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# Glomalean and septate endophytic fungi in *Hypopterygium* mosses (Bryopsida)

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**Abstract** – Although mosses are considered not to form mutualistic associations with glomalean (VAM) fungi, vesicles in mosses are known to occur. Vesicle-forming glomalean endophytes have been found in different species of the genus *Hypopterygium* collected mainly in the tropics. The glomalean mycobiont has been characterized by its morphological-anatomical features using light microscopy. The aseptate hyphae penetrate the cell walls of the moss stem and form large vesicles in some of them. Arbuscles have not been observed. Phyllidia and the majority of the moss rhizoids are free from colonization. Other endophytic fungi, characterized by septate hyphae, belonging to the *Rhizoctonia*-like and the so called dark septate (DSE) morphotypes, are also present in the plantlets.
To prove the symbiotic nature as well as taxonomic status of the vesicular and septate endophytes needs further studies. Ecological significance and possible role of these associations in nutrient transport between mosses and roots of vascular plants has been discussed.

***Hypopterygium* / glomalean fungi / VA-endophytes / dark septate (DS) fungi / mosses**

## INTRODUCTION

Endophytic fungi can be detected in all terrestrial plant groups including the gametophytes of the hepatics and mosses (Selosse & Le Tacon, 1998). In thalloid hepatics (e.g. *Marchantia, Lunularia, Preissia, Conocephalum, Pellia, Fossombronia*) intracellular colonization of arbusculum-forming VAM fungi (Glomales) has been demonstrated (Smith & Read, 1997 ; Ligrone & Lopes, 1989). From the genera of the jungermannialian families (*Marsupella, Saccogyna, Ptilidium*) and from thalloid taxa restricted to the Aneuraceae (*Cryptothallus, Aneura, Riccardia*), basidiomycetes with dolipore septa but without clamps, forming intracellular coils similar to the pelotons of orchid endomycorrhizae, have been described (Pocock & Duckett, 1984, 1985). Duckett *et al.* (1991) found ascomycetous fungi, identified by the presence of simple septa and Woronin bodies, in the flagelliform axes of British liverworts of the jungermannialian suborders Lepidoziineae and Cephaloziineae. These fungi are supposed to be identical to the mycorrhizal fungal partner of some Ericaceae /*Hymenoscyphus ericae*/ (Duckett & Read, 1995). Endophytic ascomycetes live in the rhizoids of the foliose liverwort *Cephaloziella exilifolia* even in the continental Antarctica (Williams *et al.*,

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1994). These associations are supposed to be mutualistic symbioses (mycothalli). Up to now, about 300 ascomycetous species, known to grow obligately on bryophytes, belonging to more than 80 genera (*e.g. Bryodiscus, Bryosphaeria, Epibryon, Octospora, Potriphila*) have been found (Döbbeler, 1997). The majority of them live on mosses (Bryopsida) (Döbbeler, 1997). Although vesicles of vesicular-arbuscular mycorrhizal (VAM) endophytes have also been observed in mosses (Parke & Linderman, 1980), these symbioses have been claimed not to be mutualistic. In contrast to liverworts, mosses are considered not to form mutualistic associations with fungi.

The aim of this study was to investigate and morphologically characterize the fungal endophytes in *Hypopterygium* mosses noticed first in the course of the ecologically-aimed elaboration of the samples collected by the expedition of the Botanical Department of the Esterházy Károly College, Eger organized to La Reunion in 1996. Based on this finding, other tropical material has also been drawn into the investigation.

The species of the Hypopterygiaceae family live in moist, dark places in mountain and lowland rain-forests and cloud-forests all over the world as well as in wet regions of the atlantic shores. Their distribution is mainly tropical, while a few of the species live in temperate climats. They can be found in different altitudes from the sea level to the 3000 m high mountains on soil, stones of streams and falls, on rocks, trunks, bark and other decaying wood.

The plantlets have a characteristic, horizontal "rhizome-like" primary stem from which vertical secondary stems, bearing the phyllidia, emerge. These secondary stems are branched (dendroid, flabellate or umbellate) above stipe causing a tree-like habit. The moss is perennial and produces new secondary stems every year on the "rhizome".

## MATERIALS AND METHODS

### Moss samples

The moss samples examined in this study represent different species of the genus *Hypopterygium* collected in green, living form from several, mainly tropical biotopes all over the world and dried within 1-2 days to avoid secondary fungal infection. The specimens were deposited in the Herbarium Academiae Pedagogicae, Eger, Hungary (EGR). All together 69 herbarial moss samples, representing 16 *Hypopterygium* species, were investigated for fungal endophytes. From each sample 6-15 plantlets were thorougly examined for colonization. Herbarial numbers and collection data of the positive samples are summarized in Table 1.

### *Preparing the samples for light microscopy (LM)*

As dry material was used, plantlets had to be wetted by distilled water before separating them from each other and preparing for light microscopy. Fungal structures were visualized in the intact plantlets by the staining method of Philips & Hayman (1970) modified by Koske & Gemma (1989), with further modifications as follows:

1. Samples were placed into a 20% aqueous solution of KOH and heated in a boiling water bath for 20-40 minutes. The thick, rhizome-like primary stems of

Table 1. Data of positive *Hypopterygium* samples

| *Sample (herb. Nr)* | *Species* | *Collected by* | *Date of collection* | *Country, place* | *Altitude (m)* | *Substrates* |
|---|---|---|---|---|---|---|
| 12. (2998) | *H. didictyon* C. Müll | G. Een. | 10. 06. 1966 | Tasmania, Queenstown | 330 | no information |
| 13. (8472) | *H. flavescens* Hampe | A. M. Cleef | 29. 01. 1973 | Columbia, San Juan | 3160 | trunk |
| 14. (489). | *H. flavolimbatum* C. Müll | H. Inoue | 26. 01. 1978 | Japan, Ohyatori | 400 | moist rock along stream |
| 19. (740289) | *H. filiculaeforme* (Hedw.) Brid. | B. O. van Zanten | 16. 02. 1974 | New Zealand, South Island, Lake Mahinapoua | 1-10 | rotten wood in dense shade |
| 25. (712/A) | *H. mildbraedii* Broth | A. J. and E. Sharo | 13. 07. 1968 | Tanzania, Tekukumia Falls, Meru Center Park | 2300 | trunk |
| 35 (78-2094) | *H. tamariscinum* (Hedw.) Brid. | M. Lewis | 24. 10. 1978 | Ecuador, Cordillera de Cutucú Oeste | 1200-1700 | rotten wood, primary forest |
| 69. (9637/K) | *H. laricinum* (Hook) Brid | G. Kis | 10. 07. 1996 | Réunion, Mascaranes, Forest Matarum | 1450 | moist rock |

*Hypopterygium* mosses need a more drastical clearing procedure than root hairs of plants. Bleaching in $H_2O_2$ was not necessary.
2. Rinsing several times in distilled water was followed by acidification in 2% HCl.
3. Instead of trypan blue used by Koske & Gemma (1989), staining was carried out with a 0.05% aqueous cotton blue solution at 90 °C for 10-15 minutes.

Samples were mounted on microscopic slides in 90% lactic acid, without sealing. Plantlets were examined under light microscope with low magnification for detecting fungal colonization and eliminating negative samples. For Nomarski (DIC) observations and photography, the plantlets containing fungal endophytes were removed from the slides and washed in distilled water containing 2% glycerol for two hours. Thereafter 30 µm thick longitudinal sections were made using a cryotome. Sections were mounted in 90% lactic acid and sealed with Entellan. LM-observations were carried out by an Olympus FXA microscope in normal and Nomarski (DIC) mode at 100-1000 x magnification. Half tone photos were taken by an automatic photoequipment.

Plantlets were thoroughly investigated for fungal endophytes. In *H. laricinum* vesicular colonization rates were also determined and expressed by the average vesicle number (AVN) counted on a stem (caulidium) length basis, according to Ambler & Young (1977). Average of vesicles per 100 mm in the stem was estimated by counting more than 200 vesicles under the microscope. Colonization rates of septate endophytes were calculated similarly, expressed as intracellular microsclerotia per 100 mm (AMN) in *H. flavescens* and *H. laricinum*.

The size of vesicles, the diameter and cell wall thickness of the hyphae as well as distance of septa in the septate endophytes was measured and registrated (Table 3).

## RESULTS

The rate of positive samples and tested samples as well as estimated relative colonization rates are shown in Table 2.

### Fungal colonization

Out of the 16 *Hypopterygium* species tested for colonization, seven species were positive, seven species negative and two samples (*H. viridissimum* C. Müll. and *H. vriesei* Bosch et Lac.) insufficient for investigation. The seven positive *Hypopterygium* species, containing intracellular fungal endophytes, are listed in Table 1. In Table 2, colonization rates and types within these samples have been summarized. Based on testing of several plantlets of a single specimen, no colonization could be observed in *H. ceyloniacum* Mitt., *H. debile* Reichdt., *H. muelleri*, *H. plumarium* Mitt., *H. pseudo-tamarisci* Müll., *H. setigenum* (Beuw) Hook J. Wills. and *H. tenellum* C. Müll.

In the rhizoids of the moss plantlets (Figs. 1b-c) only very few fungal hyphae could be observed. The mycobionts grow mainly inside and between the cells of the stem (caulidium). Phyllidia are free from fungi (Fig. 1a).

Glomalean (VA-type) fungi, as indicated by the presence of vesicles and aseptate hyphae, were observed in five species. (Table 2, Fig. 2a-e).

Besides the VA-colonizer, other fungi are also present in the stems of the moss plantlets. Two different septate hyphal endophytes ($T_1$, a *Rhizoctonia*-like and $T_2$, a dark septate morphotype) forming dense intracellular hyphae (Figs. 3a-b) and microsclerotia-like structures can be distinguished (Figs. 3c-e). The $T_1$ fungus could be detected only in *H. flavescens* where no glomalean colonizer was present. The $T_2$ endophyte occured in several samples, together with the VA endophyte (Table 2). Morphological characteristics of the endophytes found in *Hypopterygium* samples are summarized in Table 3.

Table 2. Colonization of the moss samples. VA = vesicular endophyte, $T_1$ = *Rhizoctonia*-like endophyte, $T_2$ = dark septate endophyte

| *Sample number* | *Positive samples/ tested ones* | *% of colonized plantlets within positive samples* | *Colonization rate in positive plantlets (–negative, + weak, ++medial, +++ strong)* | | |
|---|---|---|---|---|---|
| | | | *VA* | $T_1$ | $T_2$ |
| H. didictyon | 1/2 | 60 | ++ | – | ++ |
| H. flavescens | 1/1 | 20 | – | ++ | – |
| H. flavolimbatum | 2/5 | 20 | – | – | + |
| H. filiculaeforme | 1/2 | 25 | +++ | – | + |
| H. mildbraedii | 1/6 | 60 | + | – | – |
| H. tamariscinum | 2/5 | 10 | + | – | + |
| H. laricinum | 4/14 | 25 | + | – | + |

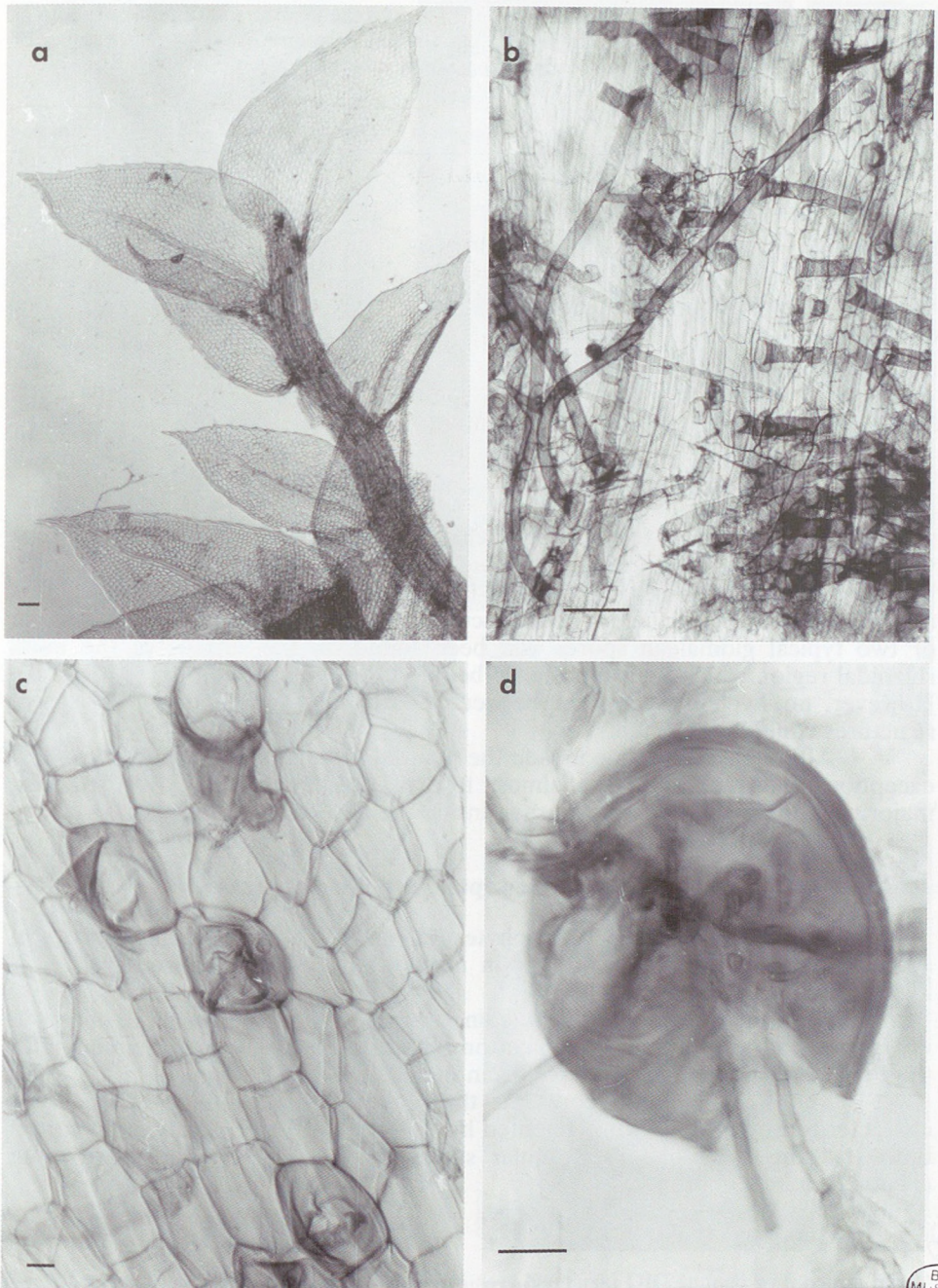




Fig. 1. The *Hypopterigium laricinum* moss (a: normal, b-d: Nomarski DIC, unstained). a. Habit of the plantlet (secondary branches) b. Surface of the rhizome-like primary stem with rhizoids. c. Epiderm of the stem and basal cells of the rhizoids. d. A glomalean spore on the surface of the moss rhizoidal region (Thin septate hyphae do not belong to the spore). Bar = a and b: 100 µm, c and d: 10 µm.

Table 3. Size of vesicles, diameter and cell wall thickness of the hyphae and distance of septa in endophytes. AVN= average number of vesicles per 100 µm of stem, AMN = average number of microsclerotia per 100 µm of stem. Minimum-maximum values are given.

| *Characterisctic* | VA *in H. laricinum* | $T_1$ *in H. flavescens* | $T_2$ *in H. laricinum* |
|---|---|---|---|
| AVM or AMN | 65.2 | 5.8 | 10.0 |
| Cross size of vesicles (µm) | 20-25 | | |
| Longitudinal size of vesicles (µm) | 25-100 (average 50) | – | – |
| Diametre of hyphae (µm) | 3-10 | 3-5 | 1-3 |
| Cell wall of hyphae (µm) | 0.5-1 | 0.4-0.6 | 0.2 |
| Distance of septa (µm) | – | 25-60 | 10-28 |

## Morphology of the glomalean endophyte

The glomalean endophyte is characterized by large, globular to oval vesicles adapted in form and size to those of the moss cells (Figs. 2 a-c) and by a branching intercellular network formed by aseptate hyphae variable in thickness (Fig. 2d, Table 3). Nevertheless, no arbuscules could be observed. In two cases one or two typical glomalean spores had been found on the surface of the moss rhizoidal region which are supposed to belong to the fungal endophyte (Fig. 1d). However, no hyphal connection between the spores and intercellular fungal structures could be detected.

Although the hyphae invade the plantlets through the rhizoids, with few exceptions (Fig. 2e) these are almost free of fungal stuctures (Fig. 1b). The majority of the endophyte can be seen inside the caulidium.

## Morphology of the septate hypal endophytes

The two other fungal endophytes ($T_1$ and $T_2$), having been observed in addition to the glomalean colonizer in moss cells, are characterized by septae hyphae without clamps.

Both the intercellular hyphae and intracellular structures of $T_1$ observed in *H. flavescens* are staining well in cotton blue. Hyphae are beaded, reminding of the hyphae of *Rhizoctonia* endophytes in orchids (Figs 3a-b).

The $T_2$ endophyte represents the so called dark septate (DSE) type characterized by thin, brown pigmented hyphae which do not stain well in cotton blue. It forms brain-like intracellular structures interpreted as microsclerotia (Figs. 3 c-e).

## DISCUSSION

The glomalean endophyte showed the typical *Arum*-type morphology according to the classical images of Gallaud (1905). Precise taxonomic status of the fungus could not be determined. Identification based upon fungal DNA-ITS

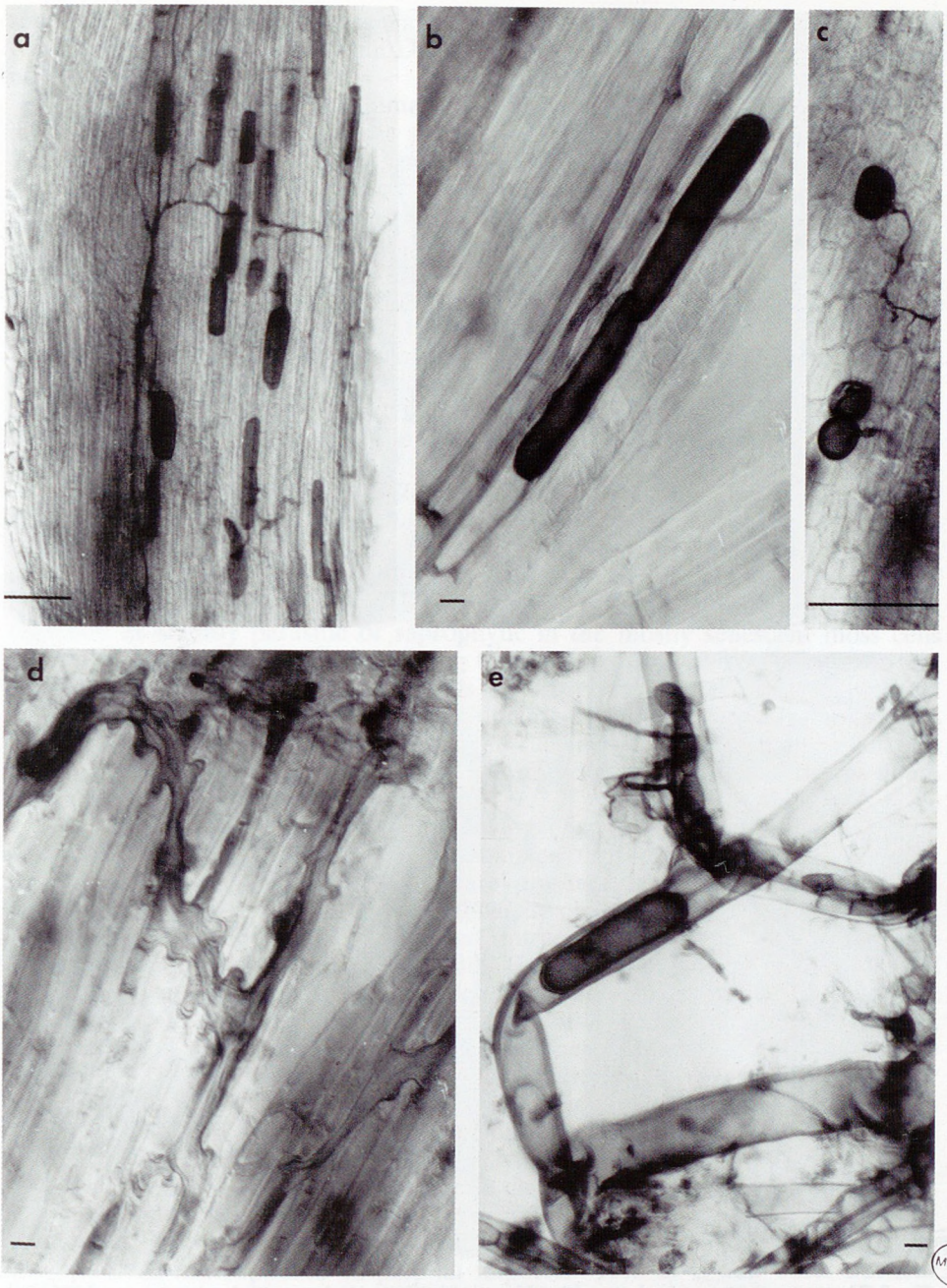




Fig. 2. Morphology of the glomalean endophyte (a-e: Nomarski DIC, stained with cotton blue). a. Longitudinal section of the primary stem with VA-colonization. b. Elongated vesicles in moss hydroid cells. c. Globular vesicles. d. Typical aseptate intercellular hyphae of the glomalean fungus of various thickness. e. A rare feature: vesicle in a rhizoidal cell, above rhizoidal colonization of the $T_1$ endophyte. Bar = a and c: 100 µm, b, d and e: 10 µm

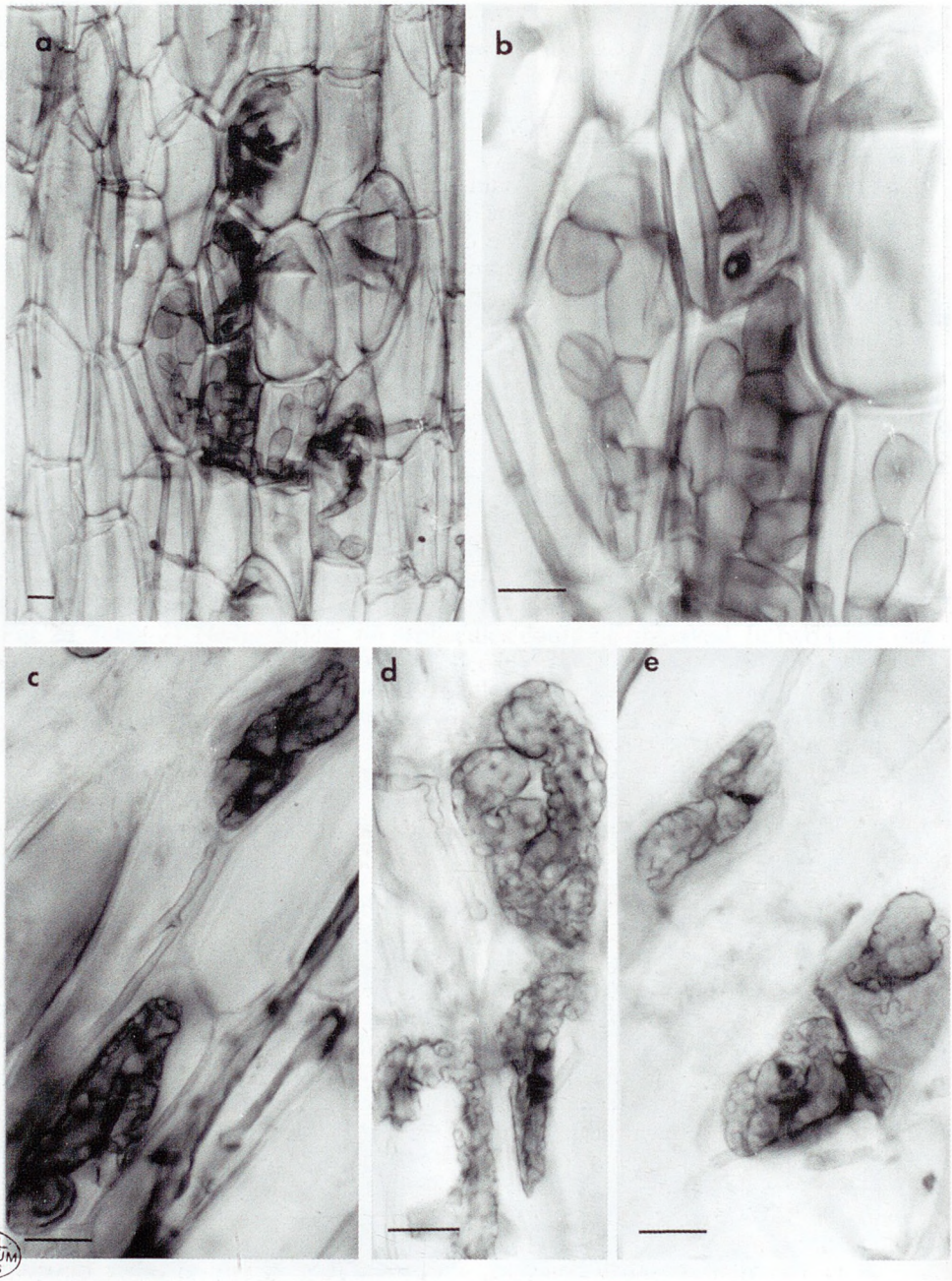




Fig. 3. The two septate endophytes. (a-e: Nomarski DIC) a-b. Intercellular hyphae and intracellular structures of the $T_1$ endophyte in *H. flavescens* (stained with cotton blue). c-e. Intercellular hyphae and brain-like microsclerotia of the $T_2$ endophyte in *H. laricinum* (unstained) Bar = all: 10 µm.

sequencing of the moss samples and glomalean spores found between the rhizoids could have been a possibility but DNA extraction from the herbarial samples was not successful.

The vesicles of the glomalean endophyte are spread almost evenly in longitudinal section of the moss stem. Parke & Linderman (1980) observed a similar localization of vesicles in the moss *Funaria hygrometrica* grown in glasshouses. Fungal colonization is mainly restricted to the non-photosynthetic, perennial primary stem which may be an indication to a long-term metabolic relationship between the partners. The consequent lack of arbuscules from the moss plantlets puts another important question about the nature of the cooperation between the partners. Although arbuscules are considered to be essential in mutualistic VAM-relationships (Gianinazzi-Pearson, 1996), they can easily remain unnoticed or overlooked because of the seasonal occurrence and transient nature of these structures. Vesicles are more persistent and more distinctly visible (Herrmann, 1985). Thus, the lack of arbuscules may indicate that the endophyte is not a symbiont in physiological sense but neither does it exlude this possibility.

Parke & Linderman (1980), who observed vesicles but no arbuscules of *Glomus epigaeus* in the moss *Funaria hygrometrica* grown among greenhouse asparagus plants, came to the conclusion that this relationship was not a mutualistic symbiosis. As the fungus infected moss cells only in the presence of asparagus roots, the mycobiont, forming VAM with the plant host, had been regarded as slightly parasitic or saprophytic in the mostly senescent moss cells (Parke & Linderman, 1980). According to Went & Stark (1968) "direct nutrient cycling" can occur from the moss cells to active mycorrhizal hosts in tropical ecosystems. Our opinion about the symbiotic nature of the *Hypopterygium* mosses and their aseptate endophytes could lead to such assumption. Such an association would have advantages in habitats of nutrient-deficient conditions where *Hypopterygium* species live.

The two septate endophytes having been detected in addition to the glomalean fungus in *Hypopterygium* samples represent two different types. As no sporocarps, spores or conidia are present these can not be identified on morphological basis. The intracellular hyphae of the $T_1$ endophyte are morphologically similar to the beaded intracellular hyphae of *Rhizoctonia*-endophytes frequently detected in orchids. The $T_2$ fungus belongs to the so called dark septate edophytes (DSE) which are widespread especially in root associations of the arctic and alpine environments (Haselwandter & Read, 1980; Currah & van Dyk, 1986). These types have been detected in more than 500 species of angiosperms, gymnosperms and pteridophytes from high latitudes, or high elevations of the temperate and tropical regions. Nevertheless, they have not been reported from mosses so far (Jumpponen & Trappe, 1998).

DSE fungi represent different types of anamorphs or teleomorphs. Sequencing of the 18S rDNA places them clearly with ascomycetes (Jumpponen & Trappe, 1998). These fungi often form brain-like intracellular structures defined as microsclerotia.

DSE are supposed to play an important role in C and other nutrient translocation in pioneer plant communities (Caldwell *et al.*, 1996). According to Jumpponen & Trappe (1998) DSE "can form mycelial connections between plant individuals of the same or even different species. These connections could be involved in photosynthate or nutrient transport as suggested for ectomycorrhizal systems (Simard *et al.*, 1997)". Similar nutrient flux also exist in achlorophyllous hepatics associated with otherwise ectomycorrhizal fungi (Read *et al.*, 2000)

Unfortunately, the study of bryophilous endophytic fungi receive no much attention and their ecological and evolutional significance is highly underestimated (Döbbeler, 1997). Nevertheless, these interactions are widespread in nature and collecting work in weakly studied areas, e.g. tropical forests, as well as revision of herbarial material can lead to new discoveries in plant-fungus relationships.

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