apex in vitro was studied further by allowing the peripheral surface hyphae to grow through oil drops on the surface of MMN agar. Growth through oil did not visibly affect the hyphal tip functions but revealed in more detail water exudation through the hyphal wall (Figs. 12, 13). At 20° C, numerous, small, spherical droplets were seen on the *ho* fungi in the oil (Fig. 12) and they grew in size and often fused. In the cold (6° C), the droplets instead retracted and were absorbed within 1 h by the hypha (Fig. 13). The process of exudation was enhanced dramatically by

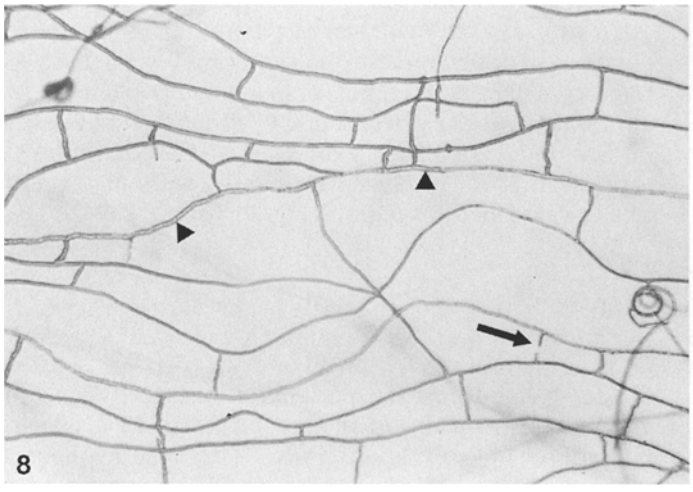
◀ **Fig. 3** **a** *Suillus bovinus, ho* in the rhizoscope; agar block (*large arrowheads*) covering roots at the site. Mycorrhizal short root tips (*arrows*) turn brown when drenched for a long time in the water of the agar. The mantle sometimes spreads into a mycelial patch (*small arrowheads*) repelling the water on the agar surface. ⊥ LECA pellets. **b** A cord drenched in the surface water of such a block also turns brown (*arrow*). The cord outside the wet agar surface (*arrowhead*) is not melanized



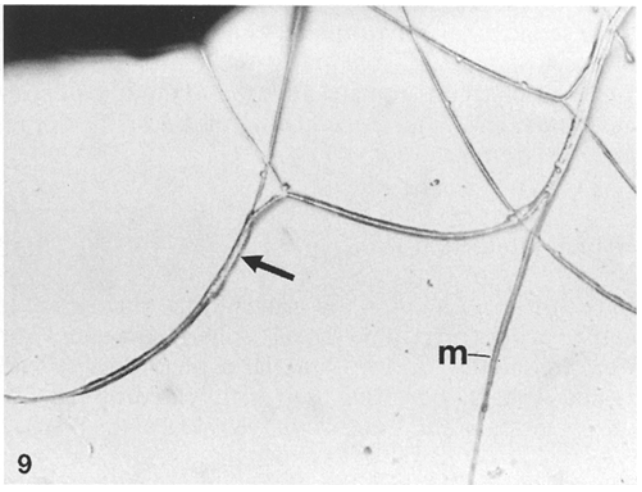




**Fig. 4** Air hyphae of *Rhizopogon luteolus* (*ho*) in vitro carrying exuded water drops. ×200

**Fig. 5** *S. bovinus* growing in an MMN agar with dispersed oil drops. The air mycelium is almost absent. Knot-like (*k*) conglomerations of hyphae form on the agar surface (cf. Fig. 2c) and send out exploiting (nutrient absorbing) tips (*et*) into the agar. ×40

**Fig. 6** *S. bovinus* (*ho*) starts forming strands on the plastic surface. Parallel hyphae are glued together in the initial strand (*s*), often pinnately branched, and sometimes form anastomoses (*a*)

first keeping the petri dish culture at +6° C for 20 min and then allowing it to warm up under the microscope while watching droplet formation. Apparently, the warming process increased turgor pressure. After inspection, the culture dishes were returned to the 20° C growth chamber and the process could be repeated the following day, often with the same hyphae, now extended by apical growth.

In a rhizoscope experiment, *S. bovinus* hyphal tips were allowed to grow onto water agar blocks with added oil drops (as in Fig. 12) and most brick pellets and peat were removed below the agar to allow for inspection in the transmission light microscope for 10 h. During that time, the shoot of the pine was exposed to cold light (400 $\mu Es^{-1}m^{-2}$) for periods of 1–4 h. Droplets appeared and grew (as in Fig. 12) during illumination. After leaving the shoot in the dark wrapped in aluminum foil for a few hours (only cold microscope light left on), some of the smaller (but not the larger) droplets were slowly reabsorbed by the hyphae (as in Fig. 13), but illumination of the leaves again made many reappear and grow.

In agar cultures of four *hi* fungi, water droplet formation (pumping) was seen inside the oil drop; the droplet surrounded the hypha (Fig. 14) and did not only sit on one side as on the *ho* hyphae (Fig. 12). Apparently, both types of hyphae have pores or other openings in the cell wall through which water was directed before forming the droplet. However, on the hydrophilic wall surface, but not on the hydrophobic, the droplet tended to spread.

## Discussion

### Interdependent fungal structures

In the present study we have tried to illuminate the morphology of the extramatrical mycorrhizal mycelium, its different structural forms (Fig. 2) and functions, using laboratory studies and field observations. Our work will now be related to general mycological work.

Wessels (1993) showed that *Schizophyllum commune* hyphae produced proteinaceous, hydrophobic substances, hydrophobins, in the cell walls of their aerial hyphae but not on submerged hyphae. A similar situation may exist in our hydrophobic fungi.

Addition of a detergent (Tween 80), thymine, and malic acid to the substrate was shown, in vitro, to enhance growth in liquid medium or to give rise to pigmented, submerged mycelia in agar of *Cantharellus sibarius* (Straatsma and Bruinsma 1986; Danell and Fries 1990) and was assumed to supply essential $CO_2$ to the mycelium. Fries et al. (1985 and references) and Sun and Unestam (submitted) noticed that lipids stimulated growth of mycorrhizal fungi in liquid culture, and Dütsch (1978) found that Tween 80 supported *Agaricus bisporus*. Those and our results (see Fig. 5 and "Hyphal growth in vitro" in Results) may in part be interpreted as follows: First, the extensive submerged growth that we found with glucose and oil may be due to the lipids removing hydrophobins from the hyphae, thereby making them less hydrophobic (Wessels 1993) and more exploiting. In addition, high concentrations of sugar may cause overloading of the metabolism with carbohydrates, which results in catabolic repression and exudation of surface tension-lowering organic acids (resembling Tween above, Fig. 2c) and other organic products from glycolysis (Lehninger 1975, pp. 438 and 980), again removing hydrophobins. Found in fungi, this repression has been called the Crabtree effect (Griffin 1981). Secondly, improved mycelial growth (see above) would be due to the switch from a water-rejecting to alternative submerged growth, simply providing a better supply of diffused nutrients than in the aerial mycelium, which is dependent on a slow transport from the exploiting, submerged hyphae. This type of switch may be very functional for a mycelium reaching a rich supply of surface tension-lowering, organic nutrients, one goal for mycorrhizal fungi in their symbiosis (Read 1991; Bending 1993).

Apparently, the extramatrical fungal mycelium of hydrophobic mycorrhizal fungi (Fig. 2) is composed of functionally diverse structures. Running pipe line, the rhizomorph-like cords soon form from hyphae of the initial mycelium extending out from the mantle. An exploratory, scouting mycelium with hyphae crossing aerial spaces (or dry surfaces) seems to create a network in which continuous cord formation takes place after exploitation has ceased (Finley and Read 1986; Read 1991). On the other hand, more peripheral exploring hyphae need the cord as a resource base for their function. They cannot exist without being anchored to a cord close by, and cords in turn can only form in a mycelial net of aerial, hydrophobic and exploring hyphae, of which most eventually disappear.

A knot structure (Figs. 1, 2, 5) is often created where an exploitable soil particle or surface (not necessarily nutritious) is found by the exploring hyphae. Finally, these knots or other aerial hyphae give rise to less hydrophobic (more but not entirely hydrophilic), exploiting hyphae which can penetrate, sometimes to a limited extent into a water-soaked substrate (Figs. 1, 5), some-

◀ **Fig. 7** Branched cord of *S. bovinus* (*ho*) seen in a longitudinal optical section. In the center is a vessel formed from a swollen and subsequently dead hypha. Septa are dissolved (*s*). The vessel is surrounded by packed live hyphae. The crystals (*o*) on the cord surface are of calcium oxalate. ×100

**Fig. 8** *Cenococcum geophilum* (*hi*) growing on a glass surface. Hyphae (often surrounded by water mantles, *arrowheads*) seldom glue together but form frequent anastomoses, from the joining of tips of hyphal branches (*arrow*), that may create pooling of resources in the mycelium

**Fig. 9** *L. laccata* (*hi*) hypha growing on a plastic surface. Water is wicking along the hyphae and forms a water mantle (*m*). Hyphae may be glued together by water when in contact (*arrow*). ×200

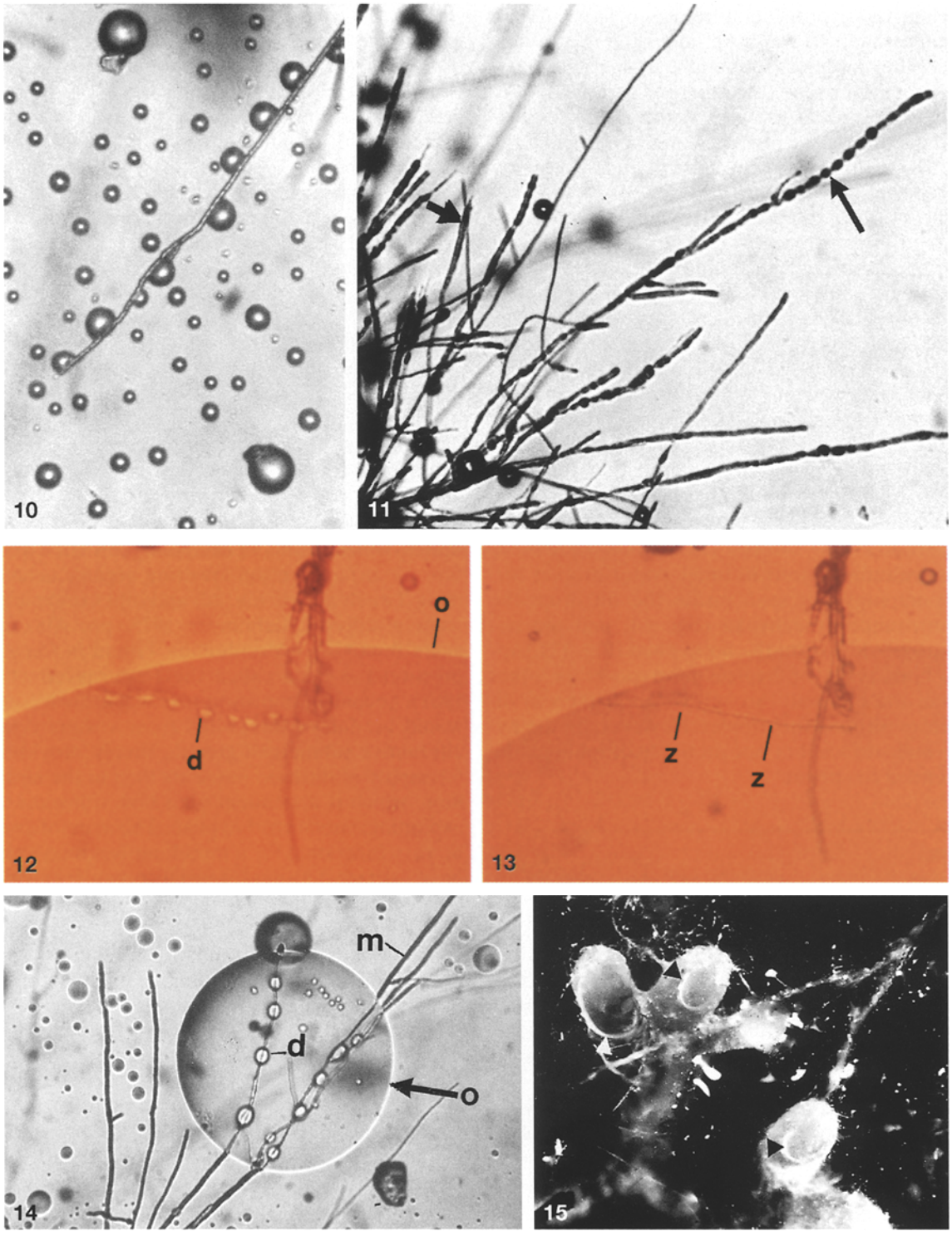



**Fig. 10** On a plastic surface exuded water attaches as distinct water drops to hyphae and plastic, both hydrophobic, even at the very apex of *R. luteolus*. ×200

**Fig. 11** *S. bovinus* hyphal tip on the water agar surface. Exuded water droplets appear as a string of beads (*small arrow*) on the hyphae or as a short, smooth water mantle (*large arrow*). The same pattern was seen on the hydrophilic glass surfaces

**Fig. 12** Hyphal tips of *S. bovinus* (*ho*) growing on the MMN agar surface. An oil drop (*o*) has been penetrated by hyphae. One of them has exuded water droplets (*d*) on its surface. Droplets do not surround the hydrophobic hypha but probably sit on the pores in the hyphal wall through which they have passed. ×240

**Fig. 13** The same hyphae as in Fig. 12 but the droplets have been retracted through the pores into the fungal cell after 1 h at 6° C. Note several diffuse, light zones (*z*), mostly between sites where drops were located. The zones may represent dissolved products released from the droplets into the oil-water interface

times to a greater extent, depending on surface tension, nutrient content etc (Fig. 2). The small, exploratory patches (with or without knots) on peat, leaves (Unestam 1991) or other wet substrates (Fig. 3a) may be highly energy consuming (Finlay and Read 1986), sometimes without yielding nutrients from their peripheral, exploiting hyphae. The larger hydrophobic mycorrhizal mat (Unestam 1991), partly seen in Fig. 1, seems to be a dense functional network of mycorrhizal roots, cords, smaller strands, often combined with patches of anastomosing hyphae and knots, and single exploiting hyphae. Thus, the creation of all extramatrical structures may be closely interdependent.

## Reaction to the wet environment

In Tween agar *S. bovinus* grew entirely submersed (Fig. 2) but such a mycelium turned immediately into the dominating aerial mycelium as soon as it was inoculated on plain MMN agar and no initial submersed growth, as in *Scizophyllum commune* (Wessels 1993), was necessary. Thus, the build up of an initial, extensive exploiting mycelium, as in *S. commune,* may not be needed. In nature, the mycorrhizal mat fungi, having an enormous body of hydrophobic structures (Unestam 1991 and references) must probably be fed water and nutrients by a limited number of exploiting hyphae, at least during dry periods, and the translocation system has to be efficient. The penetration of agar substrates shows that although hyphal tips do not suffer much from drenching or oxygen deficiency in the substrate, more complex structures are severely disturbed (Fig. 3). This prevents the whole differentiation process in water-soaked substrates and consequently only exploiting hyphae may occur there (Figs. 1, 5).

Thus, cords, mycorrhizal mantles, or mats cannot be formed when submersed and react strongly (formation of phenolic complexes?) when wetted (Fig. 3). Stenström (1991) showed that the extramatrical systems of many hydrophobic mycorrhizae disappeared and were prevented from forming by drenching the rhizoscope for only 30 s twice a week. Here again lack of oxygen can hardly be the reason. On the other hand, complex structures of hydrophobic fungi, such as the mantle, cords, and patches, may produce air pockets when flooded (Unestam 1991) which allow them to exist by holding the water back. Without these they react unfavourably to the water environment and eventually die. Death of these structures means the demise of all supported hyphae, exploring or exploiting, and all the structures have to develop again from surviving mycorrhizal root tips (Stenström 1991). It was observed by Kermit Cromack (unpublished work) that mycorrhizal mats in the forest temporarily thinned out during a rainy season but were built up again during the drier part of the year.

◀ **Fig. 14** *L. laccata* hyphae on the MMN agar surface. Water droplets (*d*) exuded from hydrophilic hyphae through pores (?) in the cell wall into an oil drop (*o*). Note that the droplets surround the hypha (in contrast to Fig. 12) and have an ovoid shape, perhaps due to the hydrophilic property of the hyphal cell wall. Outside the oil droplet, hyphae are surrounded by a mantle of water (*m*)

**Fig. 15** Forked mycorrhizal root tips with *Thelephora terrestris* (*hi*) dipping into water drops on the petri dish lid do not turn brown when remaining dipped into water (*arrowheads*), as do hydrophobic mycorrhizae (Fig. 3)

## Hyphal assembly

The simplest and most attractive hypothesis for the mechanism of cord formation is that hydrophobic hyphae, e.g. two branches from a hypha (in an exploring mycelium), which grow in the proximity of one another in the air (Fig. 1) or on an inert surface (Fig. 6) stick together by hydrophobic forces at points of contact (Wessels 1993). Attraction between hydrophobic surfaces may be a strong force and works at nm distances (Tsao et al. 1993). The hydrophobic surface layer would glue the hyphae together and the bonds would be stabilized or even initiated by anastomoses (Fig. 6). Addition of more hyphae (branches) would create the foundation for a strand and cord (Agerer 1988). Exploring hyphae, still dispersed, would degrade after failing to find exploitable substrates and finally leave only the cord intact (Fig. 1), protected from drying by its hydrophobic surface (Wessels 1993). This reorganization may represent a redistribution of the resource flow to more important sinks (Finley and Read 1986; Cairney 1992), such as the margin of the exploiting and exploring fan and the mycorrhizal root.

Hydrophilic fungal hyphae seem to function differently; they may stick together, glued by the surface tension of their exuded water (Fig. 9) and may even form anastomoses (Fig. 8), but extensive cords are not readily formed, at least not in the rhizoscope or in vitro.

## The tip of the hydrophobic hypha

Hyphal tips of hydrophobic fungi grow preferentially in the air (Fig. 2). However, when they contact a soaked substrate they show a more hydrophilic appearance (Figs. 2, 5, 11, Table 1), although not at all like a hydrophilic species (Figs. 2, 8, 9, 14). They only penetrate and exploit such a substrate with short hyphae, perhaps stimulated by the surface tension-lowering contents of intermediate metabolites of microbial origin (see "Interdependent fungal structures").

## Nutrient transport and water exudation

Jennings and Bravery (1991) proposed for *Serpula lacrimans* and other saprophytes that formation of water

drops on hyphal tips in vitro indicated acropetal nutrient transport together with water in the fungus, from the food source via the cords out to the turgid, peripheral, sugar-requiring hyphae. They suggested that the surplus water plus solutes were exuded by ultrafiltration through the cell membrane while the nutrients were kept by the cytoplasm. We found strong evidence that many peripheral hyphae (of both *ho* and *hi* fungi) continuously exuded such water drops, and the localized droplets (Figs. 12–14) made us believe in the presence of pores or openings through the cell wall. Such cell wall pores are normally small, allowing passage only of molecules smaller than 20000 Da (Wessels 1993 and references), and therefore few enzymes. The contents of these droplets are now being analyzed (Sun and Unestam, submitted). The formation of calcium oxalate crystals on hyphae and cords (Fig. 7) may take place by exudation of low concentrations of soluble oxalic acid (Duchesne et al. 1989) through the pores and precipitation on the hyphal surface with calcium ions diffusing from the soil.

Enzymes, cell wall precursors and other large molecules are released by exocytosis at the less rigid apex of hyphal tips (Wessels 1993). Lysis of soil proteins, that may be important in ectomycorrhizal nitrogen uptake (Read 1991), would then take place around the growing tip when in liquid contact with soil particles. Such contact with rather dry but hydrophilic soil particles (Unestam 1991) would be supported by the release of water from the pores (and the tip), through which peptides and amino acids might diffuse in the particle to be taken up again by the cells via the selective transport system of the plasmalemma. However, on the cellular level, the system may be more complicated. We have found a number of low molecular organic compounds in the pore exudate. When water is pressed via the cell membrane and through pores or other openings in the hydrophobic cell walls, small molecules, e.g. sugars and peptides, may always be included since ultrafiltration (Jennings and Bravery 1991) or excretion from the fungal cell normally only excludes high-molecular-weight compounds (Wessels 1993 and references). At the same time, sugars, amino acids and other small metabolites may be selectively and actively reabsorbed through the membrane transport system (Alberts et al. 1989) and thereby concentrated in the cytoplasm while non-absorbed secondary metabolites and other dissolved (unwanted) chemicals remain outside. If this is true, it is very reminiscent of a kidney function (described by Sun and Unestam, submitted) that may take place in the actively metabolizing hyphae of both hydrophobic and hydrophilic mycorrhizal fungi (see Figures 12 and 14). Formation of oxalate crystals ("kidney stones") on the cord (Fig. 7) or hyphal surface seems to be a result of this process.

From our observations and the results of other researchers, we propose seven different translocation systems for solutes in the extramatrical mycelium of ectomycorrhizal symbionts. *Long distance:* (1) A temperature-dependent bulk flow of dissolved sugar, e.g. through the vessels of the cord, out to the growing hyphal tip (Figs. 12, 13; Sun and Unestam, submitted), driven mainly by the turgor and sink of the apex (Jennings and Bravery 1991) and the gradient from the source in the green leaves via the short roots. A bulk flow of water and dissolved molecules from soil, mostly via vessels, into the mantle and cortex is also evident (Brownlee et al. 1983), although the importance of this route is controversial (Cairney 1992). (2) Metabolically driven peristaltic transport of dissolved nutrients such as polyphosphate in a vacuole chain (Ashford 1993). (3) Apoplastic wick transport of water and its solutes in hydrophilic cell walls, over considerable distances when in humid soil air (Unestam 1991 and Fig. 12). High hydraulic conductivity in soil containing disconnected, hydrophilic, mycorrhizal hyphae (as found by Lutger Eltrop, unpublished work) may therefore be carried out this way (our suggestion). *Short distance:* (4) Diffusion or cytoplasmic streaming, even in opposing directions (Olsson and Jennings 1991), in the symplast due to chemical gradients of specific compounds (Cairney 1992). (5) Organized metabolically driven transfer of molecules via cytoplasmic structures such as fibrils (Wessels 1993) and endoplasmic reticulum (as in soybean; Grabski et al. 1993). *Very short distance:* (6) Exocytosis of enzymes and other large molecules through the hyphal apex driven by hyphal growth (Wessels 1993). (7) Bridging between hyphae and soil via exuded water and gel. Both *L. laccaria* and *S. bovinus* produce such gels on the submersed hypha in our liquid cultures (unpublished result). Many of these systems (at least 1, 2, 4, and 5) will depend on accumulation of dissolved molecules by active uptake through the cell membrane, at the apex as well as the root-fungus interface. This mechanism is used by most living cells (Alberts et al. 1989).

Much of this is in agreement with Cairney's review (1992) on translocation in ectomycorrhizal and saprotrophic rhizomorphs. With radioactive tracers we have found no wicking along *S. bovinus* hyphae or strands growing on a plastic surface (to be published). If the hyphal walls contain and are cemented together by hydrophobins, neither the interhyphal space nor the cell walls are likely to wick water and participate in any apoplastic translocation in *ho* cords and hypae. Instead, the vessels may represent more or less water-tight pipes with repelling hydrophobic walls (which do not impede the flow of water molecules in the vessels but render an even higher efficiency) incapable of longitudinal translocation. If so, contacts and interchanges between the cord cortex and the vessel should be via intracordal cell wall pores and anastomoses.

We do not know much about the direction in which these routes are used and to what extent they function under varying circumstances, between soil and root, between different parts of a mat etc. Sugars, minerals, and water may be pooled to varying extents in the extramatrical mycorrhizal network and differentially redistri-

buted to different sinks. Thus, in the mycorrhizal system, carbon from the green parts of a seedling is translocated down in sequential bulk flows to the growing exploring and exploiting hyphal tips. But the feeder roots are sinks of water and cannot deliver much liquid to this acropetal carbon translocation; instead, water has to be taken from the basipetal inflow (Read 1991) and be shunted over to the outgoing carbon flow. The carbon will thus be diluted and the gradient increased towards the peripheral hypha (exploring and exploiting). There, the carbon will be used for growth and respiration and the excess water may be exuded through pores (Figs. 12, 14) and mixed with soil water, if available. This simplistic model may be most valid for the hydrophobic mycorrhiza.

## Ecological approaches

It seems that hydrophobicity among the mycorrhizal fungi contributes to S-selected (stress tolerant) properties of the examined forest fungi, since it may protect the fungus and the root from drought and to some extent against flooding stress (Unestam 1991). Drought demands a very efficient water transport (metabolically driven) in well-isolated, hydrophobic pipes. The short peripheral flow via hyphae and out through their cell walls is probably a limiting link in the chain of translocating structures. At the same time the formation of hydrophobic mats and patches probably excludes many other microorganisms in the same area (Unestam 1991), a C-selected (competitive) quality.

To utilize minerals from debris and soil particles, often very dry for example in a mat, the peripheral young hyphae may wet their environment by means of their exuded water, therepy supporting ion diffusion and uptake. Wetting will also support the activity of extracellular enzymes (released by exocytosis), phosphatases, phytases, proteases, phenoloxidases etc., which are of importance for mycobiont function in the peripheral, exploiting mycelium (Jennings and Bravery 1991). The *hi* fungi do not form proper mats but only discrete spider webs and have quite different strategies, often being ruderal, R-selected). They do not avoid or protect against waterlogging but seem often to prefer it (Table 1), and their activity and growth must suffer badly from drought. Their wicking translocation capacity of water may, however, be advantageous in a humid environment (e.g. in nurseries or new plantations with remaining slash) and increase hydraulic conductivity in soil, even without metabolic energy consumption.

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## References

Agerer R (ed) (1987–1991) Color atlas of ectomycorrhizae. Einhorn, Schwäbisch Gmünd

Agerer R (1988) Studies on ectomycorrhizae. XVII. The ontogeny of the ectomycorrhizal rhizomorphs of *Paxillus involutus* and *Thelephora terrestris* (Basidiomycetes). Nova Hedwigia Kryptogamenkd 47:311–334

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1989) Molecular biology of the cell, 2nd edn. Garland, New York London

Ashford AE, Shepherd V, Orlovich D (1993) Phosphorus transfer and vacuoles in mycorrhizas. Abstracts, 9th North American Conference on Mycorrhizae, University of Guelph, Ontario, Canada, p 70

Bending GD (1992) Distribution and function of the ectomycorrhizal mycelium. Abstracts, 9th North American Conference on Mycorrhizae, University of Guelph, Ontario, Canada, p 143

Brownlee C, Duddridge JA, Malibari A, Read DJ (1983) The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. Plant Soil 71:433–443

Cairney JWG (1992) Translocation of solutes in ectomycorrhizal and saprophytic rhizomorphs. Mycol Res 96:135–141

Danell E, Fries N (1990) Methods for isolation of *Cantharellus* species, and the synthesis of mycorrhiza with *Picea abies*. Mycotaxon 38:141–148

Duchesne LC, Ellis B E, Peterson RL (1989) Disease suppression by the ectomycorrhizal fungus *Paxillus involutus:* contribution of oxalic acid. Can J Bot 67:2726–2730

Dütsch GA (1978) Submerged culture of *Agaricus bisporus* in a syntheic medium. Bull Br Mycol Soc 12:119

Finley RD, Read DJ (1986) The structure and function of the vegetative mycelium of ectomycorrhizal plants. I. Translocation of $^{14}$-C-labelled carbon between plants interconnected by a common mycelium. New Phytol 103:143–156

Fries N, Bardet M, Serck-Hansen K (1985) Growth of ectomycorrhizal fungi stimulated by lipids from a pine root exudate. Plant Soil 86:287–290

Grabski S, Feijter AW de, Schindler M (1993) Endoplasmic reticulum forms a dynamic continuum for lipid diffusion between contiguous soybean root cells. Plant Cell 5:25–38

Griffin D (1981) Fungal physiology. Wiley, New York

Jennings DH, Bravery AE (eds) (1991) *Serpula lacrymans:* fundamental biology and control strategies. Wiley, New York

Lehninger AL (1975) Biochemistry, 2nd edn. Worth, New York

Olsson S, Jennings DH (1991) Evidence for diffusion being the mechanism of translocation in the hyphae of three molds. Exp Mycol 15:302–309

Rayner ADM (1991) The challenge of the individualistic mycelium. Mycologia 83:48–71

Read DJ (1991) Mycorrhizas in ecosystems. Experientia 47:376–391

Stenström E (1991) The effects of flooding on the formation of ectomycorrhizae in *Pinus sylvestris* seedlings. Plant Soil 131:247–250

Straatsma G, Bruinsma J (1986) Carboxylated metabolic intermediates as nutritional factors in vegetative growth of the mycorrhizal mushroom *Cantharellus cibarius* Fr. J Plant Physiol 125:377–381

Tsao Y-H, Evans DF, Wennerström H (1993) Long-range attractive force between hydrophobic surfaces observed by atomic force microscopy. Science 262:547–550

Unestam T (1991) Water repellency, mat formation, and leaf-stimulated growth of some ectomycorrhizal fungi. Mycorrhiza 1:13–20

Unestam T, Stenström E (1989) A method for observing and manipulating roots and root-associated fungi on plants growing in nonsterile substrates. Scand J For Res 4:51–58

Wessels JH (1993) Wall growth, protein excretion and morphogenesis in fungi. Tansley review no 45. New Phytol 123:397–413