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Ascophyllum and its symbionts. VI. Microscopic Characterization of the *Ascophyllum nodosum* (Phaeophyceae), *Mycophycias ascophylli* (Ascomycetes) Symbiotum

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Optical microscopy of recently living and cleared material of the fucoid, *Ascophyllum nodosum* (L.) Le Jolis, revealed novel aspects of its interaction with the ascomycete *Mycophycias ascophylli* (Cotton) Kohlmeyer and Kohlmeyer (previously *Mycosphaerella ascophylli* Cotton). Most host cells are associated with hyphae by lateral attachment of cell walls. Hyphae form extensive networks throughout the host thallus and show considerable differentiation in the various host tissues. In the base of epidermal cells, hyphae form multicellular rings around each host cell to produce a continuous network. In medullary regions, long, relatively unbranched and longitudinally aligned hyphae occur, with radial branches extending into cortical regions. Scattered in the inner cortex of host tissue are numerous multicellular nodes of smaller, polygonal to irregular shaped cells with five or more radiating arms of hyphae. Individual hyphal cells show a variety of specializations including swellings and appressoria-like attachments to some host cells. These observations provide the morphological basis for the mutualistic symbiosis supported by recent experimental work. We conclude that this association is best described by the term "symbiotum."

Key Words: anatomy, *Ascophyllum*, Fucaceae, *Mycophycias*, mycophycobiosis, symbiosis, symbiotum

INTRODUCTION

Ascophyllum nodosum (L.) Le Jolis can be considered the most successful marine intertidal alga in the North Atlantic Ocean. It has a wide geographic distribution across several climatic zones on both sides of the Atlantic Ocean. It occurs in a wide range of habitats from estuaries and salt marshes to all but highly wave exposed habitats. In many sites it can be the dominant organism where it forms continuous stands in which overlapping fronds completely cover the substratum (Baardseth 1970; Chapman 1995). The basis for this success is difficult to explain; however, several studies suggest that the interaction of *A. nodosum* with its obligate endosymbiotic fungus, *Mycophycias ascophylli* (Cotton) Kohlmeyer and Volkmann-Kohlmeyer (=*Mycosphaerella ascophylli* Cotton) (Cotton 1909; Kohlmeyer and Kohlmeyer 1972, 1979; Garbary and Gautam 1989; Kohlmeyer and Volkmann-Kohlmeyer 1998) provide evidence for the success of the host fucoid (Garbary and MacDonald 1995; Garbary and London 1995; Garbary and Deckert 2001).

Fungi are often found in intimate association with photosynthetic organisms, frequently as parasites. Remarkable interactions have been documented in which the association is so interdependent that it can be considered a composite organism. Grasses of the genera *Festuca* and *Lolium* associate with endophytic fungi of *Neotyphodium* spp. in combative-biochemical mutualisms that, to the host, confer resistance to herbivory, contribute to drought tolerance, improve competitive ability and enhance growth (Bacon and Hill 1996). The mycobiont in this case has forgone sexual reproduction and is solely transmitted vertically through host lines (Clay 1988). *Neotyphodium* is thus dependent on host fitness for the successful maintenance of its own germ line. Its influence, however, extends well beyond the host into the general community (Clay 1990; Clay and Holah 1999; Omacini *et al.* 2001; Rudgers *et al.* 2004) and it can therefore be considered a keystone species (Power *et al.* 1996).

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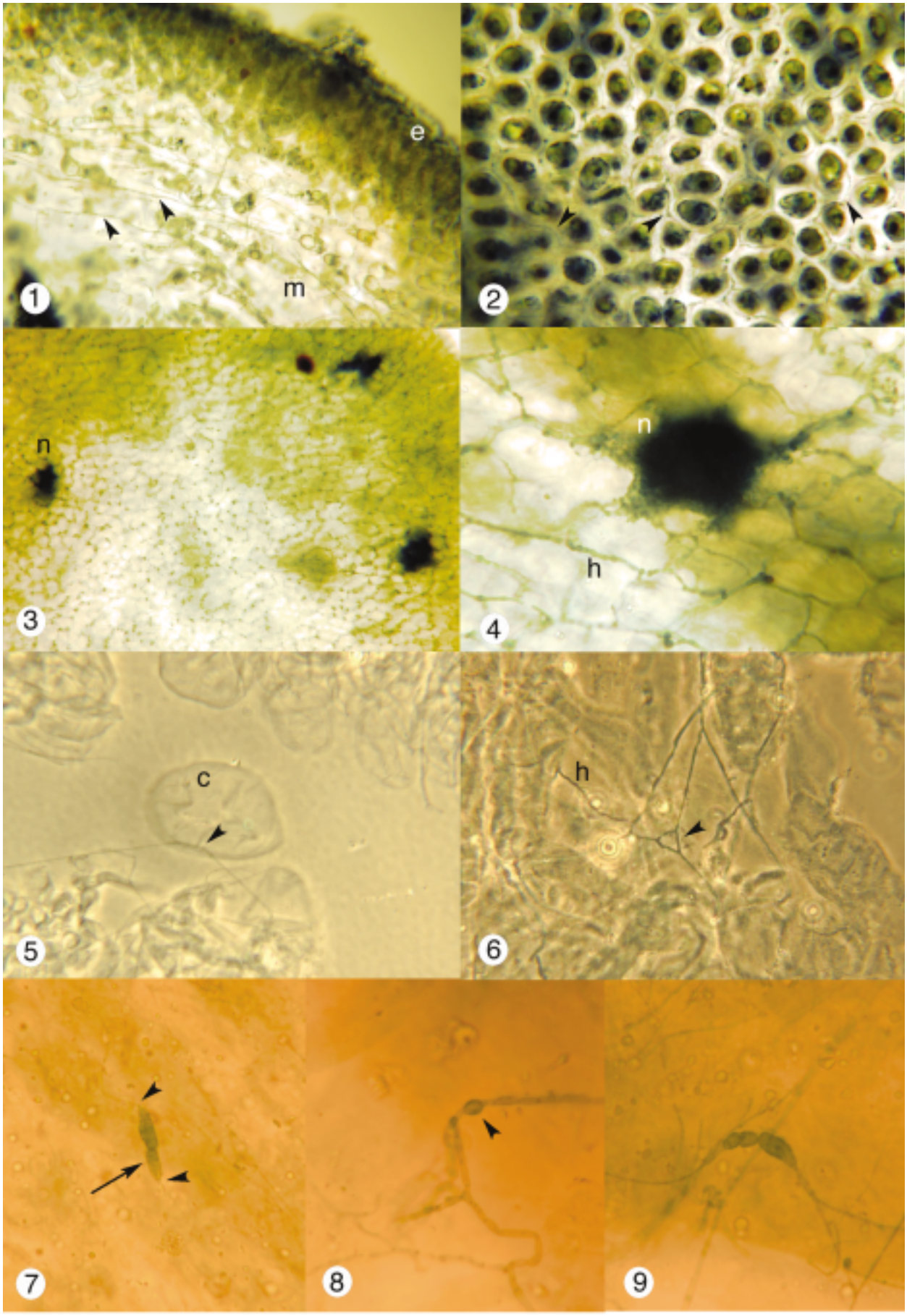
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The adaptive success of the association between species of eukaryotic algae and fungi, and occasionally cyanobacteria, to form lichens is exemplified by the continued success of these ancient composite organisms. Lichens tolerate rapid and extreme environmental fluctuations in temperature and moisture. In tandem, the mycobiont and photobiont often colonize habitats too harsh for either symbiont on its own, and where few other organisms succeed (Nash 1996). In lichens, the fungus provides a fairly stable environment for the algal cells, which, in turn, supply the fungus with fixed carbon. In addition, if cyanobacteria are present, the fungus receives nitrogen. The thallus characteristic of any particular lichen species is only produced when the alga and fungus live symbiotically (Büdel and Scheidegger 1996), and axenic culture of the symbionts is only possible in contrived laboratory conditions (Stocker-Wörgötter 1995). They are, in other words, ecologically obligate.

Kohlmeyer and Kohlmeyer (1972) coined the term "mycophycobiosis" to describe symbioses between species of macroalgae and fungi in which the photobiont is the exhabitant and the fungus is contained within the algal thallus. The term refers to interactions potentially ranging from parasitic to mutualistic. These biological systems have not been studied extensively and are often ignored in reviews of fungal-plant interactions (e.g., Issac 1992; Schulz and Boyle 2005), or even in the context of reviews of marine fungi (e.g., Fell and Newell 1998). Recently, the mycophycobiosis involving the green alga *Prasiola crispa* ssp. *antarctica* (Kützing) Knebel has been reconfirmed as a lichen as *Turgidosculum complicatum* Kohlmeyer and Kohlmeyer (Lud *et al.* 2001).

The adaptive significance of the *Ascophyllum-Mycophycias* symbiosis has been demonstrated. The growth of *Ascophyllum* zygotes is enhanced by infection of *Mycophycias* ascospores within the first week of life. Although other fungi have been described from *A. nodosum* (Kohlmeyer and Kohlmeyer 1979), labelling of fungal cultures using fluorescent antibodies suggested that only one mycobiont is regularly present (Fries and Thoren-Tolling 1978). *In vitro* experiments show that the endophyte increases desiccation tolerance of the alga (Garbary and London 1995), an important consideration in the intertidal habitat of *Ascophyllum*. The phenology of gamete release for the alga and spore release for the fungus are closely tied. Uninfected zygotes exhibit altered morphological development and decrease in several growth parameters in the laboratory compared to zygotes infected with the endophyte (Garbary and MacDonald 1995). Other potential benefits to the host, such as protection from herbivory (seen in terrestrial systems) are not yet demonstrated for *Ascophyllum*.

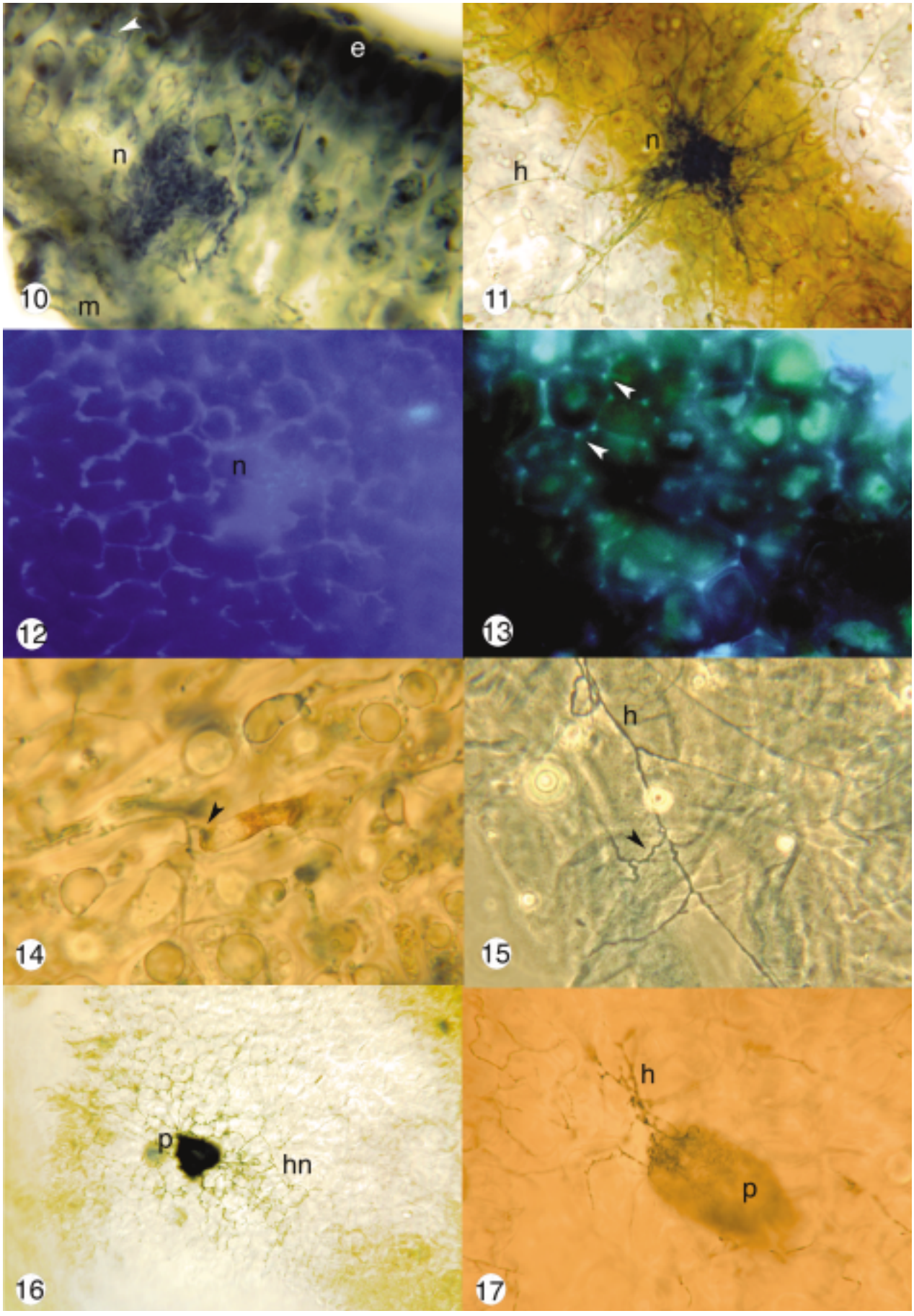
The primary objective of this study was to investigate the occupation of *A. nodosum* by *M. ascophylli* by applying novel microscopic techniques to this system. These allow visualization of the hyphal organization within the algal thallus. By clarifying morphological interactions between the species we hoped to better evaluate the causal basis for recently described interactions between the symbionts, and to better place this symbiosis in the context of other fungal-plant interactions, especially among terrestrial plants.

MATERIALS AND METHODS

Collection and sample preparation

Ascophyllum nodosum with its endophytic ascomycete, *Mycophycias ascophylli* were collected in August 2000 at Caribou Harbour, Pictou Co., Captain's Pond, Antigonish Co., and Tor Bay Provincial Park, Guysborough Co., all in Nova Scotia, Canada. Whole or portions of fronds were collected from the intertidal zone. Fresh material was hand-sectioned and mounted on microscope slides in lactoglycerol (1:1:1, lactic acid:glycerol:water) containing trypan blue (0.05%) and allowed to stain at least 1 h before viewing. Optimum staining was achieved after several hours.

Figs 1-9. The *Ascophyllum nodosum*, *Mycophycias ascophylli* symbiotum. Figs 1-4, 7-9, brightfield microscopy; Figs 5-6, phase contrast microscopy. **Fig. 1.** Portion of longitudinal section of *A. nodosum* with outer epidermal (e) region and inner medullary (m) region showing longitudinally aligned (arrow heads) and radially aligned hyphae. X 200. **Fig. 2.** Paradermal section through cortex of *A. nodosum* showing hyphal networks (arrow heads) surrounding each host cell. X 350. **Fig. 3.** Optical section through cleared tissue of *A. nodosum* showing abundant hyphal networks and three hyphal nodes (n). X 200. **Fig. 4.** Paradermal section of cleared material of *A. nodosum* through hyphal node (n) showing numerous connections to filamentous hyphae (h). X 800. **Fig. 5.** Slightly squashed preparation of cleared material of *A. nodosum* showing hyphal connection (arrow head) to cortical cell (c). X 1450. **Fig. 6.** Hyphal detail in cleared material showing hyphal anastomosis (arrow head). X 950. **Fig. 7.** Swollen hypha with crosswall (arrow) connected at either end to normal hyphae (arrow heads). X 700. **Fig. 8.** Portion of hypha with conspicuous swelling (arrowhead). X 1000. **Fig. 9.** Differentiated swollen hypha connected to normal hyphae. X 1000.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Samples of *A. nodosum* were cleared using a protocol modified slightly from Stone (1987). Thalli were divided as follows: (distal apex, young stem, basal stem, bladder, lateral appendages, and holdfast. These were treated whole (small laterals) or sectioned into large (ca. 10 mm long) or small pieces (ca. 2 mm long x 5 mm wide). Roughly 10 small or five large pieces were placed into scintillation vials and immersed in 10 mL of 1 M KOH and kept in an oven at 60°C. The clearing solution was changed daily until specimens appeared only slightly coloured. They were then rinsed twice in de-ionized water and either mounted directly onto microscope slides similar to whole mounts, or placed into 40% ethanol until mounting. Cleared material was stained with trypan blue as described above.

Fluorescent labelling and microscopy

Wheat germ agglutinin (WGA) bound to the fluorochrome Alexa Fluor 350 (absorbance 346 nm, emission 442 nm, Molecular Probes) was used to visualize hyphae of *M. ascophylli* in cleared specimens of *A. nodosum*. Cleared algal tissue stored in 40% ethanol was rinsed thoroughly in phosphate buffered saline (PBS pH 7.4), incubated in WGA in PBS (10 μg mL^{-1}) for 30 minutes, rinsed three times in PBS, and mounted in the buffer on microscope slides for ultraviolet excitation. Nuclei were stained with 4'-6-diamidino-2-phenolindole (DAPI, excitation 358 nm, emission 461 nm) by immersing samples in DAPI (0.5 μg mL^{-1}) and exposing them to microwave radiation in a conventional 700W microwave oven at full power for 5 seconds (Goff and Coleman 1990). They were then rinsed and mounted in distilled water for viewing. Microscopy was performed on a Zeiss Photomicroscope III equipped with a Spot 2 digital camera. Images were imported into Adobe Photoshop, implemented on an Apple Macintosh G4 platform. See Garbary and McDonald (1998) for details of fluorescence microscopy.

RESULTS

Fungal hyphae were present and abundant in all parts of the thallus of *A. nodosum*, and in all tissues, including the apical meristem. Longitudinal or peridermal sections in both non-cleared (Figs 1-2, 13) and cleared (Figs 3-9, 11-12, 14-17) material showed abundant hyphae. The cells of *A. nodosum* exhibit two primary orientations: either along the radial axis in the cortical region or parallel to the long axis in the medulla. Hyphal organization corresponded to the anatomy of the alga. Multidirectional hyphal growth was observed in the medullary tissue of the primary branch axis tissue, throughout the lateral appendages, and within about 1 mm of the distal apex, although orientation here was primarily along the longitudinal axis (Fig. 1). Hyphae in these areas were regular in morphology, smoothly cylindrical, 0.5-1.0 μm diameter, with long cells (usually between 20-40 μm) and few branches.

In contrast, hyphae in the cortical regions of the primary axes and air bladders were in a reticulated network, in a plane parallel to the long axis of the frond: an upper plane and a lower, more loosely organized plane. The uppermost hyphal network was highly organized, composed of a web following the contours of the bases of the epidermal cells. Hyphae followed along the cell junctions and connected to one another at the triple-cell junctions to form polygonal units, often in hexagons (Fig. 2). DAPI staining indicated that fungal nuclei were located at the corners of the polygons (Fig. 13). The second layer was roughly reticulate but more loosely organized. The hyphal network was connected to areas of hyphal aggregation or nodes (Figs 3-4, 10-11): densely clustered hyphal plates (20-100 μm diameter) that were in turn connected to the hyphae of the medullary region (Fig. 11). Network hyphae were irregularly shaped with swellings and constrictions and the cells were shorter than the medullary hyphae.

Figs 10-17. The *Ascophyllum nodosum*, *Mycophycias ascophylli* symbiotum. Figs 10-11, 14, 16-17, brightfield microscopy; Figs 12-13, fluorescence microscopy; Fig. 15, phase contrast microscopy. **Fig. 10.** Transverse section through thallus of *A. nodosum* with extensive hyphae (arrow head) and well developed hyphal node (n) immersed between epidermis (e) and medulla (m) of host. X 400. **Fig. 11.** Paradermal section of cleared host showing transverse view of base of hyphal node (n) connected to radiating hyphae (h) of the medulla. X 500. **Fig. 12.** Cell walls of fungal hyphae labelled with fluorescent lectin (WGA) probe. Note the well developed hyphal node (n). X 500. **Fig. 13.** DAPI stained preparation showing nuclei in hyphae (arrow heads). **Fig. 14.** Hyphal attachment to host cortical cell with appressorium-like hyphal swelling (arrow head). X 500. **Fig. 15.** Hyphal development showing long, straight sections (h) and shorter s-shaped hyphae (arrow head). X 500. **Fig. 16.** Cleared and stained material showing well developed hyphal network (hn) and transverse section through upper part of pseudothecium (p). X 150. **Fig. 17.** Cleared and stained material showing basal connection of pseudothecium (p) to hyphal network (h). X 300.

Hyphal networks were present in all parts of the thallus (main axes, lateral branches, bladders, and receptacles), although they were somewhat disorganized at the apical meristems. Hyphae of the medulla were more regularly cylindrical with less branching and longer cells. These hyphae were oriented parallel to the longitudinal axis of the frond with occasional connections to the reticulate mycelium. Other hyphae were oriented along the radial axis and extended to the surface of the algal frond. Hyphae were often seen strongly attached to host cells (Fig. 5) and hyphae in squash preparations were frequently connected by anastomoses (Fig. 6) or short sections of sinuous hyphae (Fig. 15). Infrequent hyphal swellings that were either septate or nonseptate were observed (Figs 7-9).

The hyphae displayed strong labelling by WGA-bound fluorochromes confirming presence of N-acetylglucosamine (chitin) or N-acetylneuraminic acid (sialic acid) residues in cell walls (Fig. 12). DAPI staining confirmed the presumed monokaryotic condition of cells (Fig. 13). Five to eight nuclei were in each net-like unit surrounding the cortical cells. Occasionally but infrequently, hyphae were attached to host cells by appressorium-like swellings. These host cells appeared dark in colour and necrotic (Fig. 14).

Pseudothecial ascocarps (Figs 16-17) were produced on receptacles; the base of these ascocarps were situated deeper in the algal tissue than were the nodes and also appeared rounder in optical section. The upper necks of the pseudothecia were normally melanized, and appeared dark, even in cleared and stained specimens. The bases of the pseudothecia remained connected to the hyphal network when fully formed (Fig. 17).

DISCUSSION

Early accounts of *Mycophycias ascophylli* demonstrated a systemic infection of *Ascophyllum nodosum*, and similar observations have been made on the related fucoid, *Pelvetia canaliculata* (L.) Decaisne and Thuret (Kohlmeyer and Kohlmeyer 1979; Kingham and Evans 1986). The nature of the interaction between these species was unclear; however, the obligate nature of the symbiosis and the absence of a conspicuous parasitism were used to imply a mutualistic symbiosis (Kohlmeyer and Kohlmeyer 1972, 1979). Smith and Ramsbottom (1915) initially queried whether or not *P. canaliculata* was a lichen, and Kohlmeyer and Kohlmeyer (1972) asked the same question with respect to *A. nodosum*. Although Kohlmeyer and Kohlmeyer pointed out the lichen-like attributes of this system, they used the more neutral term "mycophycobiosis" to describe this association and similar ones in other marine algae [i.e., *Mycophycias* (as *Mycosphaerella*) in *Apophlaea*, Kohlmeyer and Hawkes 1983]. Experiments demonstrated that infection by *M. ascophylli* modified the morphology of developing zygotes of *A. nodosum* and increased their protection from desiccation (Garbary and London 1995; Garbary and MacDonald 1995). Although the dominance of the *A. nodosum* thallus as the exhabitant preclude the determination of this system as a 'lichen' according to Hawksworth (1988), thallus habit in lichens is occasionally dictated by the photobiont (Budel and Scheidegger 1996). In addition, the lichen *Verrucaria tavaresiae* Moe was recently described in which the photobiont is the crustose brown alga *Petroderma maculiforme* (Wollny) Kuckuck (Moe 1997; Sanders *et al.* 2004).

Nevertheless, we feel that the nature of the morphological and ecological integration in the *Ascophyllum-Mycophycias* symbiosis makes this equivalent to the "symbiotum" described for the grass/endophyte interaction (Schardl *et al.* 1991). The term "mycophycobiosis" of Kohlmeyer and Kohlmeyer (1972, 1979) should be reserved for those marine algal-fungal associations where the nature of the symbiosis is unclear. Based on the ability of the fungus to modify development of its algal partner, the association previously considered to be a mycophycobiosis between *Prasiola crispa* ssp. *antarctica* and its fungal symbiont, *Turgidosculum complicatum* is now considered to be a lichen (Lud *et al.* 2001). Based on the examples provided by *V. tavaresiae* and *T. complicatum*, the *Ascophyllum-Mycophycias* symbiosis also may be considered a lichen. Thus the terms, lichen, mycophycobiosis and symbiotum are not necessarily mutually exclusive.

The *A. nodosum-M. ascophylli* symbiotum has additional species that may be obligately or facultatively associated with it. The red alga, *Vertebrata lanosa* (L.) Christensen [=*Polysiphonia lanosa* (L.) Tandy], is an obligate epiphyte that is almost always associated with *A. nodosum* (see Garbary *et al.* 1991; Tian and Garbary 1992; Garbary and Deckert 2001). The interaction of the rhizoid of *V. lanosa* with the host symbiotum suggests complex interactions of both the fungal and algal components with an invading species. Potentially more important to the symbiotum is the association with epiphytic bacteria. Fries (1988) reported that when *A.*

nodosum thalli were treated to make them axenic, hyphae of *M. ascophylli* grew out through the thallus wall and parasitized the host cells leading to host mortality. This suggests that a complex balance exists among the bacterial flora, *A. nodosum* and *M. ascophylli*.

We believe this is the first account of a highly specialized hyphal organization in an algal-fungal relationship of the sort referred to as a "mycophycobiosis". It is also the first report of this type of organization and hyphal differentiation in the *Ascophyllum-Mycophycias* system. The design of the hyphal network, which rings the perimeter of every cell in the frond at that plane, and represents a considerable energy investment on the part of the fungus (and the host), implies a specialized function. One of the demonstrated benefits of this particular symbiosis is the ability to tolerate periodic desiccation as the alga is exposed to air during tidal fluctuations. The mechanism is not known, but may involve the production of osmoregulators by the fungus, as has been proposed as a mechanism of drought tolerance in grass symbiota (West *et al.* 1990). A hyphal network like the one seen in *Ascophyllum-Mycophycias* would allow close monitoring of cell water relations and rapid response to detected changes.

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