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Epiphytic Algae and Fungi on Spruce Needles

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Abstract

The occurrence, increase and ultrastructure of epiphytic algae and fungi on aging spruce needles is described. The ultrastructure of these microorganisms is discussed with emphasis on their interrelationships concerning their nutrition. The influence of the epiphytes on the vitality of the spruce needles is discussed.

Keywords: ultrastructure, endocells, epiphytes, haustoria, spruce needles

1. Introduction

In recent years an increase in the amount of microorganisms on the surface of leaves and needles has been observed (Mathé, 1985; Ellenberg et al., 1986; Göransson, 1988). On annual leaves such organisms are shed with the leaves at the end of the season. On perennial needles, however, thick layers of microorganisms may accumulate over several years (Carroll, 1979; Steffens, 1987). On spruce trees bacteria, algae and fungi occur on needles in a moist microclimate with light wind and rather low irradiation (Burg, 1990). The occurrence of these microorganisms is thought to be associated to forest decline (Mathé, 1985; Waldner-Sander and Botzenhart, 1986). The question arises how these organisms are able to grow. Where do they get their nutrients from, are they epiphytes in the strict meaning of the word, or do they get nutrients from the tree?

"Dedicated to Prof. Dr. O.L. Lange on the occasion of his 65th birthday"

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In this paper we report on the occurrence and on the ultrastructure of these epiphytic microorganisms in order to obtain evidence as to whether they have an effect on the health of the needles and hence the tree.

2. Materials and Methods

Branches with epiphytes on their needles of different age were taken from spruce trees (*Picea abies* (L.) Karst.) in different areas in the northwestern part of Germany. For sampling details see Burg (1990).

For light microscopical observations, the epiphytes were scraped off the needles and covered with a drop of tap water.

For scanning electron microscopical observations, the needles with their epiphytes were air dried and sputter-coated with gold.

For transmission electron microscopical observations, the epiphytes were scraped off the needles and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) at 4°C for 3 hr and postfixed in 2% buffered osmiumtetroxide for 2 hr. The fixed material was dehydrated in a graded ethanol series and embedded in Epon according to Luft (1961). The ultrathin sections were cut with a Reichert ultracut E microtome using glass or diamond knives. The sections were stained with uranyl acetate and lead citrate.

Cellulose was identified by labelling with enzyme-gold complexes. The colloidal gold was prepared according to Frens (1973). Twenty mg cellulase, which was a 1,4-(1,3;1,4)-β-D-glucan-4-glucanohydrolase (EC 3.2.1.4) purified from *Trichoderma viride* (Yakult, Japan), were dissolved in 2 ml double distilled water, dialyzed against double distilled water and diluted to 3000 μl/ml. Ten ml gold sol, pH 5.5 adjusted with 0.2 M K_2CO_3, was coated with 250 μl cellulase solution ($\approx$ 75 μg enzyme/1 ml gold sol) and further stabilized with 1 ml of 10% bovine serum albumin (BSA, Sigma). After centrifugation at 17,000 rpm (Sorval SS-34-rotor) for 30 min at 4°C, the pellet was resuspended in 1 ml of 0.05 M citrate buffer, pH 5.0, containing 1% BSA. Labelling was performed in a moist chamber at room temperature by floating ultrathin sections collected on nickel grids upside down on drops of 0.05 M citrate buffer, pH 5.0, containing 1% BSA and 0.5% gelatin, for 5 min and in cellulase-gold sol (1:10 diluted) for 15 min. Sections were thoroughly washed with buffer, rinsed with double distilled water and finally conventionally contrasted.

Controls following Bendayan (1984): (1) Sections were treated with uncoated colloidal gold or with non-enzymatic protein A-gold suspension. (2) Preceding section labelling, the enzyme-gold sol used was first incubated with an equal portion of the corresponding substrates, carboxymethyl-cellulose

(Serva) in concentrations of 1 mg/ml. Polygalacturonic acid from orange, pectin from *Citrus*-fruits, and mannan from *Saccharomyces cerevisiae* (Sigma) were checked also. (3) Preceding section labelling, the substrates were enzymatically digested by cellulase solution. This was performed on sections mounted on gold grids using 1% cellulase solutions in citrate buffer, pH 5.0 for 24 hr at 40°C.

3. Results

Occurrence of microorganisms

The crust of microorganisms on the surface of the spruce needles occurs as a green to brown crumbling layer. Most of the algae are unicellular green algae belonging to the Chlorococcales (Fig. 1a). Most of the fungi are thin filamentous fungi belonging to the Ascomycetes (Fig. 1b).

The colonization by microorganisms starts at the end of the first season of a new needle in late autumn. Small colonies of algae are scattered on the upper surface of the needle, while fungal hyphae form a widely branched net (Fig. 2a). Algae and fungi are located very often close to the stomata and even in them (Fig. 2a). With increasing age of the needle the layer of microorganisms becomes increasingly thicker (Table 1). The algae rapidly propagate by autospores (Fig. 1a) and form thick clusters on the upper side of two-year-old needles (Fig. 2b). The fungal hyphae grow further and invade the lateral sides of the needles (Fig. 2b). At this stage of colonization most of the algae are still alive as shown by their bright green colour in the light microscope and by the red autofluorescence of the chlorophyll in the fluorescence microscope.

Three-year-old needles are almost totally covered by algae and fungi on the upper, as well as on large areas of the lateral sides, and even underneath (Figs. 2b and 2c). Most of the algae still appear as bright green cells, but colourless and collapsed cells are also present (Fig. 1b). The growth of the

Table 1. Thickness of the layer of epiphytes and portion of the covered surface of spruce needles in different ages. The data have been gained after measuring 286 needles.

	1-year-old	2-year-old	3-year-old	4-year-old
Thickness (μm)	< 30	50–100	70–120	90–130
Covered surface (%)	< 10	45–65	60–70	65–80

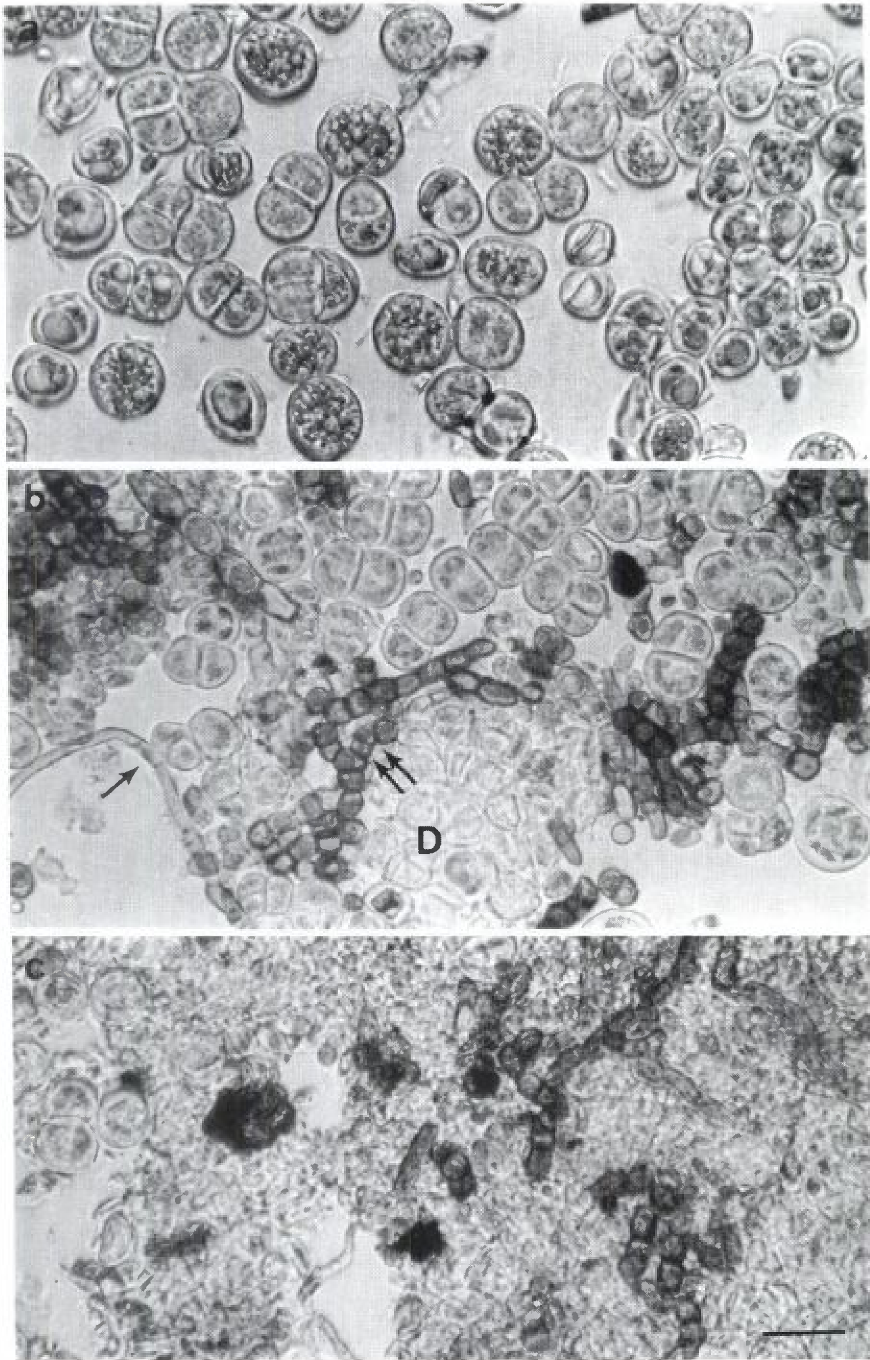

Figure 1

Figure 1. Epiphytes from spruce needles of different ages. Scale = 10 μm.
(a) Unicellular green algae in different developmental stages from a 1-year-old needle.
(b) Algae and filamentous fungi (arrow), some of which are darkly coloured (double arrow), from a 3-year-old needle. Next to apparently vital algae, dead cells (D) are present.
(c) Epiphytes including a high portion of dead organisms from a 5-year-old needle.

2d). The layer of microorganisms increases in thickness as does the proportion of needle surface it covers (Table 1). Simultaneously the proportion of dead organisms appears to be higher (Fig. 1c).

Ultrastructure of the microorganisms

The electron micrographs confirm the diversity of closely associated microorganisms (Fig. 3). Each of the unicellular algal cells is characterized by a large chloroplast (Fig. 4a). In some algae pyrenoids with pyrenoglobuli were observed within the chloroplast. Several storage bodies differentially contrasted occur around the chloroplast in all algal cells. The cell wall always appears as a rather thin, darkly stained layer (Figs. 3 and 4). An additional layer varying in thickness and of low contrast has been observed inside the dark one (Fig. 4b). Numerous cellulase-gold particles evenly distributed over the electron-dense outer layer, but not over the inner layer, indicate the presence of cellulose in the outer layer only. Outside the cellulosic layer, fine fibrillar material may be present. Close to a neighbouring fungus, it may become denser (Fig. 4a).

In the ultrathin sections, three different types of fungal hyphae have been distinguished (Fig. 5). First there are those hyphae with rather thick walls which appear to be two-layered in transmission electron microscope (Fig. 5a). The outer layer is of high contrast and about 30 nm thick, while the inner one is of lower contrast and about 110 nm thick. The second type also has two-layered walls but they are usually thinner (Fig. 5b). The outer layer, with a thickness of about 20 nm, is of high contrast, while the inner one, about 80 nm thick, is electron-lucent. Cellulase-gold labelling, specific to the inner wall layer and concentrated to its exterior region, indicates the presence of cellulose in the translucent layer only. The third type also has an electron-lucent inner wall about 50 nm thick, but the outer wall is very darkly stained and 70 to 120 nm thick (Fig. 5c). The darkly stained material seems to be loosened into small particles at the periphery of the wall. The protoplasts in all the fungi – as far as they can be considered as living cells following preservation for ultrastructural studies – are characterized by a dense cytoplasm with striking clusters of ribosomes. The plasmalemma is irregularly invaginated.

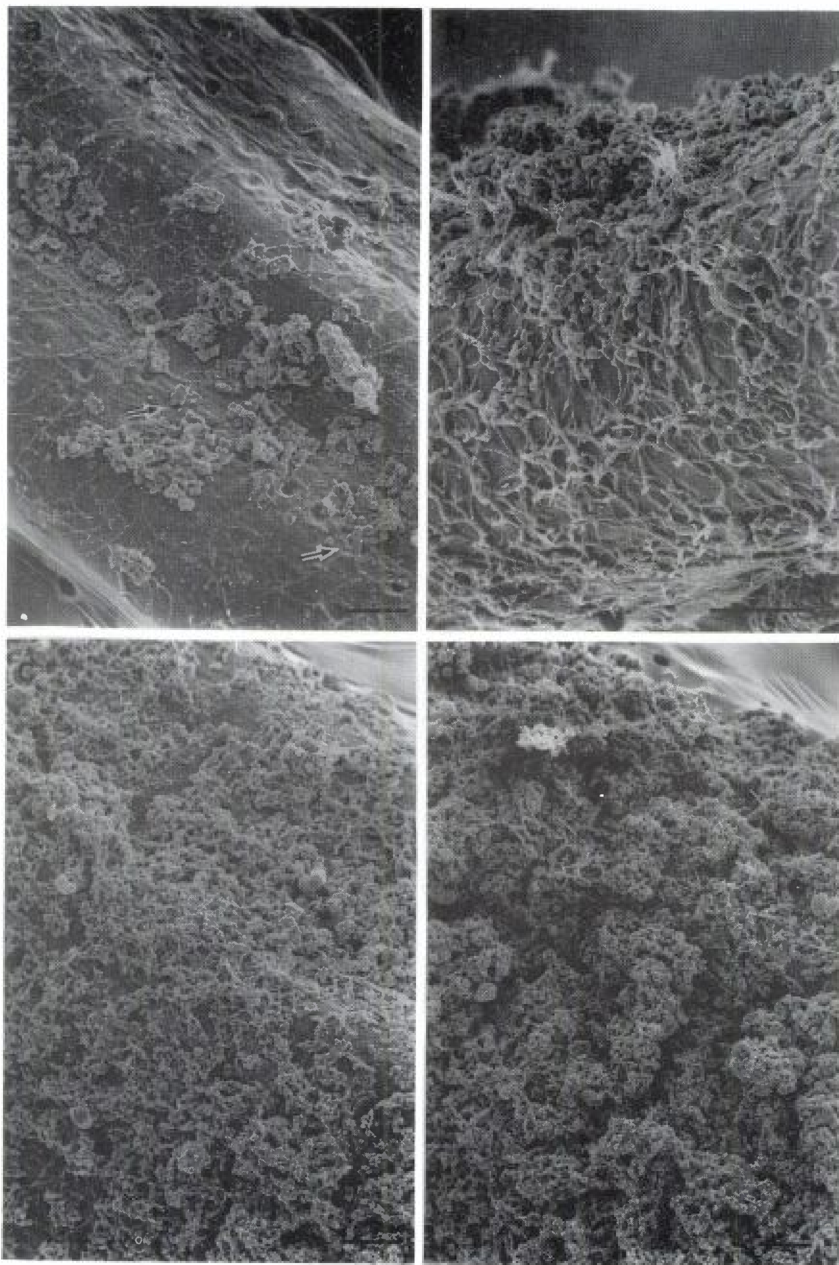

Figure 2. The increasing colonization by epiphytes of spruce needles of different ages. Scale = 100 μm.
(a) 1-year-old, (b) 2-year-old, (c) 3-year-old, (d) 4-year-old.
Note the location of the algal and fungal cells in or close to the stomata (arrows in (a).

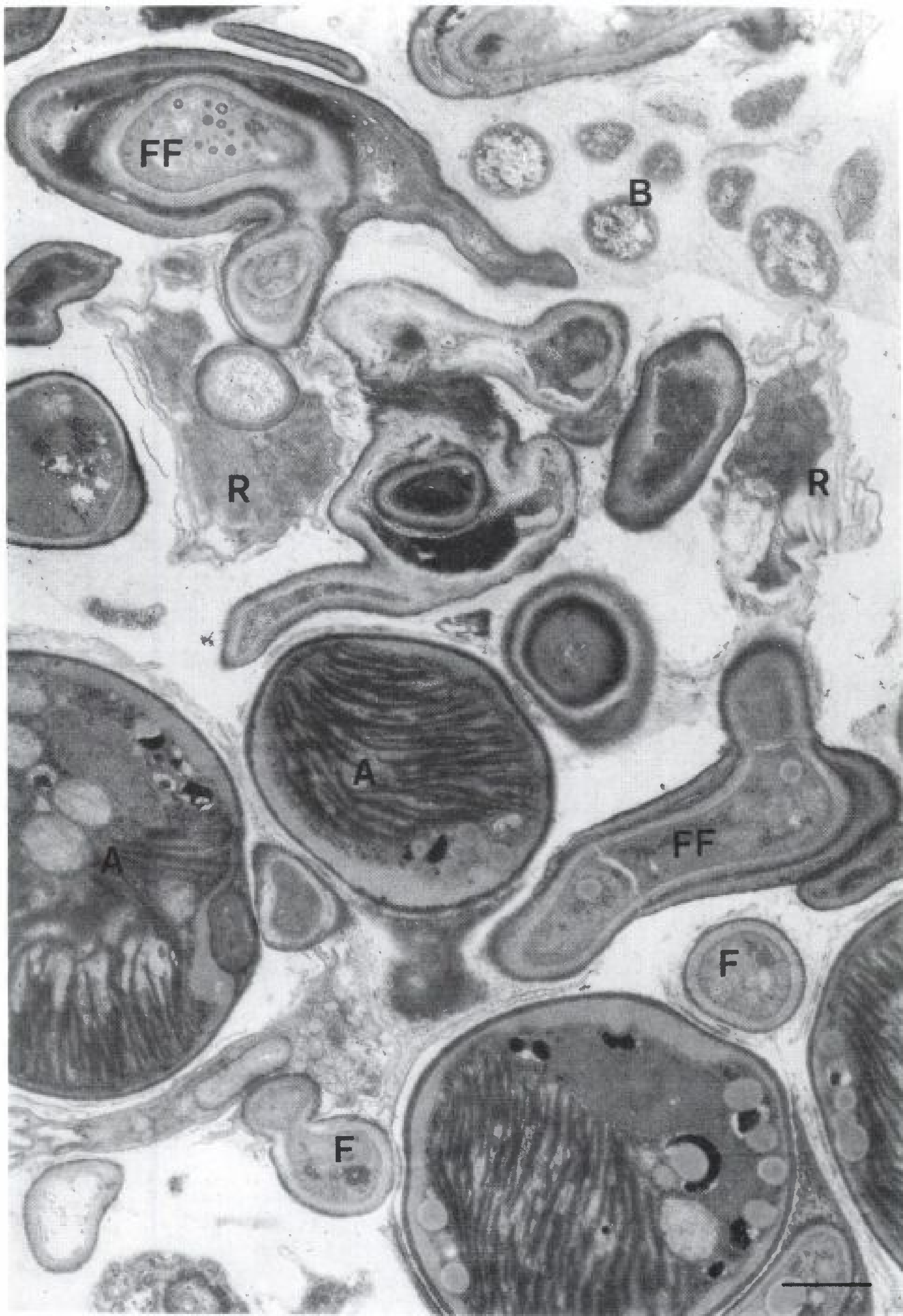

Figure 3. Ultrathin section through a cluster of epiphytes from a 3-year-old spruce needle. Among well preserved microorganisms, cellular remnants of their cells are present. A, alga, F, fungus, FF, fungus within fungus, B, bacteria, R, remnants, Scale = 1 μm.

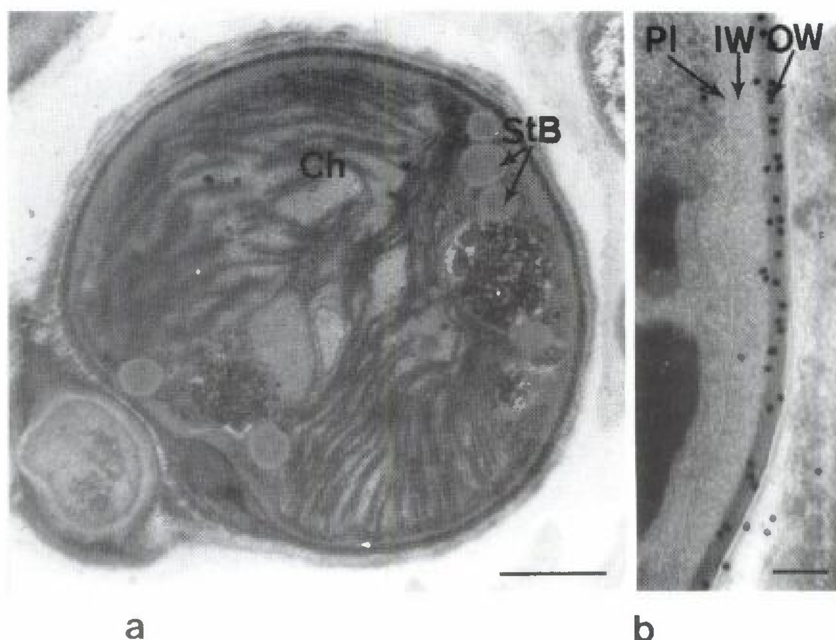

Figure 4. (a) A unicellular alga with typical ultrastructure. Ch, chloroplast, StB, storage bodies. Scale = 1 μm.
(b) Part of an algal cell wall after labelling with cellulase-gold sol. OW, outer wall layer, IW, inner wall layer, PI, plasmalemma. Scale = 0.1 μm.

Occasionally the nucleus, and in most sections mitochondria and groups of concentric bodies have been observed.

Structural interrelationships between the microorganisms

All the described algal and fungal cells appear in close proximity. Fungal cells can also be observed either in algal cells or within other fungal cells (Figs. 6 and 7), and fungal haustoria are very often present in the algae. These haustoria occur in algal cells with well preserved ultrastructure (Fig. 6a) as well as in cells with completely disorganized protoplast which are considered as dead. Sometimes such dead algal cells are full of storage bodies (Fig. 6b). There may be only one haustorium in an algal cell but several have also been found. In the contact region where the haustorium has penetrated into the

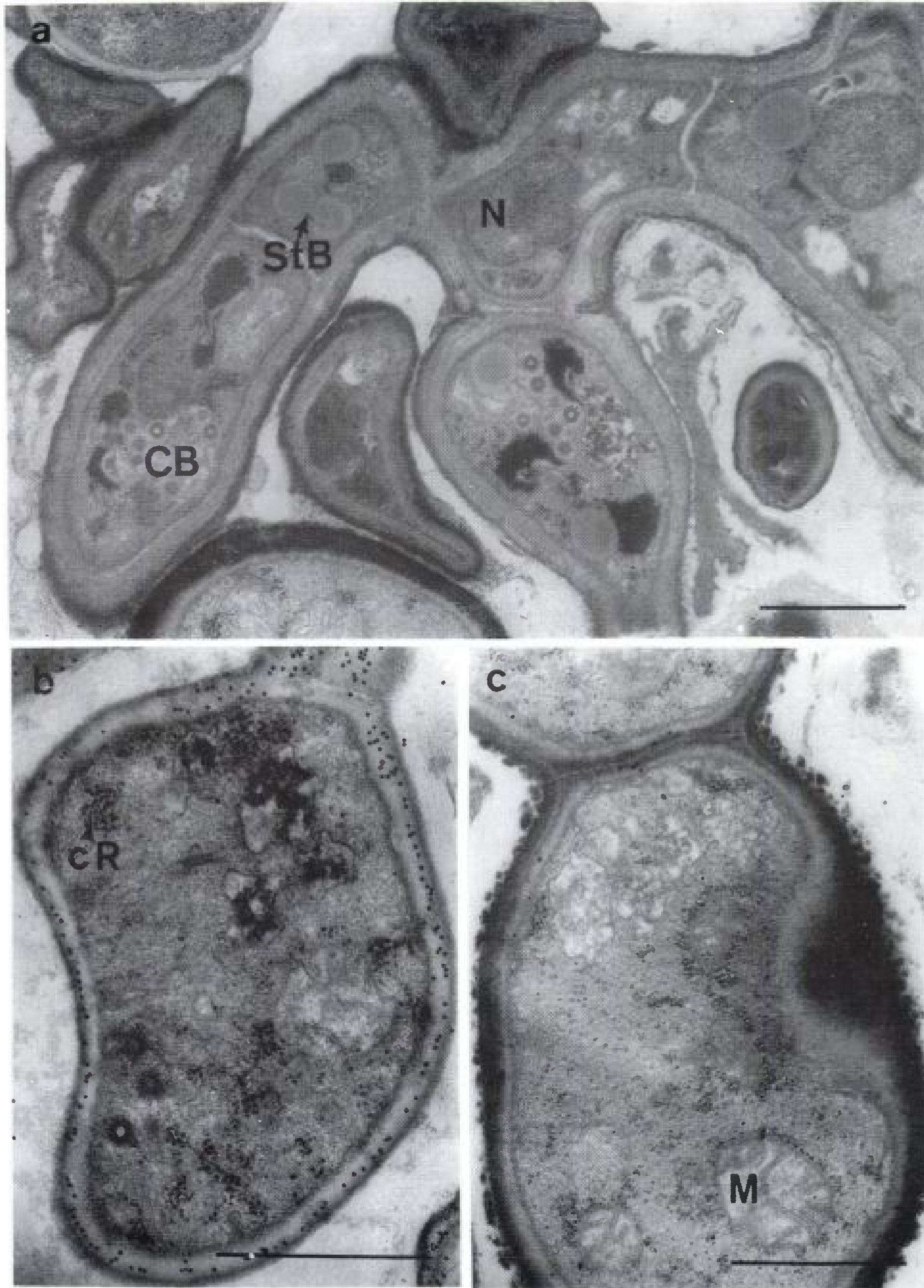

Figure 5. Different types of epiphytic fungi according to the differentiation of their cell walls. The fungus in (b) contains cellulose indicated by labelling with cellulase-gold sol. N, nucleus, M, mitochondrium, CB, concentric bodies, cR, clusters of ribosomes, StB, storage bodies. Scale = 1 μm.

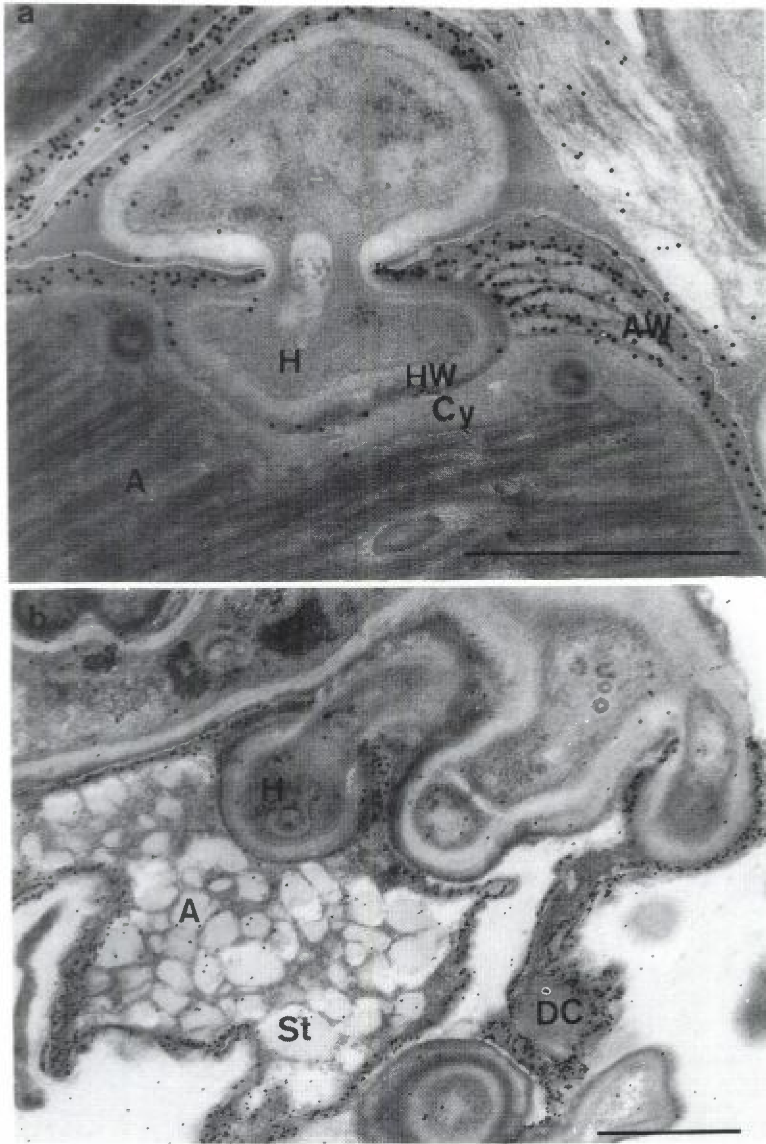

Figure 6. Fungal haustoria in algal cells. Scale = 1 μm.

(a) A haustorium (H) in a well preserved algal cell (A). Where the haustorium has penetrated, the hyphal wall (HW) lies closely to the cytoplasm (Cy) of the alga. The few gold particles in this host-fungus-interface may indicate cellulosic remnants of the algal cell wall.

(b) A haustorium in an algal cell (A) in which only storage bodies, most probably starch (St), are left.

Application of cellulase-labelled gold particles indicates the presence of cellulose around intact cells, a dead cell and remnants of cellulose from digested cells (DC).

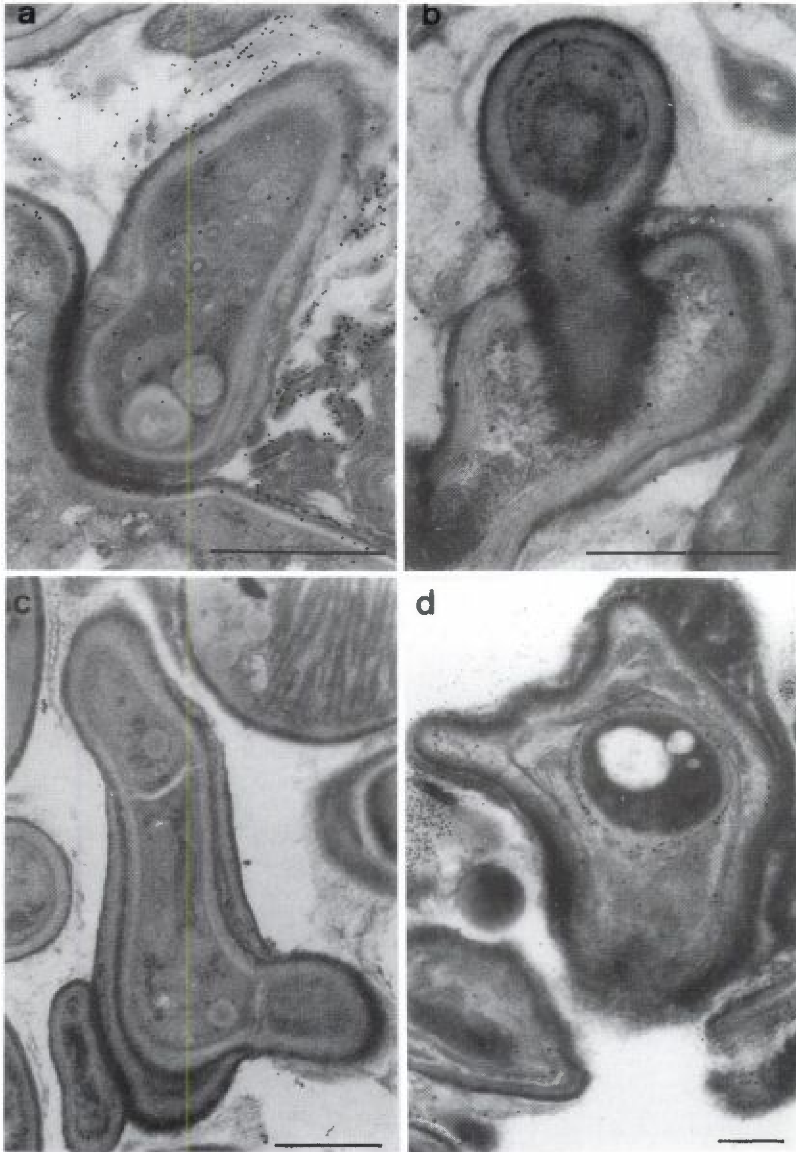

Figure 7. Diversity of fungi as endocells. Scale = 1 μm.
(a) A fungus with thick walls has deformed a hypha of a fungus with cellulose-containing cell walls.
(b) A fungus with thick walls after penetration in another one with an equivalent cell wall.
(c) Intrahyphal hypha in length view
(d) Cross sectioned fungus with cellulose-containing cell walls within another fungus with darkly stained cell walls.
(a), (b), and (d) after labelling with cellulase-gold sol.

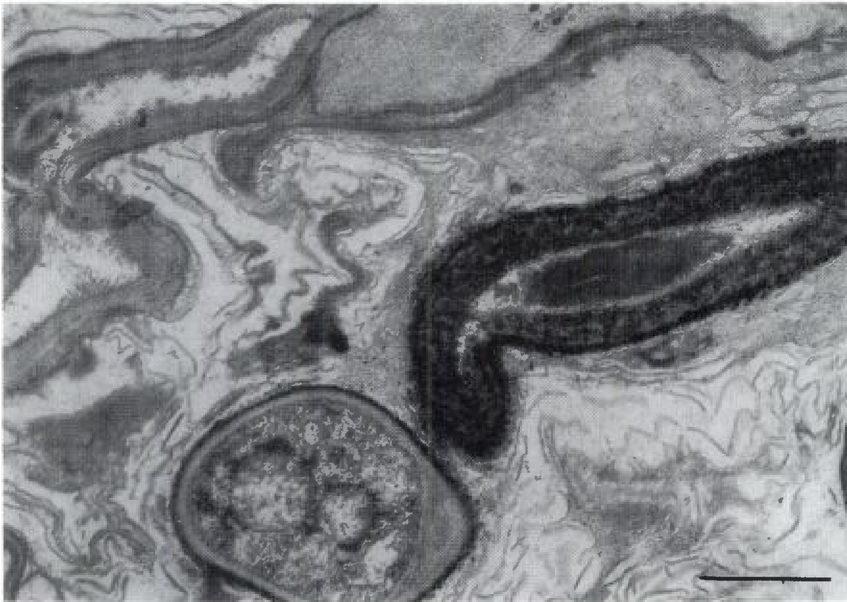

Figure 8. Remnants of epiphytes in different degrees of disintegration. Scale = 1 μm.

alga, the cellulose of the algal cell wall appears almost completely vanished as indicated by the very low labelling with cellulase-gold particles (Fig. 6a). In contrast, the haustorial wall appears of almost the same thickness as in the other parts of the fungal hyphae.

Fungal cells were observed within other fungi (Fig. 7). All of the different hyphal types were observed to occur in each other. Around the enclosed fungus, usually only a small border of the protoplast of the host hypha can be observed. It is impossible to comment on the vitality of such a host hypha.

The groups of bacteria are low in number in all observed ultrathin sections. The bacterial cells are embedded in a fibrous, very loose network (Fig. 3). Some of these cells were well preserved in their ultrastructure, while others were disorganized indicating that they are dead.

Within the layer of epiphytes from three-year-old and older needles, there is an increasing amount of dead organic material. In many cases the remains of algae and fungi are still recognizable in this dead material. The protoplasts may be clumped and the cell walls split into thin fibres (Figs. 3, 6b and 8). Very often only more or less dense fibrous or laminar material is left between the well-preserved cells. The very dark outer layer of one of the described fungal types is obvious (Fig. 8). The presence of cellulase-gold particles indicates

shapeless cellulosic material between the other unidentified remnants (Figs. 6b and 7a).

4. Discussion

The diversity in epiphytic microorganisms on spruce needles observed with the electron microscope corresponds to the results gained by Steffens (1987) with the light microscope. It is no problem to distinguish bacteria, unicellular green algae, and fungi and to determine the percentage of the single groups in proportion to the whole amount of epiphytes after counting in a Thomachamber (Steffens, 1987). In this context, a detailed knowledge of the different genera is not important, since we lay emphasis on the interactions between heterotrophic and autotrophic groups.

The quantity of algae on the needles increased greatly because they occurred in an appropriately moist microclimate (Burg, 1990) probably obtaining the needed minerals from depositions in the air. In particular, this seemed to be the case for nitrogen, since the occurrence of epiphytic algae correlates with the nitrogen emission (Göransson, 1988). In such eutrophic conditions, autotrophic algae were able to multiply rapidly. The thickness of the epiphytic layer on the observed needles was much greater than the 22 μm thick layer reported previously for tropical plants (Juniper and Jeffree, 1983).

The heterotrophic fungi seem to have at least three different sources of nutrients. First, they penetrate the algal cells forming haustoria, known as organs of nutrient absorption. Secondly, the intrahyphal hyphae (endocells), as previously described in endophytic fungi of spruce needles (Suske and Acker, 1989) and here in epiphytes, suggest a parasitism among the epiphytic fungi. This agrees with the view of most of the investigators who see the intrahyphal hyphae as a result of abnormal conditions for growth (Benhamou and Ouellette, 1987). Thirdly, fungi may utilize the dead organic material, indicating a saprotrophic nutrition.

All these observations give evidence that algae, fungi, and to a certain extent also bacteria, live in symbiotic to parasitic associations on the needle. But there are also indications that some of these epiphytes may hurt or even damage the outer epidermal wall beneath them. Evidence for this is derived from the observation that within the cell wall polysaccharides and lignin were identified in reduced amounts as compared with non-affected cells. In such situations lipophilic substances have been detected in increased amounts, and interpreted to be a spruce defense reaction in response to a fungal attack. In healthy trees such degradations of the needle surface has only been observed a few times (Tenberge, 1989; Tenberge and Peveling, 1991). Therefore the structural

influence is considered negligible as is the benefit the fungi may derive from the needle as a source of nutrition.

Another effect caused by the growth of epiphytes seems to be more important for the host tree. The quantity of the photosynthetic active irradiation usable by the needle has been shown to be reduced following colonization by epiphytes. Indeed, the rate of photosynthesis in spruce trees is decreased by epiphytes by approximately 4% of optimum (Burg, 1990). With respect to single needles enveloped in a thick, compact layer of epiphytes, the reduction in the photosynthetic rate was found to be up to 40% (Burg, 1990). Interference gás exchange was postulated by Juniper and Jeffree (1983).

In conclusion, the algae and fungi mainly live in symbiotic relationships on the needle without causing significant damage to the epidermal cell wall. However, for trees already weakened in health, the effect of these microorganisms on the vitality of the trees may be more important especially considering the cumulative effect of the reduced photosynthetic rate in covered needles.

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