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The Microtubule Cytoskeleton in Hyphae of *Uromyces phaseoli* Germlings: Its Relationship to the Region of Nucleation and to the F-Actin Cytoskeleton

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Summary

The microtubule and F-actin cytoskeleton of nondifferentiated germlings of *Uromyces phaseoli* was studied using immunofluorescence methodologies. The microtubules were oriented mostly parallel to the longitudinal axis of the hypha. Microtubule depolymerizing agents, such as cold, demecolcine, griseofulvin and nocodazole, were effective in destroying the microtubule network, but not the F-actin system. Repolymerization of microtubules, following release from these agents, occurred first in the hyphal apices and not near the nuclei or spindle pole bodies. It was concluded that the microtubule nucleating region in such fungal cells is located in the apical regions. Enhanced microtubule arrays were visualized following incubation of the cells in taxol, an agent known to favor microtubule polymerization.

Keywords: Cytoskeleton; Immunocytochemistry; Microfilaments; Microtubules; Microtubule organizing center; *Uromyces phaseoli.*

1. Introduction

Recent investigations in our laboratories have shown that treatments known to depolymerize microtubules initiate events which lead to nuclear division and/or cell differentiation in the bean rust fungus, *Uromyces phaseoli* (Pers.) Wint. (Staples and Hoch 1982). Such treatments included exposure to drugs (*e.g.*, demecolcine, griseofulvin, nocodazole), ultrasonic sound, and heat shock. More recently we noted that cold shock and high atmospheric pressure treatments were also effective (unpublished). Alternatively, microtubule stabilizing treatments [*e.g.*, exposure to D_2O (Staples and Hoch 1982) and taxol (unpublished)] prevented these differentiation-related events to varying degrees. We have recently reported that high levels of exogenously supplied cAMP, known to promote microtubule polymerization and stabilization indirectly in fibroblasts (Lockwood 1980, Puck 1977, Roisen *et al.* 1975), likewise prevented cell differentiation, although it did promote a single round of nuclear division (Hoch and Staples 1984). We have thus hypothesized that an intact microtubule cytoskeleton usually prevents the fungal cell from advancing through cell differentiation and mitosis, and that a temporary depolymerization of at least part of the cytoplasmic cytoskeleton is necessary for these events to ensue.

A necessary step toward examining our hypothesis is the visualization of the microtubule cytoskeleton. Previously, it has been studied in filamentous fungi using electron microscopy. Such studies usually have yielded an incomplete picture of the cytoskeleton because serial sections were inadequately analyzed. More importantly, the *in situ* position of the microtubule cytoskeleton most likely was poorly preserved. Recent employment of cryogenic methods using freeze-substitution has helped to overcome this deficiency (Hoch and Howard 1980, Hoch and Staples 1983a, Newhouse *et*

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al. 1983). Perhaps of greater importance, has been the inability to visualize microtubules in fungi using immunofluorescence light microscopy so commonly used in mammalian cell research. The basic problem has been the difficulty of overcoming the permeability of the cell wall to the various antibodies, as well as the lack of common antigenicity of available antibodies (principally from mammalian sources) to fungal tubulin. Here, we use indirect immunocytochemical labeling methodologies to elucidate the organization of the microtubule cytoskeleton in *Uromyces phaseoli* uredospore germlings, as affected by various microtubule stabilizing and destabilizing agents. In addition, we report on the location and appearance of the microtubule nucleating region in this filamentous fungus.

2. Materials and Methods

Uredospores of *Uromyces phaseoli* (Pers.) Wint. (race 0) were collected, stored, and prepared for germination as described previously (STAPLES and HOCH 1982).

Uredospores were uniformly dusted onto 1 cm^2 membranes of polyethylene or polycarbonate (HOCH and STAPLES 1983a, STAPLES and HOCH 1982) and onto glass microscope coverslips. The spore-laden surfaces were lightly atomized with distilled water to initiate germination, then incubated in humidified plastic petri plates for 2.5 hours at 19 °C. Following this period of germination and growth, the germlings were exposed to one of several treatments to study the stability of the microtubule cytoskeleton. Such treatments included depolymerization, repolymerization, and stabilization of the microtubules as determined by the following schedules:

1. Microtubule depolymerization. The germlings were inverted onto solutions of demecolcine (10^{-2} to 10^{-6} M), griseofulvin (5×10^{-3} to 5×10^{-6} M), and nocodazole (3×10^{-3} to 3×10^{-7} M) (Sigma Chemical Co., St. Louis, Missouri) for specific time periods between 1 and 80 minutes at 19 °C. Germlings also were subjected to cold treatments by floating the petri dish containing the membranes in an ice water bath (4 °C) for 30 or 60 minutes.

2. Microtubule repolymerization. Following exposure for 30 minutes to 5×10^{-3} M demecolcine, the germlings were rinsed in distilled water for 5 to 80 minutes. Also, following the 60-minute cold treatments as outlined above, the germlings were reincubated at 19 °C for 5 to 40 minutes.

3. Microtubule stabilization. Uredospores were atomized with 40% D_2O in distilled water or with taxol at 10 and 100 µM and incubated at 19 °C for 3, 5, and 16 hours. Germlings grown in the presence of these agents, known to stabilize microtubules (SCHIFF *et al.* 1979, SCHIFF and HORWITZ 1980), also were subjected to cold treatments as described above.

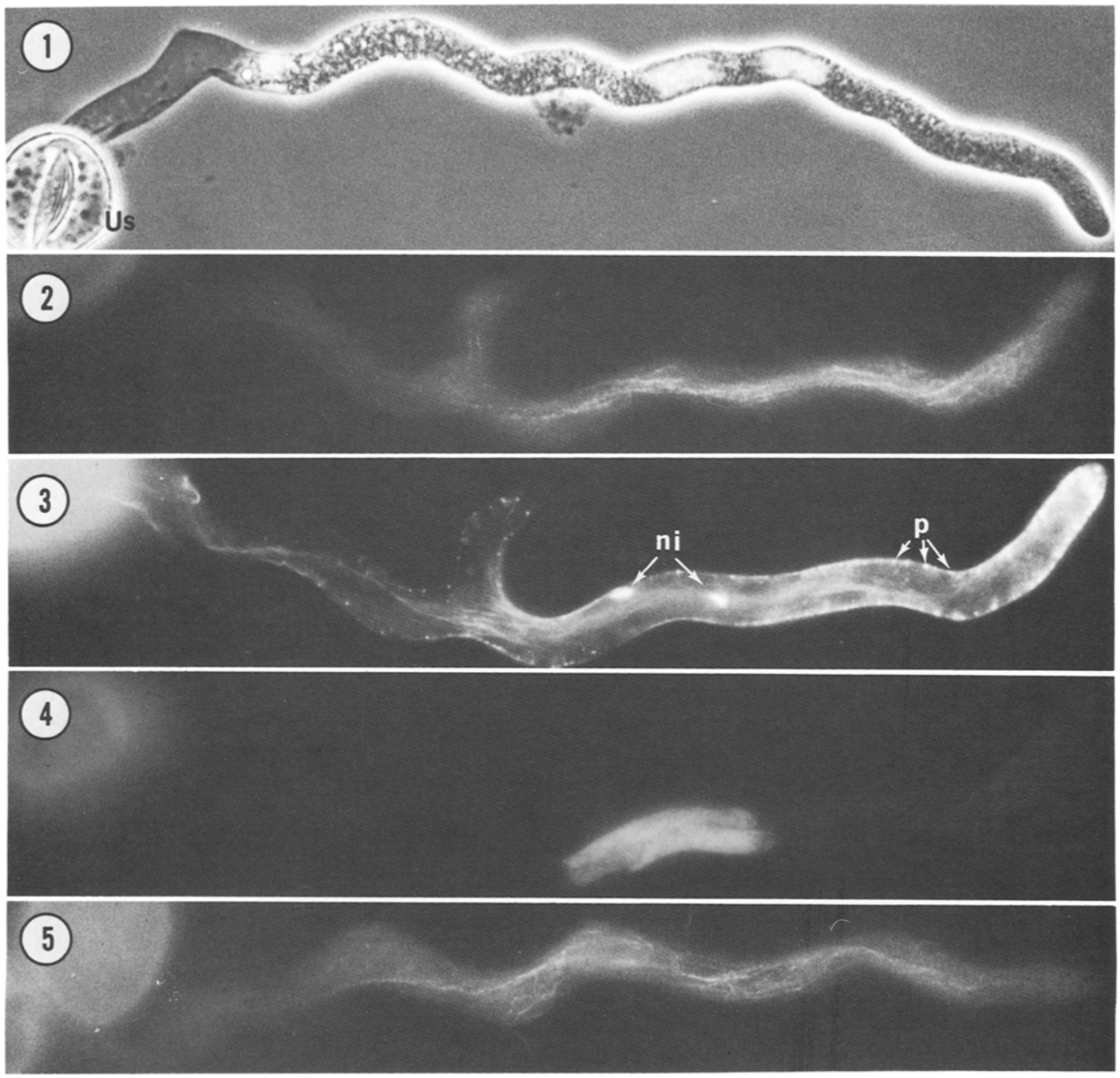
In all tests, germlings exposed only to distilled water for appropriate times served as controls.

To visualize the microtubule cytoskeleton, the germlings were fixed by inverting the membranes onto 3% formaldehyde in 50 mM phosphate (K^+) buffer containing 5 mM $MgCl_2$, pH 6.5, for 30 minutes or by plunging them into —85 °C acetone and allowing the substituent to come to room temperature. This latter method of freeze-substitution is exemplified in Fig. 8. Next, the germlings were rinsed with phosphate buffer, then treated for 15 minutes with a glusulase preparation (Endo Laboratories, Inc., Wilmington, Del.), 10 µl/ml phosphate buffer, pH 6.5, to partially digest the germling wall. The germlings were rinsed briefly with phosphate buffer, pH 6.5, then with a phosphate-buffered saline solution (PBS), pH 7.2, before being subjected to 0.1% Triton X-100 in PBS for 10 minutes to solubilize the cell membranes. Following a rinse in PBS the germlings were incubated at 37 °C for 30 minutes in a monoclonal antibody preparation (YOL 1/34) made to yeast tubulin (KILMARTIN and FOGG 1982) in PBS, rinsed with PBS containing 0.1% bovine serum albumen, and incubated with anti-rat IgG-conjugated FITC (Sigma) at 37 °C for 30 minutes. The germlings were again rinsed with PBS, stained with rhodamine-conjugated phalloin (Rh-Phn) (WIELAND *et al.* 1983) for F-actin microfilament visualization (see HOCH and STAPLES 1983b, for details concerning this staining reaction and its specificity), rinsed with PBS and further stained with the DNA-binding fluorophore, 4,6-diamidino-2-phenylindole (DAPI) (TODA *et al.* 1981), to correlate the position of the nuclei (HOCH and STAPLES 1983b). The preparations were mounted in 90% glycerol:10% phosphate buffer (0.1 M), pH 8.6, containing 0.1% n-propyl gallate (GILOH and SEDAT 1982) and examined with a Zeiss photomicroscope equipped with an epifluorescence attachment using a mercury lamp. Generally, a planapochromat, 63 x/NA 1.4/Ph, objective was used. The following excitation, dichromatic beam splitters, and barrier filters were used for rhodamine, FITC, and DAPI, respectively: 546, 580, and 590 nm; 450–490, 510, and 520–560 nm; and 365, 395, and 420 nm, respectively. Images were recorded on either Kodak Tri-X film developed at 1,600 ASA with Diafine or with Ilford XP1 400 film developed at 800 ASA with Ilford XP1 chemicals.

3. Results

Uredospore germlings of *Uromyces phaseoli* generally grow as nonbranched coenocytic hyphae (Fig. 1). As the germ tube elongates, the protoplasm which remains approximately constant in volume, migrates forward creating an emptied space in the basipetal-most regions. The two haploid nuclei are positioned in the central region of the cytoplasm most often but not always, in tandem (Figs. 1 and 4). The F-actin microfilament cytoskeleton and the ultrastructural organization of similar germlings have been described previously (HOCH and STAPLES 1983a, b).

The microtubules were oriented mostly parallel with the longitudinal axis of the hypha (Figs. 2, 5, 6, and 8). Intense fluorescence within the first 15–30 µm of the hyphal tip frequently obscured many of the individual microtubules, although by careful focusing, some could be seen within this area (Fig. 8). While microtubules often were visualized near the extreme hyphal apex following formaldehyde fixation (Figs. 2, 5, and 6), more could be seen there following freeze-substitution in acetone (Fig. 8), which preserved microtubules extending from near the hyphal tip to the more distal portion of the cytoplasm (Figs. 2 and 5). The profiles circumvented (bypassed) the two nuclei and did not



Figs. 1–5. Three-hour-old uredospore germlings of *Uromyces phaseoli*. Fig. 1. Combined phase-contrast and UV-fluorescence micrograph of a germling. The two nuclei were visualized by staining for DNA with the fluorophore, 4,6-diamidino-2-phenylindole (DAPI). Figs. 2–4. The same germling, fixed and stained with FITC-labeled antibodies for tubulin (Fig. 2), rhodamine-conjugated phalloin for actin (Fig. 3) and DAPI (Fig. 4) for microtubules, F-actin and DNA, respectively. Fig. 5. Microtubule cytoskeleton visualized by indirect immunocytochemical protocols similar to that in Fig. 2. *ni* nuclear inclusion; *p* peripheral plaques; *Us* uredospore. Fig. 1, ×940; Figs. 2–5, ×1,650

originate from or associate with a nuclear-associated microtubule organizing center [or spindle pole body (SPB)] (Figs. 6, and 21–30). The positions of the nuclei were ascertained with DAPI or by staining the F-actin-containing nuclear inclusion known to be positioned immediately subjacent the SPB within the nucleoplasm (Figs. 3 and 7) (Hoch and Staples 1983a, b).

In general, more microtubules were observed in the apical one-half of the hypha than in the more basipetal regions. F-actin microfilaments of the cytoskeleton, conversely, have been shown previously to be very abundant in the basipetal-most region of the germlings (Hoch and Staples 1983b). Scrupulous examination, however, indicated that the microtubules and microfilaments were frequently positioned along side each other (*cf.*, Figs. 6 and 7).

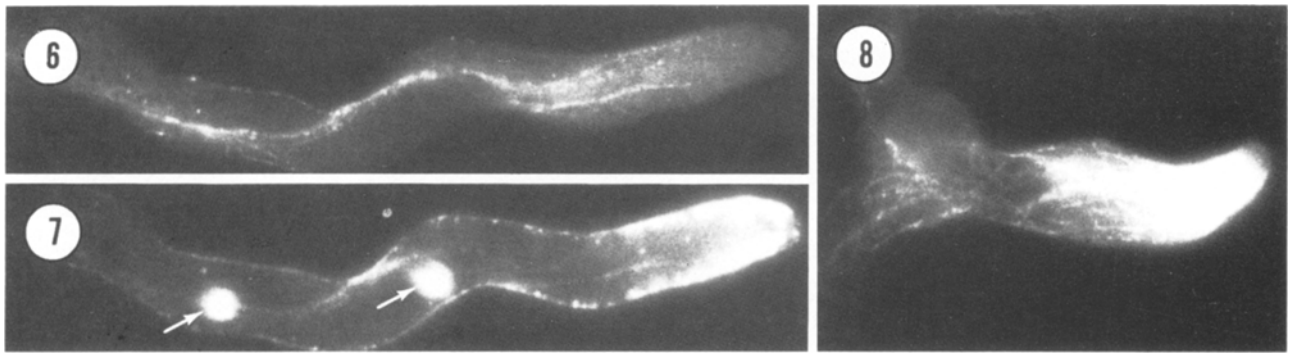
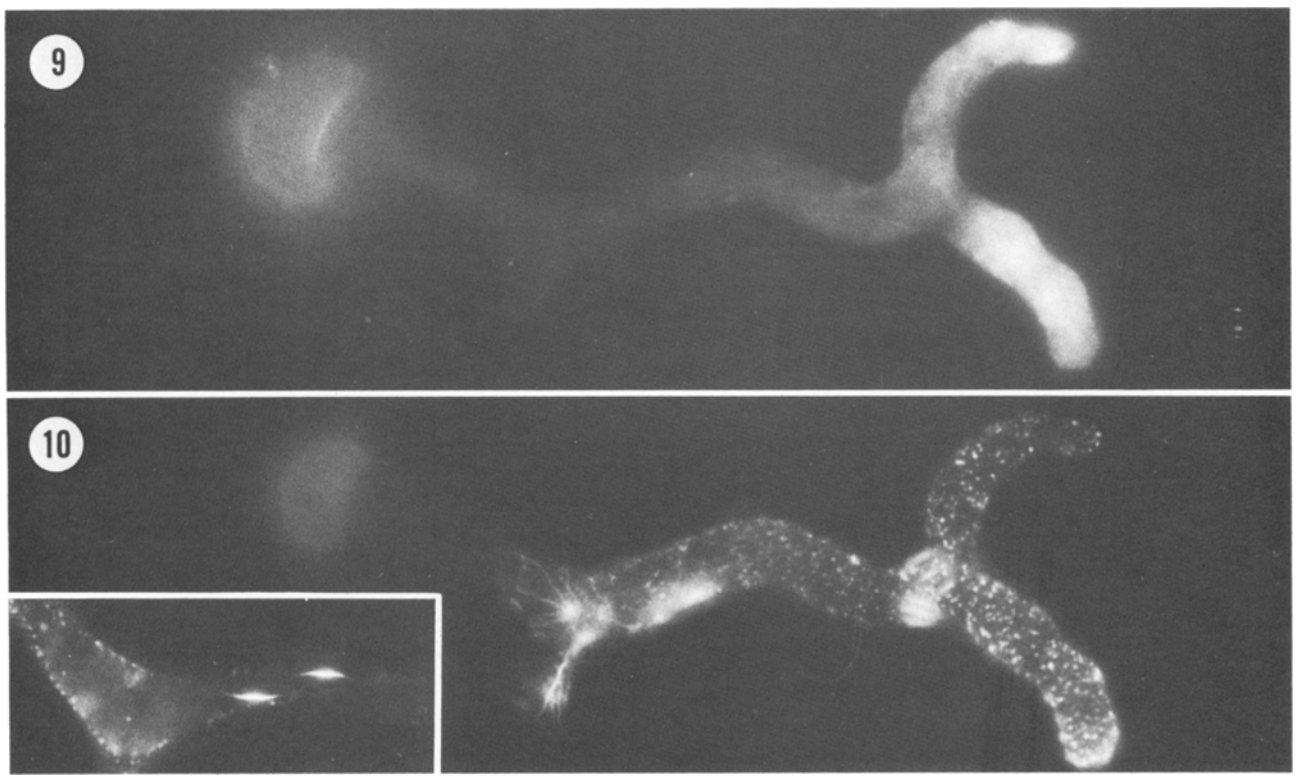


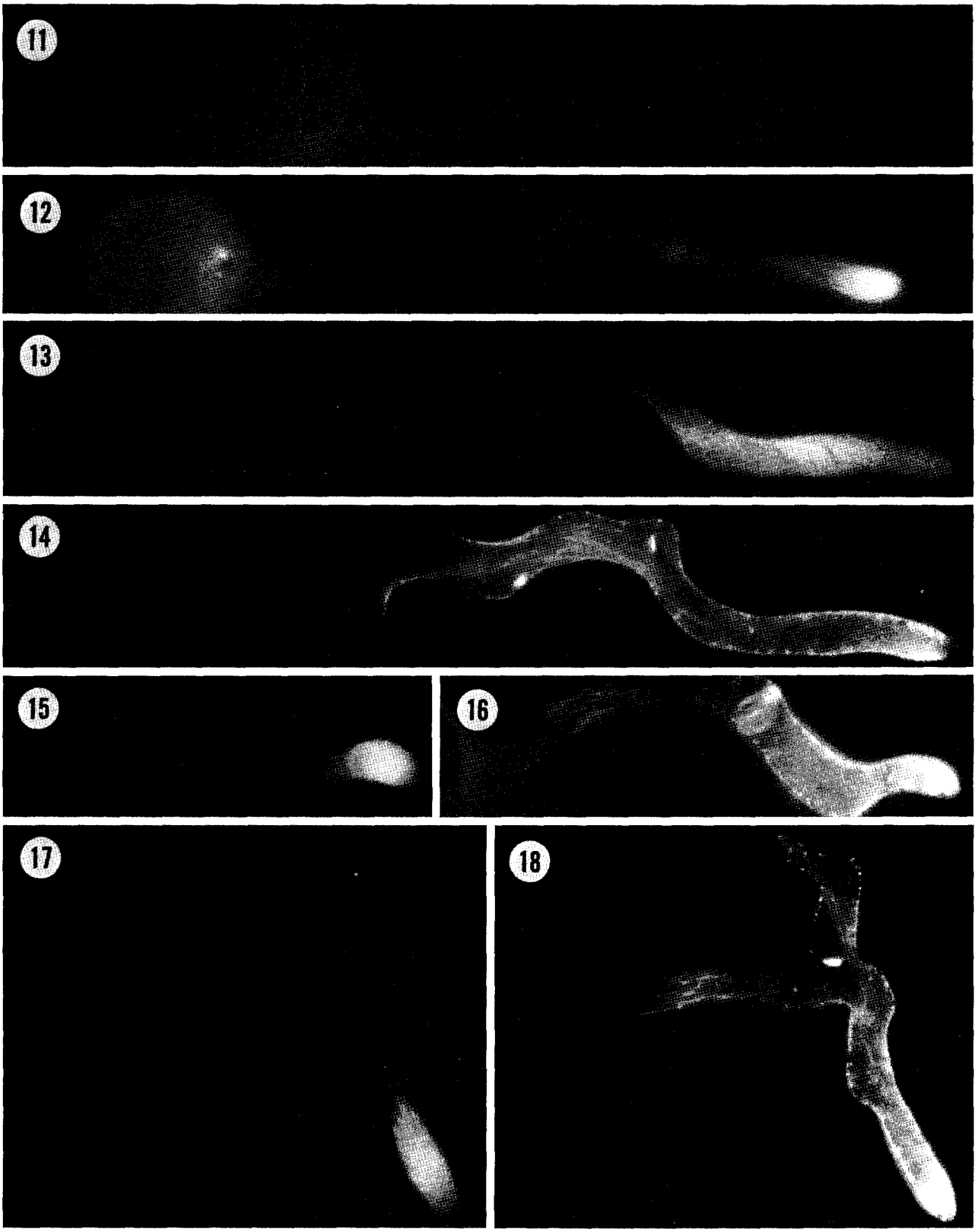
Fig. 6–8. Hyphal apices of *Uromyces phaseoli*. Figs. 6 and 7. The same cell stained for microtubules and F-actin, respectively. Many of the microtubule and F-actin microfilament profiles occupy similar sites. The two F-actin-containing nuclear inclusions (arrows) of Fig. 7 are out of the optical plane of focus. Fig. 8. Microtubules are readily seen in the hyphal apex preserved by freeze-substitution in acetone. All, × 1,650



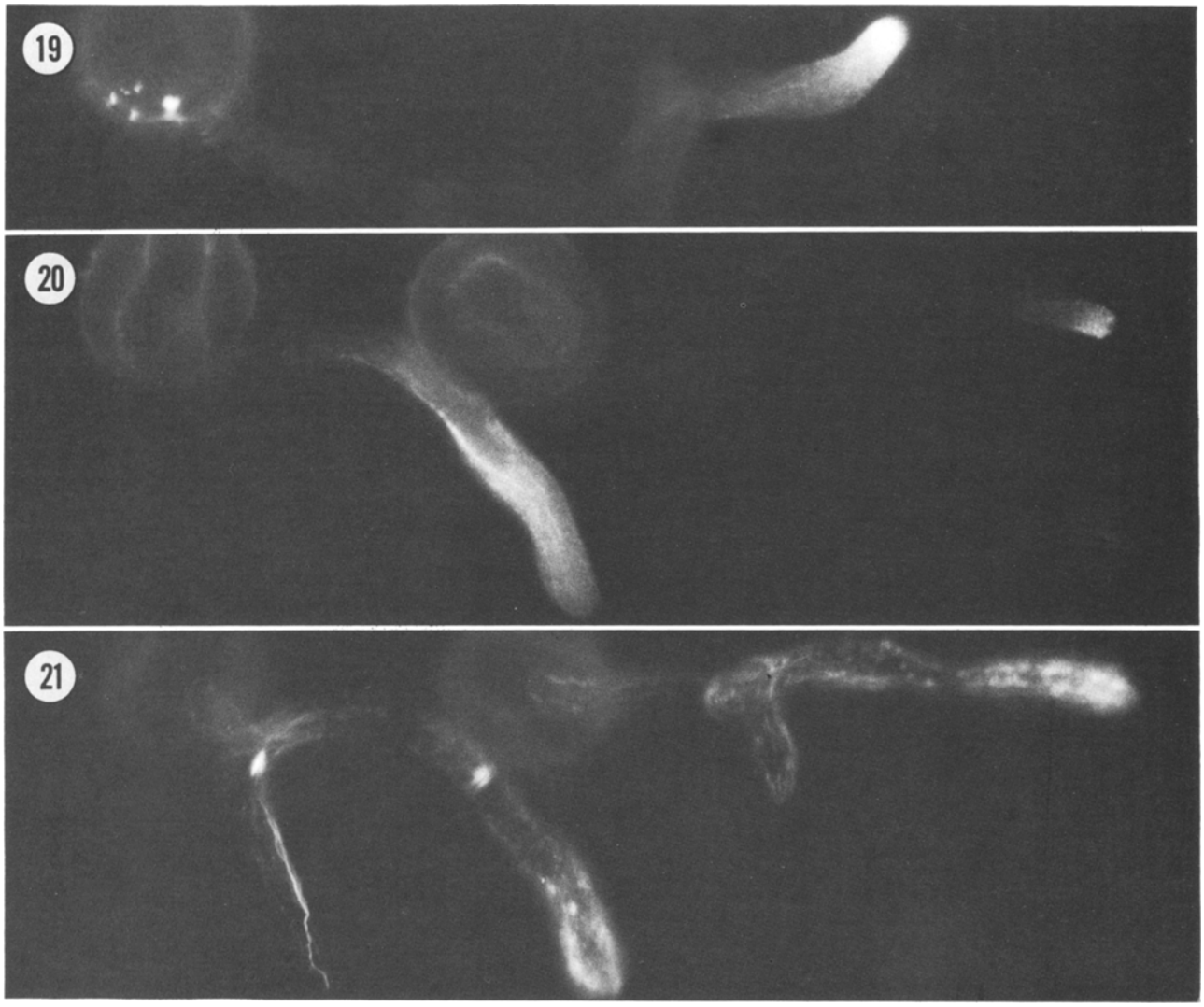
Figs. 9 and 10. Uredospores of *Uromyces phaseoli* germinated for 2.5 hours at 19 °C, then exposed to 4 °C for 1 hour prior to fixation and staining for microtubules (Fig. 9) and F-actin (Fig. 10). Microtubules were not observed. F-actin, as peripheral plaques, remained without apparent changes whereas the basipetal-most filaments appeared spider-like in profile (Fig. 10). The F-actin nuclear inclusions (Fig. 10, inset) increased slightly in length. All, × 1,650

3.1. Microtubule Depolymerization

Depolymerization of the cytoplasmic microtubule cytoskeleton was observed following exposure to cold or to the microtubule depolymerizing chemicals, demecolcine, griseofulvin, and nocodazole. Uredospore germlings exposed to 4 °C for 30 minutes contained very few, if any, cytoplasmic microtubules. Exposure for 60 minutes or longer generally resulted in depolymerization of all microtubules (Fig. 9), although ca. 10% of the germlings contained a few apparently cold-resistant microtubules positioned in the more apical regions of the hypha. F-actin in the form of peripheral plaques was not affected by the cold treatment, whereas the filaments in the more basipetal region of the cytoplasm were organized into spider-like



Figs. 11–18. Germlings of *Uromyces phaseoli* subjected to varying concentrations of microtubule depolymerizing drugs for 30 minutes following 2.5 hours of germination. Figs. 11–13. Germlings subjected to 10^{-2} M, 10^{-3} M and 10^{-4} M demecolcine, respectively, and stained for microtubules. Fig. 14. Same cell as Fig. 13 but visualized for F-actin. Figs. 15 and 16. The same cell subjected to 5×10^{-3} M griseofulvin and stained for microtubules and F-actin, respectively. Figs. 17 and 18. The same cell subjected to 5×10^{-5} M griseofulvin and stained for microtubules and F-actin, respectively. All, × 1,650



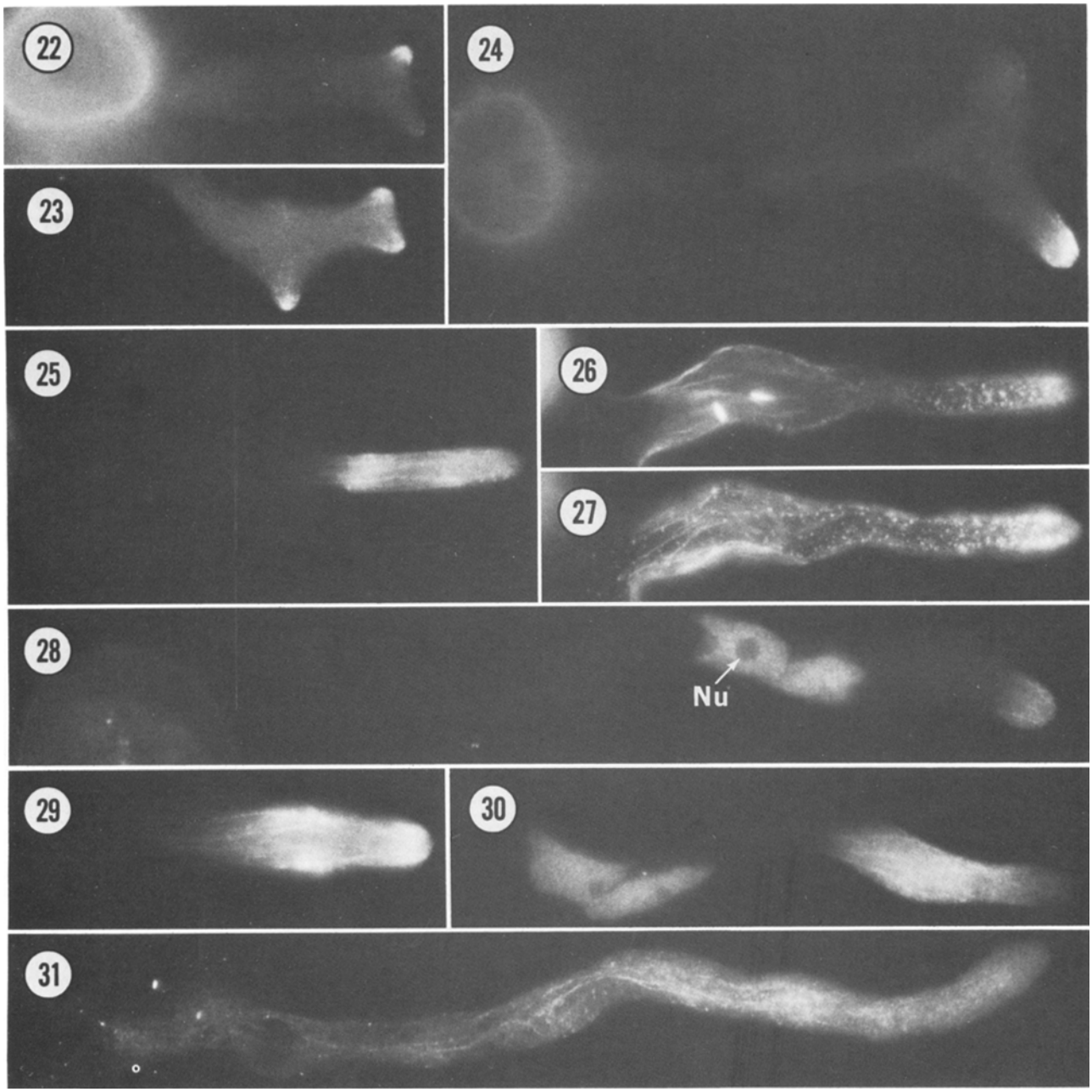
Figs. 19–21. Germlings of *Uromyces phaseoli* subjected to 3×10^{-3} M (Fig. 19) and 3×10^{-5} M (Figs. 20 and 21) nocodazole for 30 minutes following 2.5 hours of germination. Cells in Figs. 19 and 20 were stained for microtubules. Fig. 21 represents the same two germlings seen in Fig. 20 but stained for F-actin. All, ×1,650

profiles (Fig. 10), and the nuclear inclusions increased slightly in length (Fig. 10 inset). Demecolcine (10^{-2} to 10^{-4} M) (Figs. 11–14), griseofulvin (5×10^{-3} to 5×10^{-5} M) (Figs. 15 and 17), and nocodazole (3×10^{-3} to 3×10^{-5} M) (Figs. 19 and 20) were effective in depolymerizing most of the cytoplasmic microtubules. No distinct microtubules were visualized at the highest tested concentrations of these drugs; however, the number of microtubules remaining at lower concentrations of the drugs appeared to be concentration dependent. As with the cold treatments, the microtubules were seen to be most persistent in the hyphal tips (*e.g.*, Figs. 12 and 17). F-actin in the form of microfilaments (particularly in the distal regions), peripheral plaques, and nuclear inclusions mostly remained normal with the drug treatments (Figs. 14, 16, 18, and 21). F-actin microfilaments, however, in the mid to apical region of drug-treated germlings were frequently not visualized when microtubules were depolymerized.

In preliminary tests, it was discerned that 20 minutes was the minimum time in which all microtubules were depolymerized with demecolcine (10^{-2} M) (Fig. 11). No changes in microtubule abundance or pattern of distribution were noted at 1- or 5-minute exposure to demecolcine. By 10 minutes, however, most microtubules were depolymerized.

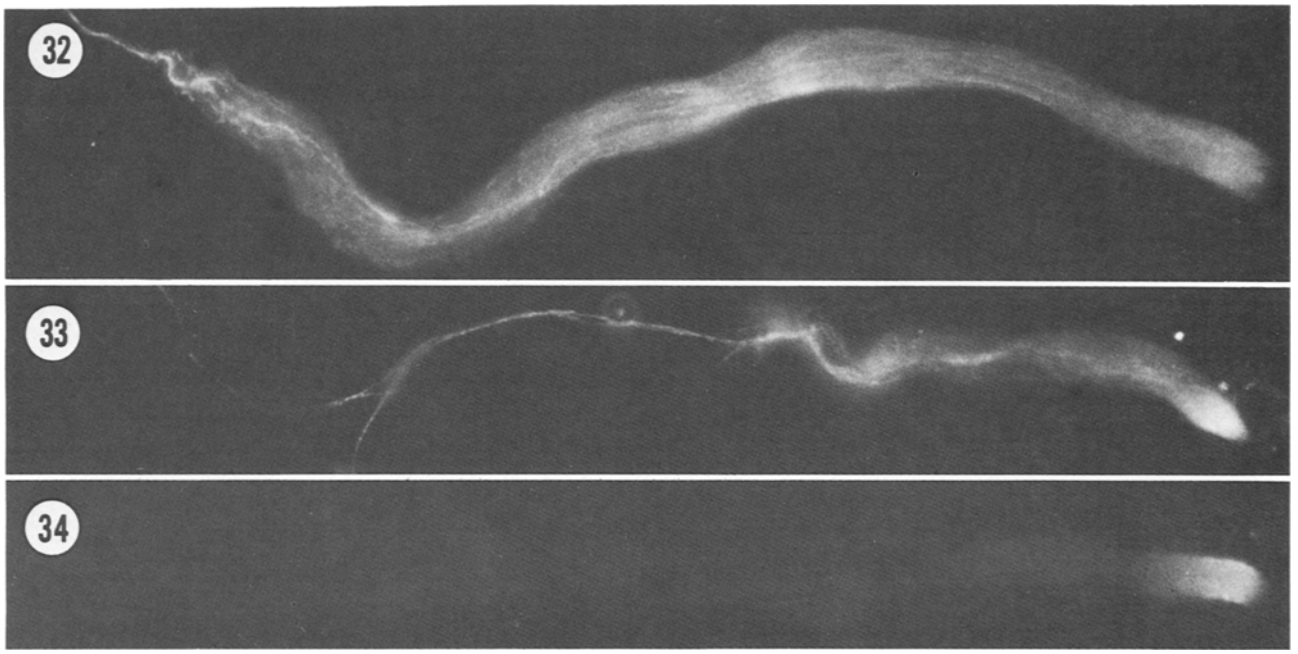
3.2. Microtubule Repolymerization

Uredospore germlings previously subjected to microtubule depolymerizing treatments were exposed to



Figs. 22–31. Cells of *Uromyces phaseoli* germinated for 2.5 hours, then subjected to microtubule depolymerizing treatments, followed by release from such treatments. Figs. 22–25. Germlings subjected to 4 °C for 1 hour, then returned to 19 °C for 8, 10, 15, and 20 minutes, respectively. Repolymerization of the microtubules began in the hyphal apices. Figs. 26 and 27. Two planes of optical focus through the same cell as that depicted in Fig. 25, but stained for F-actin. Figs. 28–31. Germlings subjected to demecolcine (10^{-3} M) for 30 minutes, then washed free of the drug and fixed after 10, 20, 40, and 80 minutes, respectively. *Nu* nucleolus. All, × 1,650

conditions which allowed microtubule repolymerization. Such treatments permitted us to determine the microtubule nucleating regions in these fungal hyphae. Microtubule nucleating regions in uredospore germlings were found to be in the hyphal apices only (Figs. 22 and 28). No repolymerization was noted from the region surrounding the nuclei (*cf.*, Figs. 25, 26, and 28). Short, 1 to 5 μm, profiles of microtubules were first observed 8 to 10 minutes following release from the depolymerizing agent [*e.g.*, shift in temperature from 4 to 19 °C (Fig. 22) or by washing demecolcine-treated cells with distilled water (Fig. 28)]. Microtubule arrays continued to increase in density and in length with time under the repolymerization conditions (Figs. 22–25 and



Figs. 32–34. Three and a half-hour-old germlings of *Uromyces phaseoli* stained for microtubules. Fig. 32. Germling grown in the presence of 100 μM taxol exhibited an enhanced microtubule cytoskeleton (*cf.*, Figs. 2 and 5). Fig. 33. Germling treated as that in Fig. 32, but subjected to 4 °C for 60 minutes. A few microtubule arrays remained. Fig. 34. Germling not treated with taxol, but subjected to 4 °C for 60 minutes. No microtubules were visualized. All, × 1,650

28–30). Microtubule arrays regained their complete profiles between 50 and 80 minutes (*e.g.*, Fig. 31).

Under the above conditions F-actin (as cytoplasmic microfilaments and peripheral plaques) remained normal and unaffected (Figs. 26 and 27). The nuclear inclusions of F-actin, which had elongated when exposed to 4 °C, reverted to their previous shorter length of 2.5 μm (Fig. 26) within 10 minutes following germling incubation at 19 °C.

3.3. Microtubule Stabilization

Uredospores germinated in the presence of water saturated with taxol (100 μM) exhibited more microtubules than controls when examined at 3 and 16 hours following initiation of germination (Fig. 32). This increased population, however, was observed in only about 30% of the germlings with the remaining cells containing normal arrays. Taxol applied at 10 μM did not appear to be effective. The F-actin structures appeared as in the water-treated germling and were not noted to be more abundant.

Germlings grown in the presence of 100 μM taxol and subsequently subjected to 4 °C for 1 hour exhibited many stabilized microtubules (Fig. 33). Again, however, only about 30% of the cells showed this response. By optically focusing through the depth of the germling, many more microtubules were visualized than are depicted in Fig. 33, although there definitely were fewer microtubules than in noncooled controls. No microtubules were observed in similar germ tubes exposed to 4 °C, not pretreated with taxol (Fig. 34).

Microtubule arrays did not stain in uredospore germlings grown in the presence of D_2O. We were, however, able to visualize microtubule arrays in human forearm fibroblasts similarly treated with D_2O. Thus far, we cannot account for this, and are able only to surmise that the D_2O interfered with the rust uredospore germling system, perhaps by inhibiting enzymatic dissolution of the cell wall.

4. Discussion

Concepts regarding the microtubule-microfilament (F-actin) cytoskeleton in filamentous fungi are fragmentary, compared to what is known for many types of mammalian cells. Such a dearth of information can be attributed to previous difficulties in readily permeating whole hyphal cells with immunochemicals. Also, until recently, appropriate immunochemicals, such as the yeast antitubulin (Kilmartin *et al.* 1982) and the F-actin-specific cytochemical phalloin (Wieland *et al.* 1983), were not available. Various sources of mammalian-derived tubulin and actin antisera tested by us did not stain the cytoskeleton of *U. phaseoli* (or of

zoospores of *Phytophthora cactorum*) and likely had low affinities for these fungal tubulin and actin proteins.

Based on electron microscopy of thin-sectioned material, it was previously shown that cytoplasmic microtubules were generally orientated parallel to the longitudinal axes of fungal hyphae. The extensiveness of the arrays of microtubules depicted in this report, however, was not previously conceived. F-actin, as a cytoskeletal component, has not been well preserved or visualized using conventional fixation protocols. Recently, however, several reports have shown abundant microfilament arrays in several fungal species preserved by freeze-substitution (Hoch and Howard 1980, Hoch and Staples 1983a, b, Howard 1981, Newhouse *et al.* 1982). With these new methodologies, immunochemicals and cytochemicals, we are arriving at a better, though still incomplete insight into the cytoplasmic microtubule-microfilament organization in many fungi.

Arrays of microtubules in the nondifferentiated germling generally extended from near the hyphal apex to a region basipetal to the nuclei. Frequently, such microtubule profiles extended to the termination of the trailing cytoplasmic processes. It was not determined if the individual arrays stained for tubulin represented one or more microtubules. Certainly, individual filaments can be discerned using immunofluorescence as has been shown by Osborne *et al.* (1978). The apparent lack of microtubules in the extreme apex of many of the figures is due primarily to the level of optical focus illustrated, but also to the method of fixation. We believe that fixation with formaldehyde, like glutaraldehyde, somewhat displaces the microtubules aftward compared to the *in vivo* state better represented in specimens preserved by freeze-substitution. The hyphal apex illustrated in Fig. 8 is representative of a freeze-substituted (acetone only) hypha.

The close association of microtubules and F-actin microfilaments seen with light microscopy was anticipated since such relationships had been reported previously in ultrastructural studies with *U. phaseoli* (Heath and Heath 1978, Hoch and Staples 1983a). Because of this association, it was also expected that treatments which depolymerized microtubules might also cause a disappearance of many, although not all, of the F-actin filaments in the hyphal tip regions. However, F-actin, as peripheral plaques, as nuclear inclusions, and as those filaments more readily seen in the basipetal regions, generally were unaffected by the microtubule drugs. Cold treatment did affect both the nuclear inclusions and the basipetally positioned filaments, as alluded to in the Results.

Microtubule organizing centers (MTOCs) most frequently have been associated with an organelle closely associated with the nucleus; *e.g.*, centrioles, basal bodies and kinetochores (Pickett-Heaps 1969, Witt *et al.* 1980). In most cell systems studied, especially mammalian cells, microtubules appear to polymerize at these nucleating sites (Brooks and Richmond 1983, de Brabander *et al.* 1981), although other cytoplasmic sites have been reported (Weber and Osborn 1981). In addition to the nuclear MTOCs, in higher plants the region of the phragmoplast probably functions as an MTOC (*cf.*, Hepler and Palevitz 1974, Euteneuer *et al.* 1982). In fungi, the SPBs (or their equivalent) usually have been considered to be MTOCs (Heath 1981). Such evidence has been based solely on ultrastructural analyses. There are many reports where microtubules have been noted not to be associated with the SPB, particularly during nuclear interphase through early metaphase (*e.g.*, Heath and Heath 1976, Hoch and Staples 1983a, O'Donnell and McLaughlin 1981). The evidence for a lack of such association has been obtained from freeze-substituted, as well as from conventionally preserved, material. During later stages of nuclear division, however, numerous microtubules are associated with the SPBs. Based on the microtubule drug and cold temperature release treatments presented in this paper, the microtubule nucleating region is in the hyphal apex. Such microtubule polymerization began in the extreme hyphal apices within minutes of release. By determining the position of the nuclei and the SPBs with DAPI and Rh-Phn, respectively, we were able to ascertain that microtubules did not polymerize in the region of the SPBs. Therefore, there is little doubt that in nondifferentiating germlings, the SPBs do not function as MTOCs. In germlings stimulated to undergo cell differentiation we would expect the SPBs to be MTOCs in addition to the apical region. We base this premise on the observations that cytoplasmic microtubules (astral) radiate from *U. phaseoli* SPBs during later stages of mitosis (Heath and Heath 1976, Hoch and Staples, unpublished) when the hyphal apex is no longer a region of polarized growth.

The amount of microtubule cytoskeleton remaining after depolymerization treatments was concentration dependent. With increasing drug concentrations, microtubule staining vanished last at the hyphal apex. This also lends credence to the idea that the hyphal apex is a microtubule nucleating site.

The properties of the hyphal apex which makes it a preferred nucleating site for microtubules are not understood. Thus far, we have not observed any ultrastructurally distinct organelle or area to which we could attribute the role of an MTOC. Alternatively, it is conceivable that in the apex, there exists an area in which Ca^{++}, GTP or other microtubule regulators are somehow tightly regulated and effective in controlling microtubule polymerization.

Frequently, low temperature or microtubule depolymerizing agents, such as griseofulvin and nocodazole, at concentrations and times that usually yielded complete microtubule depolymerization, left either a few intact microtubules or a region in the apex with diffuse staining (*cf.*, Figs. 9, 15, and 19). Drug and cold-stable microtubules have been reported for several cell types (*e.g.*, BROOKS and RICHMOND 1983, HEATH 1975, OSBORN and WEBER 1976). The diffuse fluorescence observed in some of the treatments is difficult to explain. Since it was generally limited to the hyphal apex, it possibly represents many short microtubule fragments (resistant to the treatment) which cannot be resolved individually. The staining might also represent tubulin subunits somehow bound within the area, and not readily rinsed out of the permeabilized cell. However, other areas of these cells, as well as cells representative of other depolymerizing treatments, (*e.g.*, Fig. 11) did not show any staining, and this indicates to us that tubulin subunits can be rinsed from the cell.

The drug, taxol, has been shown repeatedly to be effective in stabilizing microtubules in mammalian cells (DE BRABANDER *et al.* 1981, SCHATTEN *et al.* 1982, SCHIFF *et al.* 1980). Its effects on fungal microtubules have not been reported. While an increased population of microtubules in *U. phaseoli* was noted following exposure of the germling to this drug, many of the "stabilized" microtubules were depolymerized upon cold treatment. In cells exposed to cold but not treated with taxol, all microtubules were depolymerized. Comparatively, then, taxol did appear to stabilize the microtubules against depolymerization with cold. However, as indicated earlier, taxol was only effective in about 30% of the cells. This, plus the fact that taxol was effective only at much higher concentrations than is usually effective in mammalian cells, suggests that poorer uptake or cell reactivity of the drug may occur in *U. phaseoli*. Our inability to visualize microtubules following D_2O treatments was puzzling. We can only surmise that some property of the cell was altered so that our staining protocol was not effective. Human forearm fibroblasts similarly treated with D_2O stained satisfactorily for tubulin.

Formaldehyde was used in most experiments of this study rather than fixation with glutaraldehyde or freeze-substitution in acetone. Freeze-substitution probably yields a more realistic cytoskeletal profile and should be considered as the preferred method in the future, although it is not as convenient to use as formaldehyde. Glutaraldehyde (and acrolein) cannot be utilized for immunofluorescence studies where rhodamine and FITC are the preferred fluorophores because of fixative-induced autofluorescence of the cells. Such autofluorescence cannot be quenched with sodium borohydride as reported for other cell types (WEBER *et al.* 1978).

In this report, we have shown that the microtubule cytoskeleton in filamentous fungi can be visualized by light microscopy and that it is sensitive to microtubule depolymerizing agents. More importantly, we have demonstrated that the MTOC in nondifferentiating cells is located in the hyphal apex and not near the SPBs of the nuclei. The development of these protocols and the concepts that we now have regarding the microtubule-microfilament (F-actin) cytoskeleton in *U. phaseoli* will allow us to better pursue our objective of understanding how this filamentous network interacts with signal reception for cell differentiation.

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