Mycorrhiza (1993) 3:83-91

Mycorrhiza

Response of ectomycorrhizal fungi to benomyl and nocodazole: growth inhibition and microtubule depolymerization

Sara S. Niini, Marjatta Raudaskoski

Department of Botany, University of Helsinki, Unioninkatu 44, SF-00170 Helsinki, Finland

Abstract. The growth of seven ectomycorrhizal fungi was tested in the presence of the antimicrotubule drugs benomyl and nocodazole. The polymerization stage of the cytoplasmic microtubules in the hyphal cells was visualized by indirect immunofluorescence microscopy after a 3-h drug treatment. Nocodazole reduced the growth of all the fungi tested at concentrations of 2 and 4 $\mu g\,ml^{-1}$ and caused strong depolymerization of microtubules in all other species except *Hebeloma cylindrosporum*. Benomyl inhibited the growth and depolymerized the microtubules in the ascomycete *Cenococcum geophilum*, while in the basidiomycetes it reduced the growth and depolymerized the microtubules only in *H. cylindrosporum*. The role of the microtubule cytoskeleton and the target of the benzimidazole-derived drugs in fungal cells are discussed.

Key words: Hyphal growth - Immunofluorescence microscopy - Tubulin - Immunoblotting - Filamentous fungi

Introduction

In filamentous fungi the apical growth is dependent on localized synthesis and extension of the cell wall at the tip (Wessels 1986) that seems to be associated with a functional cytoskeleton (Salo et al. 1989). The cytoskeleton in the fungal cell consists of microfilaments (MF) and microtubules (MTs). The former structures arise through polymerization of actin monomers and the latter through polymerization of α- and β-tubulin heterodimers. MFs are regarded as necessary for the tip growth of filamentous fungi (cf. Heath 1990) but the longitudinally oriented MT tracks may also play a role (Raudaskoski et al. 1988). A role for MTs in apical growth is suggested by their extension up to the hyphal apex in most fungal species studied (Salo et al. 1989; Raudaskoski et al. 1991). MTs are also associated with vesicles similar in structure to those occurring at the hyphal apex (Kwon et al. 1991), and in some filamentous fungi apical growth is inhibited or delayed by MT-depolymerizing agents such as benomyl and nocodazole (Oakley and Rinehart 1985; Caesar-Ton That et al. 1988; Kamada et al. 1990).

Until now only a few studies have dealt with the occurrence of cytoskeletal elements in the ectomycorrhizal fungi (Raudaskoski et al. 1988; Salo et al. 1989), although these fungi are of particular interest due to their slow growth rate in pure culture and their ability to grow efficiently in association with the plant root. Benomyl is used often as a selecting agent in the isolation of ectomycorrhizal fungi from natural sources to form pure cultures since most ectomycorrhizal fungi are homobasidiomycetes and appear to be less sensitive to benomyl than ascomycetes (Taylor 1971) for reasons not yet clear.

In the present work, the growth of one ascomycete and six basidiomycetes, all ectomycorrhizal fungi, was tested on media containing benomyl or nocodazole. In order to reveal the relationship between growth and the polymerization stage of MTs during the drug treatments, the MTs in the control and treated hyphae were visualized by IIF (indirect immunofluorescence) microscopy. This technique has been applied recently in the investigation of the cytoskeleton in filamentous fungi (Runeberg et al. 1986; Salo et al. 1989; Raudaskoski et al. 1991).

Materials and methods

Source and culture of fungal isolates

The ectomycorrhizal species and their origin are listed in Table 1. All the strains used are known to form ectomycorrhizas in nature and in laboratory conditions.

The ectomycorrhizal fungi were grown in the dark at 25° C on a 1.2% agar medium modified from Melin-Norkrans MMN (Molina and Palmer 1982) in which malt extract was replaced with 2.5 $g\,l^{-1}$ glucose and only half the strength of inorganic nutrients

Correspondence to: S. S. Niini

Table 1. List of the ectomycorrhizal species used and their sources

Species	Source
Paxillus involutus (Batsch: Fr.) Fr.	Scotland Bush Estate, Institute of Terrestrial Ecology, Scotland
Suillus bovinus (L.: Fr.) O. Kuntze	Isolate 096, Prof. David Read, University of Sheffield, England
Amanita muscaria (Fr.) Hooker	Collection Prof. Veikko Hintikka, Department of Agriculture, University of Helsinki, Finland
Amanita regalis (Fr.) Maire	Obtained under the name *A. muscaria var umbrina*, mushroom collection, Department of Microbiology, University of Helsinki, Finland
Hebeloma cylindrosporum Romagnesi	A wild dikaryotic strain HC1 (Wagner et al. 1988)
Piloderma croceum Erikss. & Hjortst.	Collection Prof. Peitsa Mikola, University of Helsinki, Finland
Cenococcum geophilum (Sow.) Ferd. & Winge	Isolate SSIV-4, Dr. Robin Sen, Forest Research Institute, Finland

was used. This medium is referred to as a control medium in the present work. Stock solutions (10 mg ml^{-1}) of benomyl (99% pure, Du Pont Co.) and nocodazole (Sigma) were made in dimethyl sulphoxide (DMSO, Merck). These chemicals were added to the growth medium before pouring into Petri dishes at the final concentrations given in each experiment. DMSO in the concentrations used at different experiments had no detectable effect on the structure of the MT cytoskeleton. In the growth experiments, the DMSO concentration in 2 μg ml^{-1} benzimidazole treatments was 0.02%, which was beneficial to the growth of the fungi.

Growth experiments

The plug cultures used for the benomyl and nocodazole treatments were inoculated with discs 5 mm in diameter cut from the margins of growing colonies with a sterilized cork borer. Ten cultures were inoculated for control, benomyl (2 μg ml^{-1}) and nocodazole (2 μg ml^{-1}) treatments. All seven species were grown in the presence of benomyl and nocodazole for 25 days and the diameter of each colony was measured at days 12 and 25. The gross morphology of the hyphae was examined microscopically at days 3, 6, 12 and 25. After 7 days, four of the nongrown or poorly growing plugs in the benomyl and nocodazole treatments were transferred to drug-free medium in order to see if they were able to recover. Six plugs remained intact on the treatment medium. Smaller plugs, 1.5 mm in diameter, used in the second experiment were prepared in the same manner. The diameters of eight colonies from each treatment were measured at days 7, 14, and 21. The results are expressed as percentages of the DMSO controls.

All fungi were grown also on dialysis membranes (1 × 1 cm) overlying the culture medium. The cultures were grown until new hyphae spread from the plug on the surface of the membrane, which required 2 to 5 days depending on the species. Half of the membranes with the mycelium were then carefully lifted onto a medium containing 4 μg ml^{-1} nocodazole and another half onto a new control medium. After a 24-h treatment, the cultures were examined microscopically and the membranes were transferred back onto a new control medium. The cultures were allowed to grow further for 2 weeks. Growth was measured as the diameter at days 6 and 14.

Immunoblotting of α- and β-tubulin

For the immunoblotting of tubulins, mycelium was grown on the control medium on small pieces of dialysis membrane. Membranes with the mycelium were quickly submerged in liquid nitrogen, homogenized, and the powder transferred into Eppendorf tubes in ice. Each sample was mixed well with an equal amount of sample buffer containing 20% glycerol, 30 mM Tris-HCl pH 8.5, 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) and the following proteinase inhibitors: 25 μg ml^{-1} leupeptin, 25 μg ml^{-1} pepstatin, 10 μg ml^{-1} aprotinin, 0.33 U ml^{-1} α_2-macroglobulin, 1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone-HCl (Boehringer Mannheim Biochemicals), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 1 mM benzamidin-HCl (Sigma) (Åström et al. 1991). The samples were boiled for 10 min and insoluble material was removed by centrifugation in an Eppendorf microfuge (15000 rpm for 15 min). The samples were analyzed on 10% SDS-polyacrylamide mini-gels prepared and run in Bio-Rad's Mini-Protean II dual slab cell system. For protein determination, 10 μl of sample extract was precipitated with 100% acetone at −20° C overnight, centrifuged for 15 min, washed 3 times with cold 100% acetone and once with cold 80% acetone, dried under vacuum, dissolved in 10 μl of 1 M NaOH and diluted to 800 μl. Protein determination was made by the micro method of the Bio-Rad protein assay kit.

The procedures for protein transfer from the gel to a nitrocellulose filter and for the immunodetection of tubulins have been described by Åström et al. (1991). For the immunodetection of α- and β-tubulin the monoclonal α(DM1A)- or β(DM1B)-tubulin antibodies raised against chicken brain tubulins (Blose et al. 1984) were used at a final concentration of 1:5000. Peroxidase-conjugated immunoglobulins against mouse immunoglobulins (P 161, Dakopatts) were used as a secondary antibody.

IIF microscopy of the fungal hyphae

For IIF microscopy, all species were grown on the control medium on dialysis membranes (0.5 × 0.5 cm) starting with small inocula. The mycelium was first grown for 2 to 3 days until new hyphae were clearly emerging. The membranes with the mycelia were carefully lifted on to each of the following media: control, benomyl (4 μg ml^{-1}) and nocodazole (4 μg ml^{-1}). Treatments were carried out in the dark at 25° C for 3 h.

The freeze-substitution method developed for IIF microscopy of filamentous fungi (Raudaskoski et al. 1991) was used for the quick-freezing and low temperature fixation. Individual membrane-bound colonies were rapidly submerged in liquid Freon 22 in pierced cryotubes cooled with liquid nitrogen. The cryotubes were immediately transferred into liquid nitrogen. The samples in the cryotubes were then moved into precooled 3.7% formaldehyde in methanol and fixed at −80° C overnight. After fixation, samples were transferred to −20° C for 1 h, rinsed once with cold methanol at −20° C, warmed at 4° C for 1 h, and then brought to room temperature. Still attached to the membranes, the samples were rehydrated in a descending methanol series, rinsed with sterile water containing 1 mM PMSF and transferred to phosphate-buffered saline solution (PBS), pH 7.3 containing 1 mM PMSF. The treatment of the hyphae with cell wall-degrading enzyme preparations and detergent was unnecessary for the quick-frozen material (Raudaskoski et al. 1991) but 0.12% NovoZym (Novo, Bio-Labs) treatment for 20 min did increase the penetration of antibodies into thick-walled *Cenococcum geophilum* cells.

For the visualization of MTs, the samples were treated with the same monoclonal α-tubulin antibody as in the immunoblotting at

a final concentration of 1:500 for 1 h and rinsed 3 × 10 min with PBS containing 0.1% bovine serum albumin and 1 mM PMSF. The primary antibodies were recognized by rhodamine-conjugated anti-mouse IgG (heavy and light chains specific, Cappel Laboratories) at a dilution of 1:40 for 1 h, the samples were rinsed with PBS (pH 8.5) at least 5 times and lifted on a glass slide. Under a dissecting microscope, the hyphae were carefully separated from the membrane and the hyphae were mounted in glycerol:PBS (1:2, pH 8.5) containing 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) to visualize the nuclei. The samples were examined in a Leitz Dialux 20 microscope equipped with appropriate epifluorescence filters. Intact apical cells were searched for and their MTs were ranked into four classes: P = polymerized intact MTs, F = partly fragmented but discernible MTs, S = MTs depolymerized to tubulin spots, and D = only diffuse staining in the cells. Photography was carried out using Kodak TMax 400 ASA film.

Results

Benomyl treatment

The initiation of growth from a plug inoculum on the benomyl-containing medium appeared to be strongly dependent on the inoculum size. When a 1.5-mm plug was used, benomyl had no effect on the growth of *Piloderma croceum*, *Paxillus involutus*, *Suillus bovinus* and *Amanita regalis* while it reduced the growth of *Hebeloma cylindrosporum* and *A. muscaria*, and totally inhibited the growth of the ascomycete *C. geophilum* (Table 2). Instead, when a 5-mm plug was used, the initiation of growth on the benomyl medium was very slow. As a consequence a significant delay in the extension growth on the benomyl medium was observed in *P. croceum*, *P. involutus*, and *A. regalis* but not in *H. cylindrosporum* and *S. bovinus* (Table 2). The effect of benomyl on the growth of the small inocula was the same when the concentration was increased from 2 μg ml^{-1} to 3 μg ml^{-1}. This suggested that the slow initiation of growth in the large plugs was not due to benomyl alone but perhaps resulted from a higher amount of growth-retarding factors which may interact with benomyl and retard the growth. The effect of benomyl on the growth was reversible in all fungi studied, the plugs resuming growth when transferred from benomyl to the control medium.

Nocodazole treatment

No difference due to the size of the inocula was recorded on the nocodazole medium and, therefore, only the data for 1.5-mm plugs is given in Table 2. Nocodazole (2 μg ml^{-1}) reduced the growth of *H. cylindrosporum*, *A. regalis*, *P. croceum* and *A. muscaria* by 10%, 34%, 35% and 81%, respectively, while the growth of the homobasidiomycetes *P. involutus* and *S. bovinus* and the ascomycete *C. geophilum* was totally inhibited (Table 2). The effect of nocodazole on the growth was reversible, since the treated cultures recovered when moved back to the control medium.

The critical concentration for the nocodazole treatment seems to be 2 μg ml^{-1}. A lower concentration (1 μg ml^{-1}) had an unexpected effect; *H. cylindrosporum*, *P. croceum* and *C. geophilum* grew better than the controls, and the growth of *P. involutus* and *S. bovinus* was not affected. The growth of the *Amanita*-species only appeared to be significantly delayed (data not shown). Note that an increase in growth also occurred in *P. croceum* and in *P. involutus* on media containing 2 and 3 μg ml^{-1} benomyl and in *S. bovinus* on a medium containing 2 μg ml^{-1} benomyl (Table 2).

Dialysis membrane cultures were used instead of plug cultures in order to reveal whether nocodazole had a growth-retarding effect or if the inhibition of growth in the plug cultures resulted from inability to recover from wounding during the inoculation process. When the cultures were transferred to the nocodazole (4 μg ml^{-1})

Table 2. The effect of benomyl or nocodazole on the growth of the ectomycorrhizal fungi expressed as % of the dimethyl sulphoxide controls. Asterisks indicate significant reductions in growth as analyzed by *t*-test. ND, Not possible to determine. The data shown are means plus SEM

Drug	Benomyl	Benomyl	Benomyl	Nocodazole
Concentration	2 μg ml^{-1}	2 μg ml^{-1}	3 μg ml^{-1}	2 μg ml^{-1}
Plug size	1.5 mm	5.0 mm	1.5 mm	1.5 mm
Time	14 days	12 days	14 days	14 days
	$n=8$	$n=6$	$n=8$	$n=8$
P. croceum	211.54 ± 7.38	67.80*** ±11.86	171.15 ±34.46	65.38* ±14.46
P. involutus	147.55 ±12.53	46.25*** ± 7.50	113.99 ± 6.88	0***
S. bovinus	126.77 ±21.67	77.24 ± 4.88	85.04 ± 4.47	0***
H. cylindrosporum	89.70*** ±1.30	89.94 ± 3.35	89.27*** ± 2.44	89.70*** ± 2.68
A. regalis	96.43 ± 2.93	45.98*** ± 5.75	83.93** ± 4.29	65.86*** ± 1.36
A. muscaria	76.14*** ± 6.18	ND	34.09*** ± 2.91	18.18*** ± 1.82
C. geophilum	0***	0***	0***	2.02*** ± 2.02

Table 3. The effect of a 24-h treatment with 4 μg ml^{-1} nocodazole on the growth of the ectomycorrhizal fungi after 6 days growth recovery on control medium (expressed as % of the controls). Asterisks indicate significant reductions in growth as analyzed by *t*-test ($n=5$). ND, Not possible to determine. The data given are means plus SEM

P. croceum	71.79*** ± 6.41
P. involutus	70.83*** ± 4.58
S. bovinus	70.97*** ±11.29
H. cylindrosporum	78.21*** ± 2.39
A. regalis	78.95*** ± 3.95
A. muscaria	ND
C. geophilum	ND

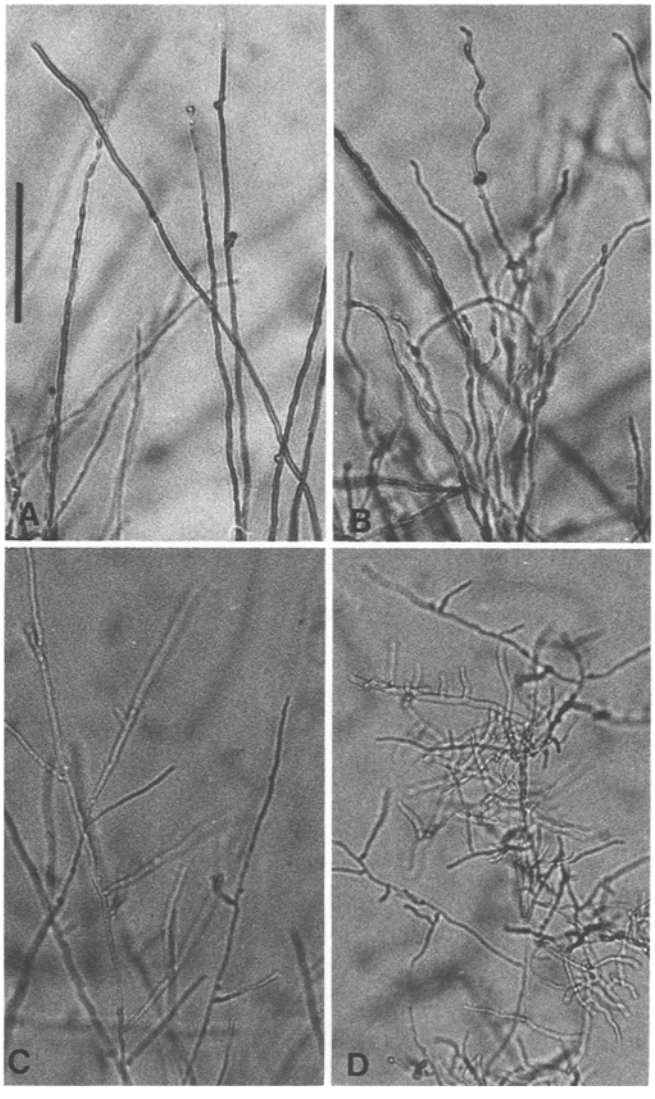


Fig. 1. The comparison of the hyphal morphology on control (**A, C**) and nocodazole (**B, D**) media at day 7. **A, B** *Amanita regalis*; **C, D** *Hebeloma cylindrosporum*. *Bar* = 100 μm

medium for 24 h and then returned to the control medium, the growth interruption by nocodazole was still seen after 6 days recovery as a reduction in diameter in most species (Table 3). In the extremely slow-growing fungi *A. muscaria* and *C. geophilum*, the 24-h growth is not measurable, which made it impossible to record any difference in growth due to the treatment in these fungi.

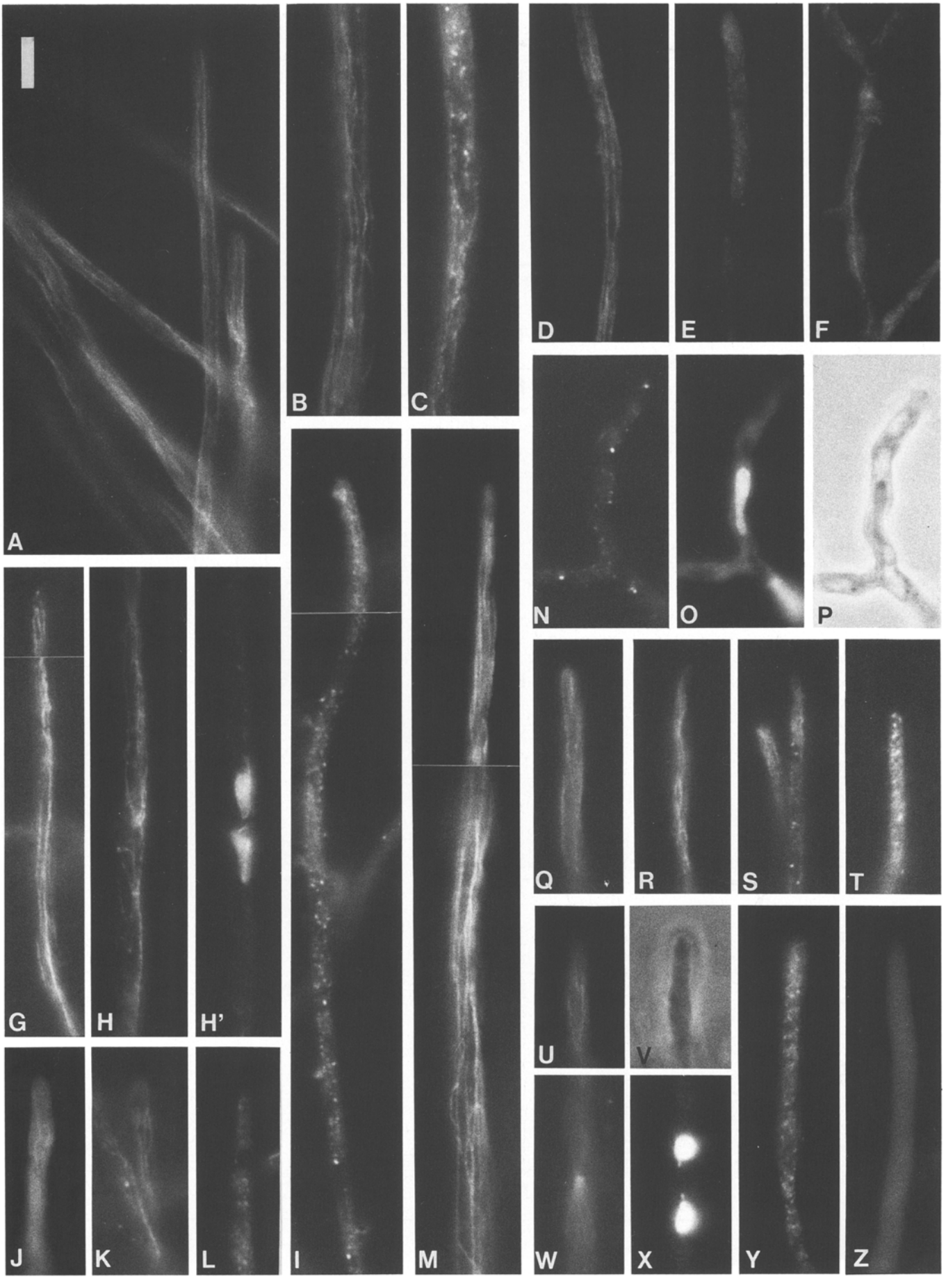
Morphology

The gross morphology of the basidiomycetes remained unaffected by the benomyl treatment, while hyphae emerging from the inoculum plugs of the ascomycete *C. geophilum* were very short and twisted. In *A. regalis* (Fig. 1A, B) and *H. cylindrosporum* (Fig. 1C, D), which were able to grow to some extent on the nocodazole medium (Table 2), the emerging hyphae branched strongly and this led to a "bushy" appearance of the colony. The effect of nocodazole on the morphology was reversible; the growth became normal after the transfer to a nocodazole-free medium.

IIF microscopy

In the control hyphae of the basidiomycetes, the IIF microscopy with the α-tubulin antibody revealed MTs as longitudinal tracks extending close to the apex (Fig. 2A, G, M, R). Occasionally, the MTs were diffuse at the hyphal tip but in the subapical region the tracks were clearly visible (Fig. 2D, J). In the ascomycete *C. geophilum*, the MTs were more diffuse than in the basidiomycetes. In this fungus, MTs were detected best close to the apex often in the mitotic spindles (Fig. 2U–X). In the hyphae of *C. geophilum*, the only thin-walled areas existed in

Fig. 2A–Z. The effect of a 3-h benomyl (4 μg ml^{-1}) and nocodazole (4 μg ml^{-1}) treatment on the MT cytoskeleton of ectomycorrhizal fungi as revealed by IIF microscopy. Thick lines between pictures separate species. **A–C** Apical cells of *Paxillus involutus* on control medium (**A**) and subapical regions of benomyl treated (**B**) and nocodazole treated (**C**) hyphae. **D–F** *H. cylindrosporum* control hypha (**D**), benomyl treated tip (**E**) and branched nocodazole-treated hypha (**F**). **G–I** *A. regalis* growing on control medium (**G**), benomyl-treated subapical region with dividing nuclei (**H** immunofluorescence and **H′** DAPI-staining of the nuclei in **H**), and a nocodazole-treated apical cell (**I**). **J–L** Apical cells of *A. muscaria* growing on control (**J**), benomyl (**K**) and nocodazole (**L**) media. **M–Q** An apical cell of *Suillus bovinus* on control medium (**M**), a branching hypha on nocodazole medium (**N** immunofluorescence, **O** DAPI staining of the nuclei and **P** phase contrast microscopy of the hypha in **N**), and an apical cell growing on benomyl medium (**Q**). **R–T** Apical cells of *Piloderma croceum* growing on control (**R**), benomyl (**S**) and nocodazole (**T**) media. **U–Z** Ascomycete *Cenococcum geophilum* hyphae and dividing nuclei on the control medium (**U** immunofluorescence of a tip, **V** phase contrast microscopy of the hypha in **U**, **W** immunofluorescence of subapical nuclei, only the lower nucleus in focus, and **X** DAPI-staining of the nuclei in **W**), a benomyl-treated hypha (**Y**), and a hypha growing on nocodazole medium (**Z**). *Bar* = 5 μm


A
B
C
D
E
F
N
O
P
G
H
H'
I
M
Q
R
S
T
U
V
J
K
L
W
X
Y
Z

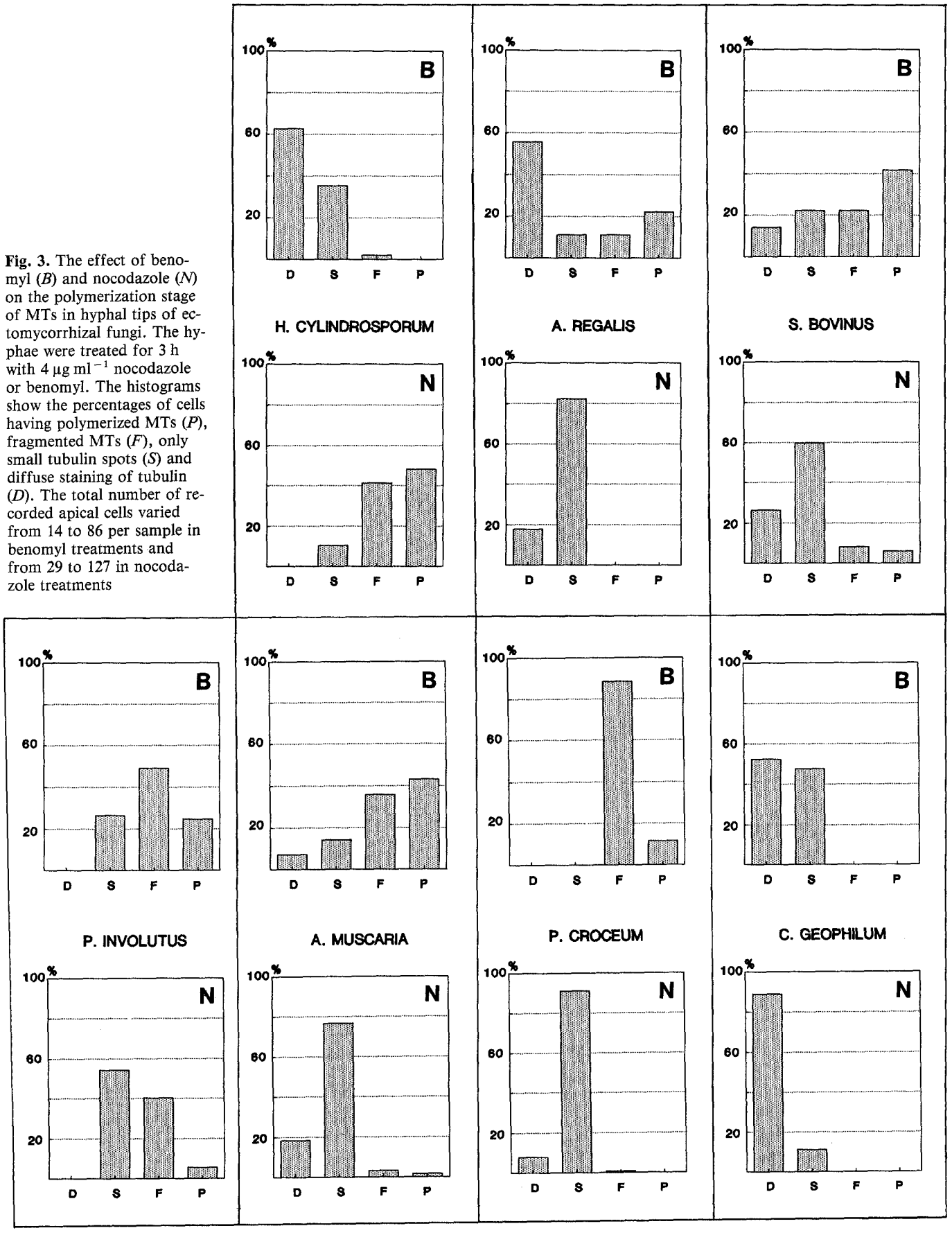


Fig. 3. The effect of benomyl (*B*) and nocodazole (*N*) on the polymerization stage of MTs in hyphal tips of ectomycorrhizal fungi. The hyphae were treated for 3 h with 4 $\mu g\ ml^{-1}$ nocodazole or benomyl. The histograms show the percentages of cells having polymerized MTs (*P*), fragmented MTs (*F*), only small tubulin spots (*S*) and diffuse staining of tubulin (*D*). The total number of recorded apical cells varied from 14 to 86 per sample in benomyl treatments and from 29 to 127 in nocodazole treatments

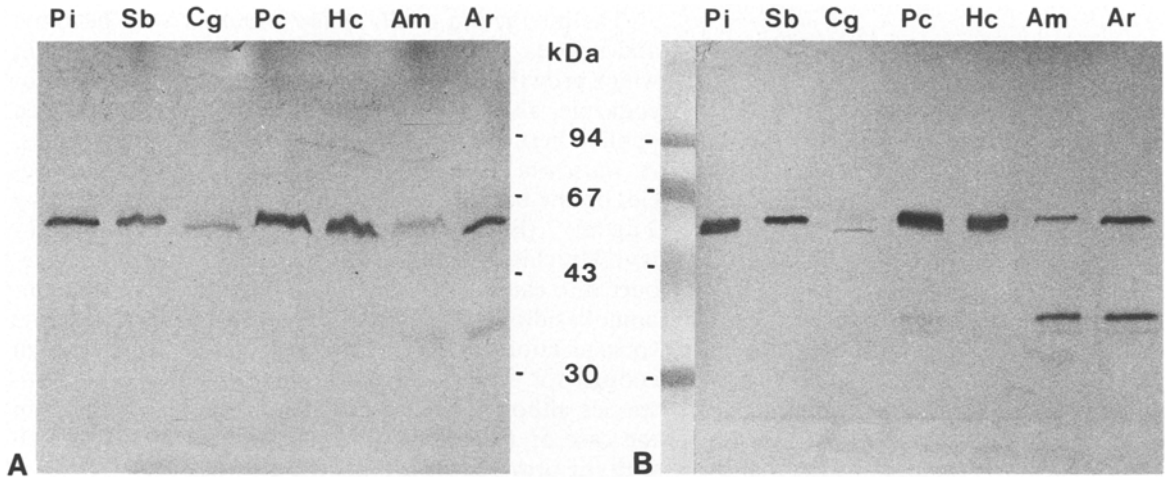


Fig. 4. The immunoblots of α-tubulin (**A**) and β-tubulin (**B**) of *P. involutus* (*Pi*), *S. bovinus* (*Sb*), *C. geophilum* (*Cg*), *P. croceum* (*Pc*), *H. cylindrosporum* (*Hc*), *A. muscaria* (*Am*) and *A. regalis* (*Ar*). Amounts of total protein loaded for the immunoblotting of α-tubulin were 9 µg (*Pi*) and (*Sb*), 15 µg (*Cg*), (*Pc*) and (*Hc*), 14 µg (*Am*) and 15 µg (*Ar*), and for immunoblotting of β-tubulin 5 µg (*Pi*) and (*Sb*), 15 µg (*Cg*), 11 µg (*Pc*), 10 µg (*Hc*), 6 µg (*Am*) and (*Ar*)

the apical region where the large antibody molecules appeared to penetrate the wall. The frequent occurrence of mitotic spindles in this area suggests that it is also the region where nuclear divisions take place.

In the drug-treated cultures, the changes in the IIF pattern of the MT cytoskeleton were more prominent in the nocodazole- than in the benomyl-treated hyphae (Fig. 2). Figure 3 presents the percentage of benomyl- (Fig. 3B) and nocodazole- (Fig. 3N) treated apical cells with polymerized (P), fragmented (F) or depolymerized (S, D) microtubules.

In the benomyl-treated hyphae of *H. cylindrosporum* (Fig. 2E) and *C. geophilum* (Fig. 2Y), no polymerized MTs were detected. Only bright tubulin spots or diffuse staining were seen in IIF microscopy (Fig. 3B). Instead, polymerized MTs and fragments of MTs (classes P + F) were recorded in 33%, 67%, 74%, 79%, and 100% of the cells in *A. regalis* (Figs. 2H, H′, 3B), *S. bovinus* (Figs. 2Q, 3B), *P. involutus* (Figs. 2B, 3B), *A. muscaria* (Figs. 2K, 3B), and *P. croceum* (Figs. 2S, 3B), respectively. The polymerized MTs were usually found in the subapical region, while the fragmented MTs and bright tubulin spots occurred in the hyphal tips. In the treated colonies of *P. involutus*, it was difficult to find intact apical cells, apparently due to damage during the sample processing. Therefore, the calculations of the polymerization had to be made from hyphae with broken tips. This explains the absence of class D from *P. involutus* in Fig. 3.

Nocodazole treatment depolymerized most MTs in the hyphae of *A. muscaria* (Figs. 2L, 3N), *A. regalis* (Figs. 2I, 3N), *S. bovinus* (Figs. 2N–P, 3N), *P. involutus* (Figs. 2C, 3N), *P. croceum* (Figs. 2T, 3N) and *C. geophilum* (Figs. 2Z, 3N). In contrast, in *H. cylindrosporum* polymerized MTs occurred in 48% of the apical cells (Figs. 2F, 3N), although they were more diffuse than in the control hyphae (Fig. 2D).

Immunoblotting of tubulins

The separation of polypeptides of crude mycelial extracts on SDS-PAGE followed by immunoblotting with α- and β-tubulin antibodies led to the detection of a single band for both tubulin proteins in all six basidiomycetes (Fig. 4A, B). The molecular weight of α- and β-tubulin in all the basidiomycetes studied was in the region of 55 kDa. In the β-tubulin immunoblots of *A. muscaria* (Fig. 4B, Am) and *A. regalis* (Fig. 4B, Ar), two additional bands, one weak and one strong, were detected. Their molecular weights were lower than that of β-tubulin. Such bands, although weaker, occurred also in the β-tubulin blot of *P. croceum* (Fig. 4B, Pc) and in the α-tubulin blots of the *Amanita*-species (Fig. 4A, Am and Ar). These bands are probably proteolytic breakdown products of tubulins induced by the extraction procedure. Interestingly, the low molecular weight bands of β-tubulin showed the same mobility in the *Amanita* species (Fig. 4B, Am and Ar) and in *P. croceum* (Fig. 4B, Pc), which suggests that the proteolytic site in the β-tubulin is conserved.

In the ascomycete *C. geophilum*, the immunoblots of α- and β-tubulin led to much weaker signals than those of the basidiomycetes, although the protein content of the loaded samples of *C. geophilum* was the same or higher than that in the samples of the basidiomycetes (Fig. 4A, B, Cg). One α-tubulin band and two β-tubulin bands with different intensities and mobilities were recorded in *C. geophilum*. The weak β-tubulin band of *C. geophilum* (seen clearly in the blots themselves but only weakly in the figure) migrated as fast as the basidiomycete β-tubulin, while the strong band migrated slightly faster (Fig. 4B, Cg). Whether the two bands represent two β-tubulin isotypes of *C. geophilum* or whether the lower band is a breakdown product of the higher one remains to be clarified.

Discussion

The investigation on the effect of anti-MT drugs on the growth of ectomycorrhizal fungi met with several difficulties. The initiation of the growth from mycelial inocula took at least 3 days, and the size of the inoculum unexpectedly interfered with the treatment when benomyl was used. During the long incubation times, the effect of the drug can decrease due to breakdown. Indeed, benomyl is known to convert rapidly in aqueous medium to methyl benzimidazol-2-yl-carbamate. This drug, however, retains anti-MT effects comparable to that of benomyl (Davidse and Flach 1977; Jung and Oakley 1990), and is known to be relatively persistent in soil, at least when it is used as a fungicide (Torstensson and Wessén 1984). Nocodazole is reported by the manufacturer (Janssen Biotech 1988) to be stable in biological media for more than 7 days. It was also difficult to record the immediate effects of the drug treatments on extension growth. The retarding effect of the nocodazole treatment on the extension growth became obvious when the diameter of the treated and untreated colonies were compared after 6 days growth. Among the slowest growing species, *A. muscaria* and *C. geophilum*, even this experimental design did not show any effect of the drug on extension growth.

In contrast to the growth experiments, it was possible to record significant changes in the MT cytoskeleton structure already after a 3-h drug treatment with the IIF microscopical studies. The application of the quick-freezing and freeze-substitution method in sample preparation for IIF microscopy (Raudaskoski et al. 1991) led to more accurate visualization of the MTs in ectomycorrhizal fungi than previously has been accomplished (Salo et al. 1989). Only in the ascomycete *C. geophilum* was the visualization of the MTs restricted to the apical parts of the hyphae. This pattern of MT fluorescence might not just reflect insufficient sample preparation but could result from a more restricted MT distribution and a higher turnover rate of tubulin in the ascomycetes than in the basidiomycetes (Salo et al. 1989). This was also suggested by weaker signals in the immunoblots of tubulins in *C. geophilum* than in the basidiomycetes.

In the ascomycete *C. geophilum*, nocodazole and benomyl were shown to inhibit the growth and to depolymerize the MTs at concentrations of 2 $\mu g\ ml^{-1}$ and 4 $\mu g\ ml^{-1}$, respectively. This was an expected result since the benomyl and nocodazole concentrations used here were comparable to those known to inhibit hyphal growth in ascomycetes *Aspergillus nidulans* and *Neurospora crassa* (Caesar-Ton That et al. 1988; May et al. 1990). In the basidiomycetes, 4 $\mu g\ ml^{-1}$ nocodazole reduced the growth in all measurable cultures but least in *H. cylindrosporum* and in the *Amanita*-species. It also caused depolymerization of MTs in all species except *H. cylindrosporum*. In contrast, benomyl had no effect on the growth or MT polymerization in the basidiomycetes except in *H. cylindrosporum* and *A. muscaria*. In *H. cylindrosporum* but not in the *Amanita*-species, all the MTs were depolymerized after 3 h on a medium with 4 $\mu g\ ml^{-1}$ benomyl.

The interaction of *H. cylindrosporum* with benomyl and nocodazole resembles that of *Coprinus cinereus* in which growth is inhibited with benomyl but not with nocodazole, although at much higher concentrations than applied here (Kamada et al. 1989). In previous studies, *A. muscaria* has been listed as a benomyl-sensitive species on the basis of more than 50% growth inhibition by 2 $\mu g\ ml^{-1}$ (Edgington et al. 1971) and 10 $\mu g\ ml^{-1}$ benomyl (Hutchison 1990). These concentrations were not reported to cause any reduction in the growth of the other homobasidiomycetes tested. This could mean that the concentration of benomyl used here was not high enough for total depolymerization of MTs in *Amanita*-species although the concentration was high enough in the case of *H. cylindrosporum*. This result agrees well with the previous report that the growth of *H. cylindrosporum* is more sensitive to benomyl than the growth of the *Amanita*-species (Hutchison 1990). In the case of nocodazole, the situation seemed to be the opposite in the two fungi; the concentration used was high enough to depolymerize the MTs in the *Amanita*-species but not in *H. cylindrosporum*. This also emphasizes that the structure of the cytoskeleton in *H. cylindrosporum* may have unique as yet unknown features.

The good correlation obtained between the reduction of growth and depolymerization of MTs especially in the nocodazole-sensitive fungi *C. geophilum*, *P. involutus* and *S. bovinus* supports the idea that intact cytoplasmic MTs are needed for the extension growth of the apical cells, providing tracks for translocation of vesicles towards the apex (Howard and Aist 1980; Raudaskoski et al. 1988). Nocodazole appears to be the drug of choice for these fungi when the function of the cytoskeleton is investigated in ectomycorrhizal associations and during nutrient translocation in strands (Timonen et al. 1993). The continuation of growth and the occurrence of intact MTs in the hyphae of *P. croceum*, *P. involutus* and *S. bovinus* in the presence of benomyl justifies further use of low benomyl concentrations in the isolation of these basidiomycetes from nature.

In fungi, the primary target for the benzimidazole-derived drugs such as benomyl and nocodazole appears to be the β-tubulin (Davidse and Flach 1977; Sheir-Neiss et al. 1978; Thomas et al. 1985; Orbach et al. 1986; Jung and Oakley 1990), although the involvement of microtubule associated proteins (Stearns et al. 1990), cell permeability, synergistically interacting genes (Molnár et al. 1985), and the size of the tubulin pool cannot be excluded. The comparison of amino acid sequences encoded by the β-tubulin genes in benomyl-sensitive and -resistant strains of the ascomycetes *N. crassa* and *A. nidulans* suggests that the regions around amino acids 6, 165 and 198 combine in the three-dimensional structure of β-tubulin to form the benzimidazole-binding site (cf. Dyer et al. 1992). Recently, it was shown by sequencing a β-tubulin gene from the homobasidiomycete *Schizophyllum commune* that amino acid 165 in the β-tubulin protein is a cysteine in an amino acid region which is otherwise homologous to those in ascomycetes (Russo et al. 1992). *S. commune* is resistant to benomyl but sensitive to nocodazole, as are *P. croceum*, *P. involutus* and

S. bovinus. When more β-tubulin sequences from homobasidiomycetes are available, it will be interesting to see if the sensitivity and resistance to benomyl and nocodazole are correlated with amino acid substitutions at specific sites in β-tubulin proteins.

Acknowledgements. This work was supported by research grants from the Nordic Joint Committee for Agricultural Research, The Niemi Foundation and the Alfred Kordelin Foundation. We are grateful to Kurt Fagerstedt, Ph. D. and Helena Åström, M. Sc. for critical reading of the manuscript.

References

Åström H, Virtanen I, Raudaskoski M (1991) Cold-stability in the pollen tube cytoskeleton. Protoplasma 160:99–107

Blose SH, Meltzer DI, Feramisco JR (1984) Ten nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. J Cell Biol 98:847–858

Caesar-Ton That TC, Rossier C, Barja F, Turian G, Roos U-P (1988) Induction of multiple germ tubes in *Neurospora crassa* by antitubulin agents. Eur J Cell Biol 46:68–79

Davidse LC, Flach W (1977) Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. J Cell Biol 72:174–193

Dyer M, Volpe F, Delves CJ, Somia N, Burns S, Scaife JG (1992) Cloning and sequence of a β-tubulin cDNA from *Pneumocystis carinii*: possible implications for drug therapy. Mol Microbiol 6:991–1001

Edgington LV, Khew KL, Barron GL (1971) Fungitoxic spectrum of benzimidazole compounds. Phytopathology 61:42–44

Heath IB (1990) The roles of actin in tip growth of fungi. Int Rev Cytol 123:95–127

Howard RJ, Aist JR (1980) Cytoplasmic microtubules and fungal morphogenesis: ultrastructural effects of methyl benzimidazole-2-ylcarbamate determined by freeze-substitution of hyphal tip cells. J Cell Biol 87:55–64

Hutchison LJ (1990) Studies on the systematics of ectomycorrhizal fungi in axenic culture. IV. The effect of some selected fungitoxic compounds upon linear growth. Can J Bot 68:2172–2178

Janssen Biotech (1988) Jansen life sciences products catalogue. Olen, Belgium, pp 35–36

Jung KM, Oakley BR (1990) Identification of an amino acid substitution in the *ben*A, β-tubulin gene of *Aspergillus nidulans* that confers thiabendazole resistance and benomyl supersensitivity. Cell Motil Cytoskel 17:87–94

Kamada T, Sumiyoshi T, Shindo Y, Takemaru T (1989) Isolation and genetic analysis of resistant mutants to the benzimidazole fungicide benomyl in *Coprinus cinereus*. Curr Microbiol 18:215–218

Kamada T, Hirami H, Sumiyoshi T, Tanabe S, Takemaru T (1990) Extragenic suppressor mutations of a β-tubulin mutation in the basidiomycete *Coprinus cinereus*: isolation and genetic and biochemical analyses. Curr Microbiol 20:223–228

Kwon YH, Hoch HC, Aist JR (1991) Initiation of appressorium formation in *Uromyces appendiculatus*: organization of the apex, and the responses involving microtubules and apical vesicles. Can J Bot 69:2560–2573

May GS, Waring RB, Morris NR (1990) Increasing *tub*C β-tubulin synthesis by placing it under the control of a *ben*A β-tubulin upstream sequence causes a reduction in *ben*A β-tubulin level but has no effect on microtubule function. Cell Motil Cytoskel 16:214–220

Molina R, Palmer JG (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. The American Phytopathological Society, St Paul, Minn, pp 115–129

Molnár A, Hornok L, Pesti M (1985) The high level of benomyl tolerance in *Fusarium oxysporum* is determined by the synergistic interaction of two genes. Exp Mycol 9:326–333

Oakley BR, Rinehart JE (1985) Mitochondria and nuclei move by different mechanisms in *Aspergillus nidulans*. J Cell Biol 101:2392–2397

Orbach MJ, Porro EB, Yanofsky C (1986) Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol Cell Biol 6:2452–2461

Raudaskoski M, Salo V, Niini SS (1988) Structure and function of the cytoskeleton in filamentous fungi. Karstenia 28:49–60

Raudaskoski M, Rupeš I, Timonen S (1991) Immunofluorescence microscopy of the cytoskeleton in filamentous fungi after quick-freezing and low-temperature fixation. Exp Mycol 15:167–173

Runeberg P, Raudaskoski M, Virtanen I (1986) Cytoskeletal elements in the hyphae of the homobasidiomycete *Schizophyllum commune* visualized with indirect immunofluorescence and NBD-phallacidin. Eur J Cell Biol 41:25–32

Russo P, Juuti JT, Raudaskoski M (1992) Cloning, sequence and expression of a β-tubulin-encoding gene in the homobasidiomycete *Schizophyllum commune*. Gene 119:175–182

Salo V, Niini SS, Virtanen I, Raudaskoski M (1989) Comparative immunocytochemistry of the cytoskeleton in filamentous fungi with dikaryotic and multinucleate hyphae. J Cell Sci 94:11–24

Sheir-Neiss G, Lai MH, Morris NR (1978) Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell 15:639–647

Stearns T, Hoyt MA, Botstein D (1990) Yeast mutants sensitive to antimicrotubule drugs define three genes that affect microtubule function. Genetics 124:251–262

Taylor JB (1971) A selective medium for the isolation of basidiomycetes from diseased roots, mycorrhizas, and soil. Trans Br Mycol Soc 56:313–314

Thomas JH, Neff NF, Botstein D (1985) Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics 112:715–734

Timonen S, Finlay RD, Söderström B, Raudaskoski M (1993) Identification of cytoskeletal components in pine ectomycorrhizas. New Phytol (in press)

Torstensson L, Wessén B (1984) Interactions between the fungicide benomyl and soil microorganisms. Soil Biol Biochem 16:445–452

Wagner F, Gay G, Debaud JC (1988) Genetical variability of glutamate dehydrogenase activity in monokaryotic and dikaryotic mycelia of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Appl Microbiol Biotechnol 28:566–571

Wessels JGH (1986) Cell wall synthesis in apical hyphal growth. Int Rev Cytol 104:37–79