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Ascophyllum and Its Symbionts. VII. Three-way Interactions Among *Ascophyllum nodosum* (Phaeophyceae), *Mycophycias ascophylli* (Ascomycetes) and *Vertebrata lanosa* (Rhodophyta)

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Ascophyllum nodosum (L.) Le Jolis has a systemic infection with the ascomycete *Mycophycias ascophylli* (Cotton) Kohlmeyer and Volkmann-Kohlmeyer with which it establishes a mutualistic symbiosis. In addition, *A. nodosum* is the host for the obligate red algal epiphyte, *Vertebrata lanosa* (L.) Christensen. Using light and electron microscopy we describe morphological and cytochemical changes occurring as a consequence of rhizoid penetration of *V. lanosa* into cortical host tissue. Rhizoids induce localized cell necrosis based on physical damage during rhizoid penetration. Host cells adjacent to the rhizoid selectively undergo a hypersensitive reaction in which they become darkly pigmented and become foci for hyphal development. Light and electron microscopy show that *M. ascophylli* forms dense hyphal aggregations on the surface of the *V. lanosa* rhizoid and extensive endophytic hyphal growths in the rhizoid wall. This is the first morphological evidence of an interaction between *M. ascophylli* and *V. lanosa*. We speculate that *M. ascophylli* may be interacting with *V. lanosa* to limit tissue damage to their shared host. In addition, the fungus provides a potential pathway for the transfer of materials (e.g., nutrients and photosynthate) between the two phototrophs.

Key Words: ascomycetes, *Ascophyllum nodosum*, epiphytism, *Mycophycias ascophylli*, marine fungi, mycophycobiosis, rhizoids, symbiosis, symbiotum, *Vertebrata lanosa*

INTRODUCTION

The epiphytic habit is common in marine algae. Indeed, hundreds of species have been recorded as epiphytes on seagrasses, kelps or even *Codium* species (Round 1981). Many of the epiphytes are obligate or facultative, and may be associated with one to many host species. Interactions between host and epiphyte vary, and range from the completely benign and haphazard to obligate mutualistic or parasitic associations. Whether or not a given epiphyte individual or species is parasitic depends upon the precise interaction of the epiphyte with the host, i.e. mechanism of attachment, extent of growth, and ability to extract nutrients from the host. In addition, relationships vary in terms of host specificity, with some epiphytic species being restricted to single hosts, and others growing on many hosts (Wahl 1989; Wahl and Mark 1999).

Ascophyllum nodosum (L.) Le Jolis is an abundant intertidal marine alga in the northwestern Atlantic Ocean, and within its range, vast expanses occur where it is the dominant species (Baardseth 1970; Cousins 1984). *Ascophyllum nodosum* is unusual among marine algae, other than crustose forms, because single fronds can be extremely long lived (e.g., over 20 years). *Ascophyllum* has a modular construction in which many erect axes are produced from a basal disk (Cousins 1982; Åberg 1989). Because of the high standing crops and long-lived nature of the individual fronds, *A. nodosum* would appear to be an excellent substratum for epiphytes (Garbary and Deckert 2001). Thus, it is not surprising that this species, along with some other Fucales, have an epidermal shedding process in which the entire outer layer of wall material is shed along with its associated cover of epiphytes (Filion-Myklebust and Norton 1981; Russell and Veltkamp 1984). Long-lived epiphytic species on *A. nodosum* such as *Vertebrata lanosa* (L.) Christensen [formerly *Polysiphonia lanosa* (L.) Tandy, see Choi *et al*. 2001] must be able to penetrate through the epidermal layer and attach, to avoid being shed with the epidermis (Garbary *et al*. 1991). The process of spore attachment by

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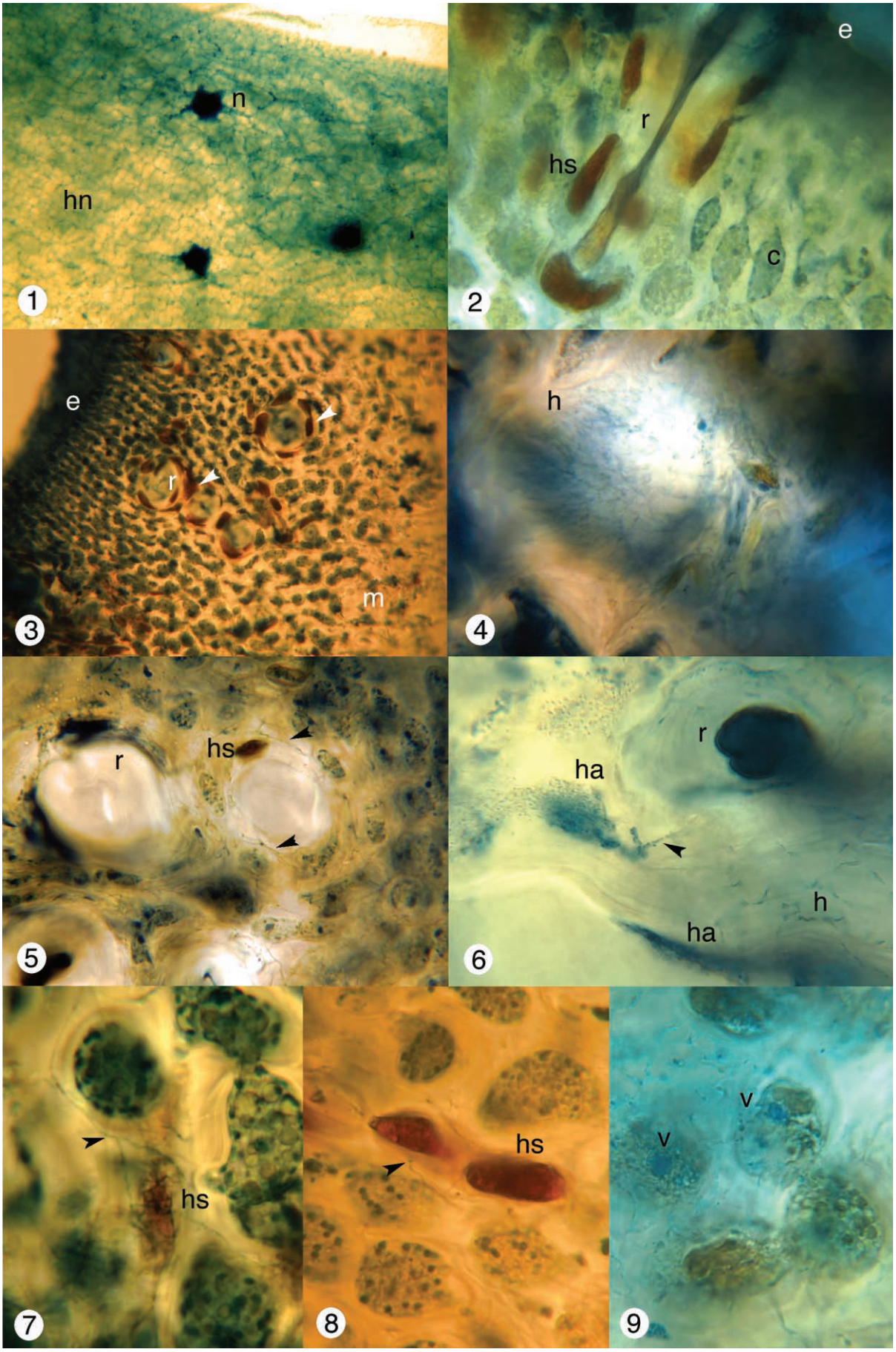
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V. lanosa and development of its penetrating rhizoids was described previously by Rawlence (1972) and Rawlence and Taylor (1970, 1972).

A further complication with *A. nodosum* is the presence of a systemic colonization by the ascomycete fungus, *Mycophycias ascophylli* (Cotton) Kohlmeyer and Volkmann-Kohlmeyer (1998) (formerly *Mycosphaerella ascophylli* Cotton). This fungus is universally present in *A. nodosum* plants in nature as well as in the related fucoid *Pelvetia canaliculata* (L.) Decaisne and Thuret (Kingham and Evans 1986). The symbiosis is initiated soon after zygote formation of A. *nodosum* (Garbary and MacDonald 1995; Garbary and Deckert 2001). There is no conspicuous parasitism associated with the fungus, and the relationship has been assumed to be mutualistic. Kohlmeyer and Kohlmeyer (1972, 1979) suggested that this symbiosis has many analogies with the lichen symbiosis. Experimental evidence to support this came from two fronts. First, Garbary and MacDonald (1995) showed that germinating zygotes of *A. nodosum* were morphologically different and grew faster when infected with *M. ascophylli* compared with uninfected zygotes. Second, Garbary and London (1995) demonstrated that the presence of *M. ascophylli* in developing embryos protects them from desiccation. More recently, Deckert and Garbary (2005) showed details of hyphal elaboration and association with host cells that make the comparison of the lichen analogy even more compelling than that suggested by Kohlmeyer and Kohlmeyer (1972). Given the description of the crustose brown alga *Petroderma maculiforme* (Wollny) Kuckuck as the lichen, *Verrucaria tavaresiae* Moe (Moe 1997; Sanders *et al*. 2004), the basis for considering *A. nodosum* a lichen is stronger than ever.

The presence of *M. ascophylli* in *A. nodosum* raises the possibility that the fungus may provide protection from parasitic invasion by bacteria, other fungi, and algal epiphytes, or produce secondary compounds that protect the host from herbivory. All of these would make the *Ascophyllum-Mycophycias* system analogous to the grass systems with their fungal endophytes [review by Bacon and Hill (1996)]. The term "symbiotum" is used to refer to this kind of association (Schardl *et al*. 1991), and we feel that this is appropriate for the *A. nodosum-M. ascophylli* association (Garbary and Deckert 2001; Deckert and Garbary 2005).

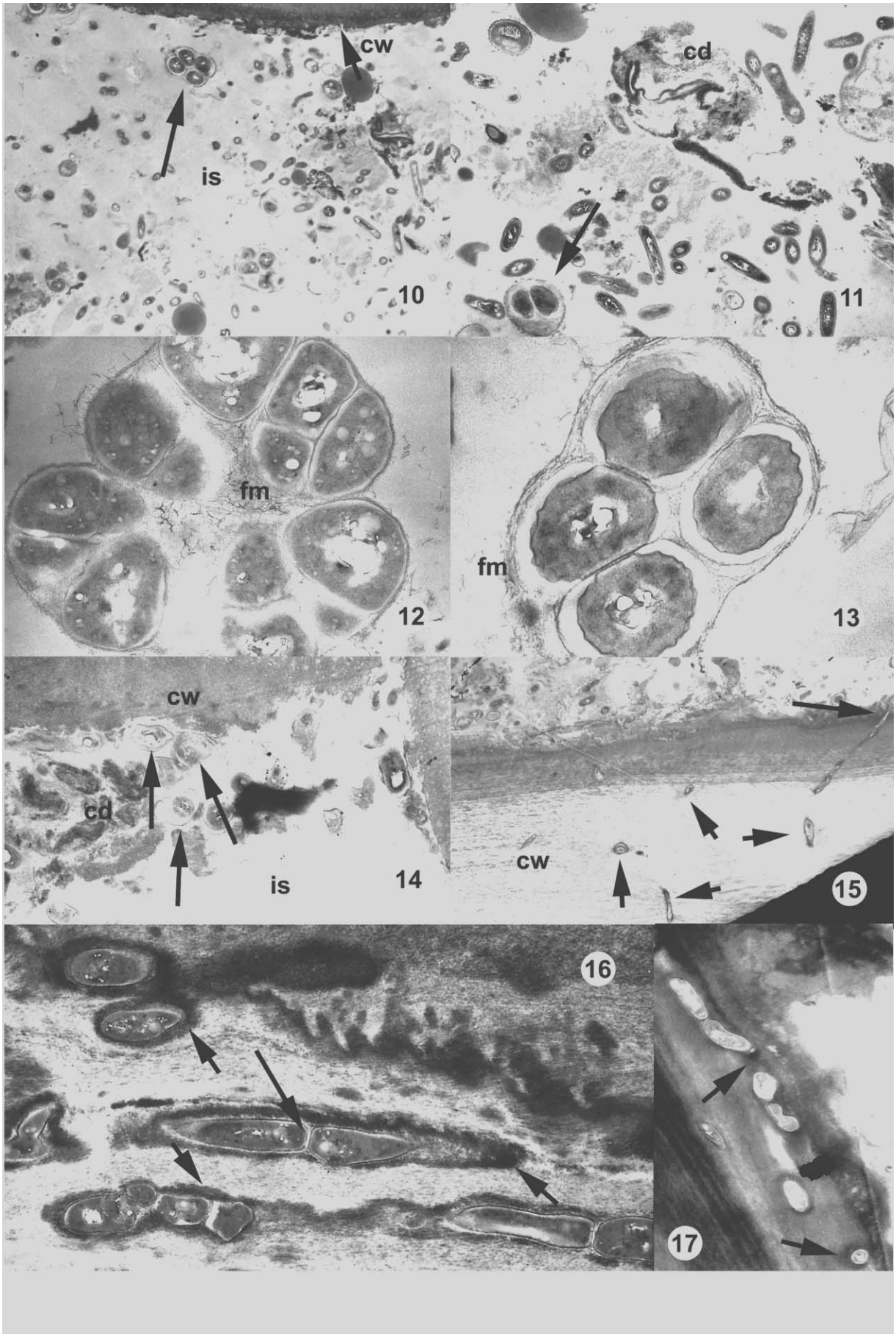
Elsewhere we described the basic structure of the *Ascophyllum* symbiotum in the absence of other associated algae (Deckert and Garbary 2005). Here we examine the morphological and cellular interactions of an obligate epiphytic species, *V. lanosa* on *A. nodosum*. *Vertebrata lanosa* is largely host-specific (however, see Rindi and Guiry 2004 regarding occurrence on *Fucus vesiculosus* L.), and the biology is highly integrated with that of its host (Garbary *et al*. 1991; Garbary and Deckert 2001). We use the new structural observations to explain previous physiological observations on this complex symbiosis. As a common epiphyte on *A. nodosum* virtually throughout its host range (South and Tittley 1986), *V. lanosa* provides a model system for investigating interactions of an epiphyte with the *Ascophyllum* symbiotum.

MATERIALS AND METHODS

Light microscopy

Five plants each of *Ascophyllum nodosum* with endophytic *Mycophycias ascophylli* and epiphytic *Vertebrata lanosa* were collected at Tor Bay Provincial Park, Guysborough Co., Nova Scotia (49.19°N 61.34°W) in August 2000. All plants were from the mid intertidal zone where *A. nodosum* is abundant. Ten fronds hosting the epiphyte were chosen haphazardly, and thalli were hand sectioned and stained with trypan blue (0.05 %) in lactoglycerol (1:1:1, lactic acid:glycerol:H_2O) for 0.5 to 12

Figs 1-9. Interactions of *Ascophyllum nodosum*, *Mycophycias ascophylli* and *Vertebrata lanosa*. All photographs follow staining with trypan-blue. Fig. 1. *M. ascophylli* in *A. nodosum* with host cells cleared using KOH treatment showing extensive hyphal network (hn) and three hyphal nodes (n). × 100. Fig. 2. Rhizoid (r) of *V. lanosa* embedded in cortex of *A. nodosum* and surrounded by several cortical cells (c) having undergone hypersensitive reaction (hs). × 400. Fig. 3. Transverse section of frond of *A. nodosum* showing thallus epidermis (e), medulla (m) and three rhizoids (r) of *V. lanosa* surrounded by host cells showing hypersensitive reaction (arrow heads). × 100. Fig. 4. Optical section of rhizoid of *V. lanosa* showing abundant endophytic hyphae (h) in cell wall. × 200. Fig. 5. Transverse sections of several rhizoids (r) of *V. lanosa* with cell of *A. nodosum* having undergone hypersensitive reaction (hs) and showing hyphae surrounding rhizoid (arrow heads). × 350. Fig. 6. Transverse sections through adjacent rhizoids (r) of *V. lanosa* with hyphal aggregates (ha) on rhizoid surface connected to extensive network of endophytic hyphae (h) in rhizoid wall (arrowhead). × 550. Fig. 7. Portion of cortex of *A. nodosum* with hyphal network (arrow head) and many hyphae associated with hypersensitive cell (hs). × 750. Fig. 8. Two adjacent cells of *A. nodosum* following hypersensitive reaction (hs). Note attached hypha with terminal swelling (arrow head). × 600. Fig. 9. Two cortical cells of *A. nodosum* following penetration of protoplast by hyphae and development of fungal vesicles (v) inside cells. × 650.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h prior to observation. In addition, portions of plants were cleared with 1 M KOH, changed daily, in an oven at 60°C for 48-96 h prior to staining with trypan blue (Deckert and Garbary 2005). Hand sections or whole mounts of cleared material were observed using bright field or phase contrast optics on a Zeiss Photomicroscope III. Images were captured using a Snap2 low-light, digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) in Adobe Photoshop, implemented on a Macintosh G4 platform.

Transmission electron microscopy

Field-collections of *V. lanosa* were made from Tor Bay Provincial Park in October and November 2000. The erect axes were removed and cubes of rhizoids and surrounding tissue of *A. nodosum* ca. 2 mm^3 were excised and fixed in a solution of 5% gluteraldehyde, 2% paraformaldehyde, 3% NaCl in phosphate buffer (pH 7.4) for 18 h. Material was post-fixed in a solution of 1 % OsO_4, 37% NaCl in buffer for 1 h, dehydrated in an ethanol series, and embedded in either Spurr's, low-viscosity resin or in LR White resin. The blocks were trimmed and then sectioned using glass knives made with an LKB 7800 knife maker. Gold-silver sections were cut using a Porter-Blum MT-2 ultramicrotome and collected on precleaned mesh copper grids. Grids were stained with uranyl acetate for 1 min and lead citrate for 2 min. Sections were observed using a Phillips 300 transmission electron microscope at 60-80 kv. Images were initially captured on film; however, the negatives were digitized using a scanner and then processed using Adobe Photoshop as described above.

RESULTS

Light microscopy

In the absence of *Vertebrata lanosa*, hyphae of *Mycophycias ascophylli* form an extensive and highly regular network throughout the host thallus (Fig. 1). Groups of hyphae surround the base of each epidermal cell and form noninvasive associations with most cortical and many medullary cells of the host. Concurrent with the development of erect axes, the germinating spore of *V. lanosa* produces an initial rhizoid that penetrates through the epidermis and into the host cortex and medulla (Figs 2-3). The rhizoid is approximately 40 μm wide and the protoplast comprises the central 10-20 μm of the cell (Figs 2-3). Subsequently, multiple rhizoids develop from the basal cells of erect axes. Thus, a cluster of rhizoids attaches each individual of *V. lanosa*. The rhizoids encounter and develop an intimate association with the host fungal endophyte (Figs 4-6).

As each rhizoid pushes through the host tissue, host cells in the immediate path become pushed aside, stretched, and eventually degraded (Figs 2, 3, 5). A number of cells adjacent to each rhizoid become modified such that they turn brown in whole or in part. These cells are foci for fungal hyphae and they display increased nearby hyphal proliferation compared to normal cells (Figs 7-9). At least one hyphal tip becomes enlarged, resembling an appressorium, after contact with these cells (Fig. 8), and hyphae occasionally invade the host cell protoplast, producing hyphal vesicles (Fig. 9). Intracellular fungal structures were never observed in healthy cells, but it is not clear whether the fungus is reacting to, or initiating, cell necrosis.

As rhizoids of *V. lanosa* grow they also encounter the hyphal structures of *M. ascophylli*. One to many hyphae may grow around the exterior rhizoid wall (Fig. 5), and extensive hyphal aggregations form on the rhizoid wall (Fig. 6). Hyphal aggregations are continuous with hyphae leading into the intercellular spaces of the host, hyphae that grow on the rhizoid surface and hyphae that penetrate and proliferate inside the rhizoid wall (Fig. 6). Optical sections through all parts of rhizoids show hyphal profiles. We did not observe hyphae penetrating

Figs 10-17. Transmission electron micrographs of *Mycophycias ascophylli* endophytic in intercellular spaces of *Ascophyllum nodosum* and in the rhizoids of *Vertebrata lanosa*. Fig. 10. Numerous hyphal profiles of *M. ascophylli* in intercellular spaces (is) of *A. nodosum* in area adjacent to cell wall (short arrow) of *V. lanosa*. Long arrow indicates section through hyphal aggregate. × 2000. Fig. 11. Intercellular space adjacent to rhizoid of *V. lanosa* with numerous hyphal profiles and some cellular debris (cd). Arrow indicates section through two-celled portion of hyphal aggregate. × 4000. Figs 12-13. Sections through well developed hyphal aggregates of *M. ascophylli*. Note differentiated wall layers and fibrillar material (fm) on surface of cells. × 25,000, × 30,000. Fig. 14. Profiles of hyphae (arrows) adjacent to cell wall (cw) of *V. lanosa with* an accumulation of cell debris (cd) and considerable intercellular space (is). × 16,000. Fig. 15. Section through extensive portion of cell wall (cw) of *V. lanosa* with hypha penetrating from outside (long arrow) and several hyphal profiles (short arrows) in cell wall. × 3000. Fig. 16. Heavily infected portion of cell wall of *V. lanosa*. Note glancing section of pore apparatus in septum (long arrow) and electron dense areas of host cell wall around hyphae (short arrows). × 15,000. Fig. 17. Outer portion of cell wall of *V. lanosa* with extensive development of endophytic *M. ascophylli*. Note electron dense host cell walls adjacent to hyphae (arrows). × 6000.

into the rhizoid protoplast, and there was no indication of damage to the protoplast of *V. lanosa* as a consequence of fungal growth in the cell walls.

Transmission electron microscopy

Transmission electron microscopy (Figs 10-17) confirmed much of the interaction between *M. ascophylli* and *V. lanosa* as observed with light microscopy. Thus sections through the intercellular spaces surrounding the rhizoid of *V. lanosa* showed numerous cell profiles of *M. ascophylli* in various planes. The hyphal network so apparent in parts of *A. nodosum* not infected with *V. lanosa* (see Deckert and Garbary 2005) was not visible in the spaces surrounding the rhizoids. In the intercellular spaces around the rhizoids the hyphae had no particular orientation (Figs. 10, 11), although hyphal aggregates of 2-4 cells were common (Figs 10, 11, 13) with larger aggregates (Fig. 12) being less frequent. All cells in these aggregates were round to oval to polygonal, and they were never cylindrical. Hyphal aggregates had a specialized cell wall that included an outer layer of electron dense fibrillar material that continued into the intercellular spaces surrounding the cells (Figs. 12, 13). It is unclear if the hyphal aggregates develop from the hyphal nodes in the host cortex (Fig. 1), or if they are specialized structures that form only in association with the rhizoids of *V. lanosa*. Little cellular detail was apparent within the free hyphae; however, hyphae in the aggregates commonly had less electron dense spherical structures as well as irregularly shaped clear areas that we interpret as storage bodies. Many hyphae were tightly adpressed against the rhizoid of *V. lanosa* (Fig. 14) and many were associated with cell debris (Figs. 11, 14).

The cell wall of *V. lanosa* was highly organized with numerous transversely aligned fibrillar regions, and it typically had an electron dense outer layer (Fig. 15). Hyphae were occasionally observed running into the wall of *V. lanosa*, and hyphal profiles within the host cell wall were numerous. In some sections extensive hyphal development occurred parallel to the rhizoid surface (Figs 16, 17). An electron dense layer typically surrounded hyphae endophytic in the rhizoid cell walls (Figs 16, 17).

DISCUSSION

Here we report novel details of the association between *Mycophycias ascophylli* and *Vertebrata lanosa*, and between *Ascophyllum nodosum* and *V. lanosa* using both light and electron microscopy. Previously, the relationship between *V. lanosa* and *A. nodosum* was studied extensively from ecological, physiological and ultrastructural perspectives (review by Garbary and Deckert 2001); however, the association between *V. lanosa* and *M. ascophylli* was entirely overlooked. Light and electron microscopic studies by Rawlence (1972) and Rawlence and Taylor (1970, 1972) showed details of rhizoid morphology and development, and also pointed out the host necrotic cells. However, these studies failed to observe the hyphae of *M. ascophylli* either in the host tissue or associated with the rhizoid of *V. lanosa*. Thus, there was little insight into the potential interaction between *V. lanosa* and *A. nodosum* that might be associated with *M. ascophylli*.

Carbon fixed by *A. nodosum* as well as inorganic nutrients can be transported to the epiphyte from the host or to the host from the epiphyte (Citharel 1972; Penot 1974; Penot and Penot 1974; Harlin and Craigie 1975; Ciciotte and Thomas 1997). Rates of exchange vary from no more than can be accounted for by diffusion to an apparently directed transfer of photosynthate in either direction. Regardless, *V. lanosa* appears to be an independent photosynthetic organism. The settlement success of *V. lanosa* on *A. nodosum* versus other fucoids has been explained based on fluid dynamics and the occurrence of appropriate settlement sites (Lobban and Baxter 1983; Pearson and Evans 1990). The relative host specificity of *V. lanosa* for *A. nodosum* was explained by Garbary et al. (1991) and Tian and Garbary (1992) as a result of ecological factors that restricted recruitment onto *A. nodosum* rather than a biochemical dependency of *V. lanosa* for *A. nodosum*. This is consistent with the recent observations of *V. lanosa* epiphytic on *Fucus vesiculosus* in Ireland (Rindi and Guiry 2004).

Our observations on the *A. nodosum*, *V. lanosa* and *M. ascophylli* system suggest a new interpretation of the interactions among the three species. First, *V. lanosa* is a minor parasite of *A. nodosum*. As previously reported by Rawlence and Taylor (1972) some host cells are damaged by rhizoid penetration. Thus cortical and medullary host cells in the direct path of the rhizoid become distorted and necrotic.

A second phenomenon that is likely part of the parasitic syndrome is the transformation of selected host cells adjacent to the rhizoid that become deep brown. This colour is attributed to an increase in polyphenolics that was demonstrated in similar responses in fungal-land plant interactions (e.g., Stone *et al*. 1994; Deckert *et*

al. 2001). Cells and tissues of *A. nodosum* (and other fucoids) typically have high levels of these compounds (e.g., Pearson and Evans 1991; Schoenwaelder 2002), and these levels can be further increased by damage (e.g., Pavia *et al*. 1997). The transformed cells of *A. nodosum* are invested and occasionally penetrated by hyphae. It is unclear if the fungus or the host induces the transformation of these cells. Regardless, the appearance of these transformed host cells is suggestive of the well-known hypersensitive reaction in terrestrial plants where hosts attempt to limit the growth and establishment of microbial parasites (e.g., Adaskaveg 1992). A characteristic of hypersensitive responses in some systems studied is the prerequisite of fungal presence; wounding alone is not sufficient to initiate the response (Kuc 1983). In addition to providing direct chemical deterrence, localized cell necrosis also may interrupt the symplastic flow of nutrients toward the rhizoid. This coordinated response suggests that *A. nodosum* and *M. ascophylli* are acting like a single organism to restrict the invading rhizoids of *V. lanosa*. Cooperation by the symbionts would have the advantage of bringing to bear two different detection and defensive systems with an enhanced suite of potential elicitors and response behaviour.

The association between *M. ascophylli* and the rhizoid of *V. lanosa* may be a strategy of the fungus to restrict rhizoid and epiphyte growth. Deckert and Garbary (2005) suggested that the dense aggregations of fungal hyphae (the nodes) in the cortex of *A. nodosum* might function as collection and redistribution centres for nutrients and other compounds of specialized function, such as osmoregulators that were gathered from the surrounding hyphae (especially the net hyphae in the host cortex). If this is the case, then the hyphae penetrating the thick rhizoid wall may be reabsorbing nutrients that would otherwise pass into the rhizoid protoplast and then into the rest of the *V. lanosa* thallus. It is possible also that these wall hyphae may be withdrawing nutrients from *V. lanosa*. Thus *M. ascophylli* may be restricting nutrient transfer to *V. lanosa*.

Variable development of *M. ascophylli* in rhizoids of *V. lanosa* may explain the diversity of results reported in nutrient exchange experiments between *A. nodosum* and *V. lanosa*. Thus the apparently incongruent results on nutrient exchange of Harlin and Craigie (1975) who reported transfer rates no more than could be accounted for by diffusion, and Ciciotte and Thomas (1997) who reported considerable active transport in both directions (ca. 10% of total 14C label), may be explained by differential infection of the *V. lanosa* rhizoids by *M. ascophylli*. In studies of phosphate transfer, Penot *et al*. (1993) showed one directional movement from *A. nodosum* into *V. lanosa*. Accordingly, *M. ascophylli* in some situations may be acting as a harvest symbiont with *V. lanosa*. It would be of interest to examine uptake rates of various compounds into *V. lanosa* after antibiotic treatment that restricts fungal metabolism.

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