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Microtubules and microfilaments are both responsible for pollen tube elongation in the conifer *Picea abies* (Norway spruce)

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Summary. In *Picea abies* (Norway spruce), microtubules and actin microfilaments both form a dense matrix throughout the tube mainly parallel to the direction of elongation. In these conifer pollen tubes the organization of this matrix is different from that in angiosperms. This study tests our hypothesis that differences in cytoskeletal organization are responsible for differences in tube growth and physiology. Pollen grains were germinated in media containing cytoskeletal disruptors and analyzed for germination, tube length, tube branching, and tip swelling. Disruption of microtubules significantly inhibits tube elongation and induces tube branching and tip swelling. Tip swelling is probably caused by disruption of the microtubules in the tip that are perpendicular to the direction of elongation. Confocal microscopy indicates that colchicine and propyzamide cause fragmentation of microtubules throughout the tube. Oryzalin and amiprophosmethyl cause a complete loss of microtubules from the tip back toward the tube midpoint but leave microtubules intact from the midpoint back to the grain. Disruption of microfilaments by cytochalasins B and D and inhibition of myosin by N-ethylmaleimide or 2,3-butanedione monoxime stops tube growth and inhibits germination. Microfilament disruption induces short branches in tubes, probably originating from defective microfilament organization behind the tip. In addition, confocal microscopy coupled with microinjection of fluorescein-labeled phalloidin into actively growing pollen tubes indicates that microfilament bundles extend into the plastid-free zone at the tip but are specifically excluded from the growing tip. We conclude that microtubules and microfilaments coordinate to drive tip extension in conifer pollen tubes in a model that differs from angiosperms.

Keywords: Conifer; Microtubule; Microfilament; Myosin; *Picea abies*; Pollen tube.

Introduction

Pollen tubes are a tip-growing system in higher plants that delivers sperm cells for fertilization. Angiosperm pollen tubes are also an established model system for investigating tip growth, where the actin-myosin system is an important component of elongation and tip extension (reviewed in Derksen et al. 1995b, Li et al. 1997). However, a tip-focused calcium gradient may be the actual mechanism driving vesicles into the plasma membrane (reviewed in Taylor and Hepler 1997). Microfilaments are found throughout angiosperm pollen tubes in a net axial array (reviewed in Pierson and Cresti 1992, Li et al. 1997), but their presence in the elongating tip is controversial (Taylor and Hepler 1997). Microfilaments are found in chemically fixed material, but recent evidence indicates that in living material microfilaments are excluded from the extreme apex (Miller et al. 1996, Kost et al. 1998). Pollen tube organelles move along actin cables in vitro, and this motility is blocked by the myosin inhibitor N-ethylmaleimide (NEM) (Kohno and Shimmen 1988). Myosins are also found throughout pollen tubes and distinct subclasses colocalize with different organelles (Tang et al. 1989, Miller et al. 1995, Yokota et al. 1995). Disruption of microfilaments with cytochalasins inhibits germination, tube growth, and organelle motility and causes the accumulation of vesicles at the tip (Pierson and Cresti 1992). Cytochalasin treatment also induces tip swelling (reviewed in Steer and Steer 1989).

The role of microtubules in angiosperm pollen tube growth remains controversial. Microtubules are found throughout the tube primarily in the cell cortex in either net helical or axial arrays but are either excluded from the tip or found sparingly (Pierson and Cresti 1992, Li et al. 1997). Microtubule disruption

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alters organelle zonation in the tube (Taylor and Hepler 1997) and inhibits elongation in some studies (Heslop-Harrison et al. 1988; Joos et al. 1994, 1995; He et al. 1996), while having no effect in others (Franke et al. 1972, Åström et al. 1995). Microtubule disruption by benomyl, a fungicide, inhibits germination and causes tip swelling (He et al. 1996). Both kinesin-like and dynein-like molecules are found in pollen tubes, with kinesin localized in the tip (Cai et al. 1993) and dynein throughout the tube (Moscatelli et al. 1998). However, the roles of these putative microtubule motors in tube growth and tip extension have not been established (Li et al. 1997, Taylor and Hepler 1997).

Pollen tube growth and sperm delivery in conifers is fundamentally different from angiosperms (reviewed in Singh 1978). Conifer pollen grains land in a pollination droplet, germinate, and tubes elongate through the nucellus to the megagametophyte. Conifer pollen tubes secrete proteases (Pettitt 1985) and slowly digest through ovule tissue to reach the egg. During tube growth, the reproductive cells remain within the pollen grain. Once the tube reaches the egg, there is a resting period during which the reproductive cell forms two sperm, one of which fertilizes the egg. In members of the family Pinaceae, the gap between pollination and seed set can be quite long; up to two years in *Pinus sylvestris* (Singh 1978). Our model system is Norway spruce (*Picea abies*), where the gap between pollination and seed set is about 4 months. Pollen grains germinate in a pollination droplet at the micropyle and elongate about 300 μm through the megagametophyte to reach the egg. The detailed organization of the pollen tube cytoskeleton in vivo is not known. In vitro, the vegetative nucleus migrates within the elongating tube in a network of microfilaments and microtubules (Lazzaro 1996, 1999). The spermatogenous cell remains within the grain in vitro, enmeshed in a network of microfilaments and microtubules (Lazzaro 1998, 1999) which extend out through the grain aperture forming a net axial array down the length of the tube. In chemically fixed material, microfilaments in the tip form a 6 μm thick layer which ensheathes a microfilament-depleted core. This core extends 36 μm back from the tip and contains cytoplasm and organelles (Lazzaro 1996). Microtubules in the tip form an array beneath the plasma membrane that is perpendicular to the direction of tube growth. This array extends back 20 μm from the tip. There is an abrupt transition from a net perpendicular to a net axial organization at the edge of the enriched region. In medial sections, microtubules are present in the center of the elongating tip (Lazzaro 1999); coincident with the microfilament-depleted region (Lazzaro 1996). Cytoskeletal organization in this conifer model system differs from that seen in angiosperm pollen tubes (Pierson and Cresti 1992, Li et al. 1997) and this difference is especially pronounced at the tip. Little is known about the physiological function of the cytoskeleton in conifer tube growth. There is qualitative evidence in *Pinus densiflora* that microfilament disruption inhibits tube growth, myosin inhibition blocks germination, and microtubule disruption induces additional branching (Terasaka and Niitsu 1994).

In this study, we test the hypothesis that the differences in cytoskeletal organization in conifer pollen tubes are responsible for differences in pollen tube growth and morphology. Pollen grains were grown in vitro on agar germination media containing increasing concentrations of cytochalasins B or D, which disrupt microfilaments; NEM and 2,3-butanedione monoxime (BDM), which inhibit myosin; and amiprophosmethyl (APM), colchicine, oryzalin, or propyzamide, which disrupt microtubules. Germination, tube length, tube branching, tip swelling, and cytoskeletal organization were subsequently examined in over 33,000 pollen tubes and results were evaluated statistically. In addition, the microfilament organization was examined in the tips of actively growing pollen tubes. The effects of cytoskeletal disruption in this conifer model system are substantially different from effects seen in angiosperm pollen tubes but similar to effects seen in tip-growing protonema of mosses and ferns. The evidence presented supports our working model that tip growth in conifer pollen tubes is fundamentally different from tip growth in angiosperm pollen tubes.

Material and methods

Solvent selection

Since most inhibitors were made as concentrated stocks in non-aqueous solvent, we tested the effect of the solvents dimethylsulfoxide (DMSO) and ethanol on pollen tube elongation. Pollen grains were sown on germination media containing 1% DMSO or 0.1, 1, or 10% ethanol. DMSO significantly inhibited pollen tube elongation, while ethanol, up to 10%, had no effect on elongation or on the frequency of pollen tube branching or tip swelling (Table 1). Therefore, we did not use DMSO in our experiments and only used ethanol when preparing concentrated inhibitor stocks. The final concentration of ethanol in the media was always adjusted to 1%, which was the lowest possible percentage for the highest inhibitor concentrations.

Table 1. Effect of solvents on pollen tube morphology

Solvent and concentration (%)	Tube length (μm)	Nr. of tubes	% Tubes with		Total nr. of tubes	Repli cates
			branches	tip swelling		
DMSO						
0.0	262	56	nd[a]	nd		
1.0	158[b]	89	nd	nd		
Ethanol						
0.0	256	133	9.3	2.5	162	
0.1	250	124	9.7	3.4	176	
1.0	252	124	6.0	3.6	167	
10.0	249	77	11.5	6.3	96	

[a] Not determined

[b] Value significantly different from controls (no solvent) on the basis of Tukey's multiple com parison test (tube length) at $P < 0.05$

Inhibitor treatment

Pollen grains from *Picea abies* (Norway spruce, Pinaceae) were field collected and stored at –20 °C. These grains were sown directly on agar germination media containing increasing concentrations of APM, colchicine, oryzalin, or propyzamide, which disrupt microtubules (Hoffman and Vaughn 1994); cytochalasins B or D, which disrupt microfilaments (Cooper 1987), or NEM and BDM, which inhibit myosin (Kohno and Shimmen 1988, Baskin and Bivens 1995). Inhibitor stock solutions were made up in 100% ethanol (20 mM oryzalin, 50 mM propyzamide, 10 mM APM, 1 mM cytochalasin B, 1 mM cytochalasin D, 100 mM NEM), except for colchicine, which was a 25 mM stock in distilled water, and BDM, which was dissolved directly into media. Germination media (1% agar, 10% sucrose, 1 mM $CaCl_2$, and 1 mM H_3BO_3 in distilled water) was heated to boiling and set aside to cool. When the media temperature had dropped below 40 °C, 800 μl were transferred to microfuge tubes containing 200 μl of appropriately diluted inhibitor stock. The final inhibitor concentrations in the cooled agar germination media were up to 100 μM for APM, oryzalin, or propyzamide, 5 mM for colchicine, 100 μM cytochalasin B, 10 μM cytochalasin D, 500 μM NEM, or 50 mM BDM. Approximately 200 μl of cooling germination media was transferred to a glass slide. One glass slide was prepared for each inhibitor concentration. Once the agar solidified, pollen grains were evenly spread over the surface. Finally the slides were placed in a humid chamber for 24 h at 30 °C (Frankis and Grayson 1990).

Morphological measurements

Pollen tubes were examined on an inverted microscope equipped with a charge-coupled-device camera connected to a Macintosh computer. Images were captured in digital form and subsequently analyzed with NIH Image. For each slide, six images were captured through the ×5 objective and subsequently scored for pollen germination. In addition, for each slide ten phase contrast images containing 3 to 5 pollen grains were captured through the ×10 objective. These images were subsequently scored for pollen tube length and frequency of tube branching and tip swelling. Pollen tube length was measured as the distance from the aperture to the tube tip, and these measurements were calibrated by capturing an image of a stage micrometer. Only tubes with clear branches were scored as branched, tubes that were merely twisting in an s-pattern were scored as unbranched. Tip swelling was scored as tips whose diameter was at least 50% greater than the tube diameter near the grain aperture.

All data were analyzed for statistical significance with Systat. Data were formatted in two-dimensional matrices of replicate versus concentration. Each drug concentration was analyzed in paired comparisons to the control. Pollen tube length was analyzed with Tukey's multiple comparison test. The frequencies of germination, tube branching, and tip swelling were analyzed with Pearson's chi-square test (Zar 1984). Values were significant at $P < 0.05$. The concentration series for each inhibitor was replicated 3–6 times. Over 33,000 pollen tubes were measured in total.

Immunolabeling

Microtubules and microfilaments were examined in a subset of inhibitor treatments composed of controls, 100 μM APM, 100 and 1000 μM colchicine, 50 and 100 μM oryzalin, 100 μM propyzamide, 1 and 10 μM cytochalasin B, 0.5 and 1 μM cytochalasin D, and 50 and 100 μM NEM. Germination media with inhibitors were layered in the bottom of 1.5 cm diameter cylindrical sample wells (1 cm deep) glued onto glass slides. Pollen grains were sown on these sample wells and germinated for 24 h at 30 °C in humid chambers. Pollen tubes were then immunolabeled as described in detail by Lazzaro (1999). Following fixation, cell walls were permeabilized with either enzymatic digestion (most of the microtubule labeling preparations) or freeze shattering (all microfilament labeling preparations). The resulting samples were immunolabeled with monoclonal antibodies to either alpha tubulin (Amersham N356) or pea root actin (mAb3H11) (Andersland et al. 1994), and Cy3-conjugated secondary antibodies. Controls omitting either the primary antibody or primary and secondary antibodies were also prepared. Pollen tubes were examined with a Zeiss LSM510 confocal microscope.

Microinjection experiments

Pollen tubes growing on agar media for 24 h at 30 °C were transferred to culture slides (Pierson et al. 1994) and microinjected with 6.6 μM fluorescein-phalloidin as described in Miller et al. (1996). Immediately following injection, labeled microfilaments were imaged with a Bio-Rad MRC 600 confocal microscope. Since *P. abies*

Table 2. Effect of cytoskeletal disruption on pollen tube morphology[a]

Inhibitor and concentration (μM)	% Germinated pollen grains	Total nr. of grains	Tube length (μm)	Total nr. of tubes	% Tubes with		Total nr. of tubes	Replicates
					tip swelling	branches		
APM								
0	96	452	254	255	1.8	7.8	398	6
	95	416	235*	246	10.0*	16.8*	369	6
10	96	455	230*	241	10.3*	15.8*	348	6
50	91*	506	219*	283	10.9*	7.6	394	6
100	88*	502	193*	243	6.2*	4.2	354	6
Oryzalin								
0	96	489	267	195	1.7	5.0	242	
2	94	558	252	202	4.4*	8.7*	252	
10	94	568	225*	221	11.0*	14.0*	272	
50	94	541	208*	219	21.3*	13.6*	287	
100	91*	584	149*	236	14.2*	3.0	302	
Propyzamide								
0	93	697	284	196	2.0	4.7	254	
	94	710	270	203	1.8	11.6*	284	
10	91	652	273	212	1.3	6.0	298	
50	94	670	259*	209	5.0*	8.3*	289	5
100	92	780	242*	224	2.9	14.2*	311	5
Colchicine								
0	92	684	270	146	0.0	8.8	249	
10	94	647	256	138	0.9	14.1*	220	
50	92	603	293	129	1.4	13.7*	211	
100	92	659	277	132	0.0	17.3*	225	
500	93	633	265	148	0.0	13.3	226	
1000	91	637	255*	154	0.0	13.7*	241	
5000	91	429	214*	93	1.3	2.0	154	
Cytochalasin B								
0	95	559	262	204	0.8	3.7	241	
0.01	93	548	283	175	1.9	3.2	216	5
0.1	94	516	253*	206	0.4	6.1	246	5
0.5	93	541	206*	230	3.0	13.4*	269	5
1	92*	568	170*	241	1.6	15.1*	258	
5	89*	581	93*	267	0.3	7.2*	347	
10	71*	506	50*	214	n.d.[b]	n.d.		
50	11*	490	38*	91	n.d.	n.d.		
100	0*	402	0*	93	n.d.	n.d.		
Cytochalasin D								
0	88	625	263	158	2.3	1.7	172	
0.01	87	542	262	156	1.6	13.0*	184	
0.1	91	564	237*	162	0.5	24.1*	195	4
0.5	92	695	142*	214	0.9	45.5*	231	4
1	74*	671	108*	194	0.0	45.5*	200	4
5	22*	999	40*	93	n.d.	n.d.		
10	0*	704	0*	90	n.d.	n.d.		
NEM								
	95	248	298	105	0.7	3.7	135	
	97	256	295	116	0.7	9.6*	135	
	90	219	296	108	0.0	6.2	129	
10	94	237	286	115	0.0	6.0	134	
50	91	282	165*	143	0.0	0.5	209	
100	40*	285	76*	127	0.0	0.0	126	

Inhibitor and concentration (μM)	% Germinated pollen grains	Total nr. of grains	Tube length (μm)	Total nr. of tubes	% Tubes with		Total nr. of tubes	Replicates
					tip swelling	branches		
BDM								
	84	494	243	251	1.0	0.3	295	4
1000	83	357	243	199	1.5	2.5	197	3
5000	79*	325	247	172	1.6	0.0	185	3
10000	73*	576	206*	279	2.6	0.0	265	4
50000	0*	308	0*	167	n.d.	n.d.		3

[a] Values marked with asterisks are significantly different from controls (no inhibitor) on the basis of Tukey's multiple comparison (tube length) and Pearson's chi-square (germination, branching, and swelling) at $P < 0.05$
[b] Not determined

pollen tubes grow slowly, time lapse differential interference contrast (DIC) images of transferred pollen tubes were collected at 3–5 min intervals to insure that only actively growing tubes were injected. Time lapse DIC images were also collected after both injection and confocal scanning to insure that pollen tubes survived injection and laser scanning. Only tubes that were clearly growing after transfer to culture slides were injected, and only tubes that were clearly growing after injection and laser scanning were analyzed. Confocal images from nongrowing tubes were discarded.

Liquid-media experiments

Pollen grains were also germinated in liquid media (500 mM sucrose, 1 mM $CaCl_2$ and 1 mM H_3BO_3 in distilled water) containing more dilute inhibitor concentration series. The final inhibitor concentrations in liquid media were up to 50 μM APM, 100 μM colchicine, 50 μM oryzalin, 50 μM propyzamide, 0.5 μM cytochalasin B, 0.5 μM cytochalasin D, 100 μM NEM, or 10 mM BDM. Pollen tubes in liquid media were more sensitive to ethanol (data not shown), so the final concentration of ethanol in the system was kept at 0.1%. Pollen grains were sown on 2 ml of liquid media in small petri dishes and incubated for 24 h at 30 °C. An aliquot of pollen was then transferred to a glass slide and analyzed for tube length as described above.

Results

Pollen grains sown on agar media without inhibitors had a 92% germination rate. After 24 h, the control pollen tubes were 268 μm long. These tubes had very low frequencies of tip swelling (1.3%) and tube branching (4.5%). These values are averages of all control populations (Table 2).

Microtubule disruption

Pollen grains were sown on agar germination media containing increasing concentrations of the microtubule disruptors APM, oryzalin, propyzamide, and colchicine. Treatment with APM (50–100 μM) and oryzalin (100 μM) caused a slight but significant reduction in pollen germination, while propyzamide and colchicine did not affect germination (Table 2). All four microtubule disruptors caused a linear decline in pollen tube length (Fig. 1 A–D) and these declines were all statistically significant. Threshold concentrations for a significant reduction in tube length were 5 μM for APM, 10 μM for oryzalin, 50 μM for propyzamide, and 1000 μM for colchicine (Table 2). The effectiveness of these inhibitors varied. At the highest tested concentrations, oryzalin was the most effective inhibitor of tube length (149 μm, 56% of control length), APM was next (193 μm, 76% of control length), followed by colchicine (214 μm, 79% of control length) and propyzamide (242 μm, 85% of control length).

Disruption of microtubules with APM and oryzalin caused a significant increase in the percentage of pollen tubes with swollen tips. This was significant at 5 μM APM and 2 μM oryzalin (Table 2). The 5 μM threshold concentration for APM was the same as that for decreasing tube length. For oryzalin, the 2 μM threshold was less than the 10 μM threshold for tube length. Propyzamide and colchicine did not significantly increase tip swelling (Table 2). All four compounds significantly increased the percentage of pollen tubes with side branches. The concentration series of inhibitors did not exhibit the same threshold of significance seen with tube length and tip swelling. Instead, branching was significant at discrete concentration subsets. APM was effective at 5 and 10 μM, while oryzalin was effective at 2–50 μM. However, at 50–100 μM APM and 100 μM oryzalin there was no longer a significant increase in branching. This contrasts with the clear threshold for tip swelling with these compounds (Table 2). Propyzamide and colchi-

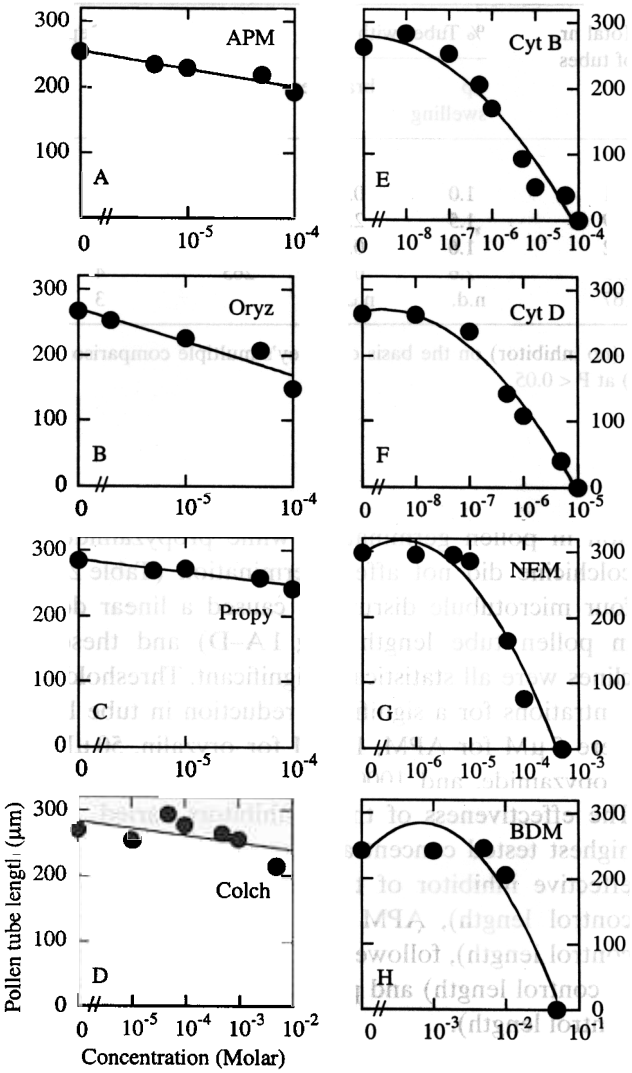


Fig. 1. Pollen tube elongation was significantly inhibited by cytoskeletal disruption. Treatment with APM (**A**), oryzalin (**B**), propyzamide (**C**), or colchicine (**D**) caused significant linear decreases in elongation. Treatment with cytochalasin B (**E**), cytochalasin D (**F**), NEM (**G**), or BDM (**H**) caused a sharp decline in elongation once concentrations passed their threshold values, and germination was completely inhibited at high concentrations. For all graphs, the *y*-axis is pollen tube length (µm) after 24 h treatment and the *x*-axis is a logarithmic scale of the inhibitor concentration (molar)

cine also caused significant branch formation. Propyzamide was effective at 5, 50, and 100 µM but not at 10 µM, while colchicine was effective at 10–100 and 1000 µM but not at 500 or 5000 µM (Table 2).

It is important to note that branching and swelling, while significant, occur in a subset of pollen tubes, while the decrease in tube length occurs throughout the population. There are other, more qualitative, morphological changes induced by microtubule disruption. Control tubes are usually long and straight, with amyloplasts spread throughout the tube, concentrated around the tube nucleus and excluded from the terminal 30 µm at the tip (Fig. 2A). The tip swelling caused by APM and oryzalin was typically coincident with the plastid-free region at the tip (Fig. 2B, D). The branches caused by all four compounds look like side branches coming off the main tube (Fig. 2C, E–G). These branches likely form from bifurcation of the tip, and once formed, they continue to grow and are morphologically similar to the main tube. In general, APM-, oryzalin-, and propyzamide-treated tubes are shorter, plastids are not evenly distributed through the tubes, and the tubes undulate in an s-shape. Colchicine treatment does not cause these changes in plastid distribution and tube shape; the tubes are just shorter and branched.

Microtubule organization was examined with confocal microscopy. Pollen tubes were treated with inhibitor concentrations that were above the threshold values for decreasing tube length. These tubes were then immunolabeled with an α-tubulin antibody. In controls, microtubules form a contiguous network of bundles parallel to the direction of elongation from the pollen grain to the tip (Fig. 3A) (Lazzaro 1999). The net parallel orientation abruptly changes about 20 µm from the tip to a net perpendicular organization. Cortical microtubules remain perpendicular to the direction of elongation all the way to the tip (Fig. 3B) (Lazzaro 1999). Treatment with APM and oryzalin specifically disrupts this band of perpendicular microtubules (Fig. 3C, D), and this is coincident with the induction of tip swelling by these inhibitors (Fig. 2B, D). Microtubules further back in the tube are relatively intact. Treatment with propyzamide or colchicine disrupts microtubules throughout the pollen tube, causing the formation of short, fragmented microtubule bundles (Fig. 3E, F).

Microfilament disruption and myosin inhibition

Pollen grains were also germinated on agar media containing increasing concentrations of cytochalasins B and D, which disrupt microfilaments; or NEM and BDM, which inhibit myosin's ATPase activity. Germination was significantly inhibited by 1 µM cytochalasin B or D and was completely inhibited at 100 µM cytochalasin B and 10 µM cytochalasin D (Table 2). Germination was also significantly inhibited by 100 µM NEM and 5 mM BDM and was completely

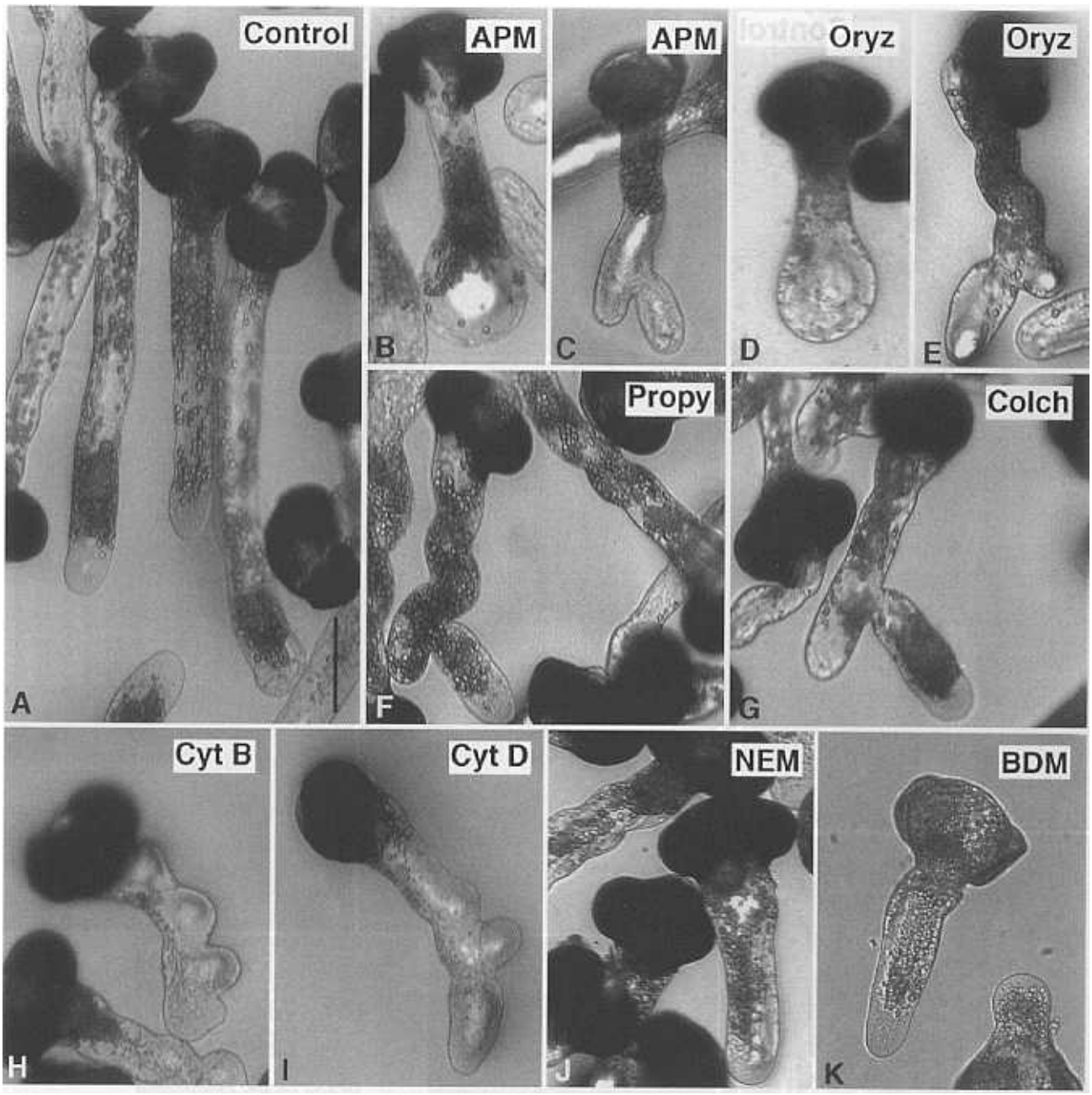
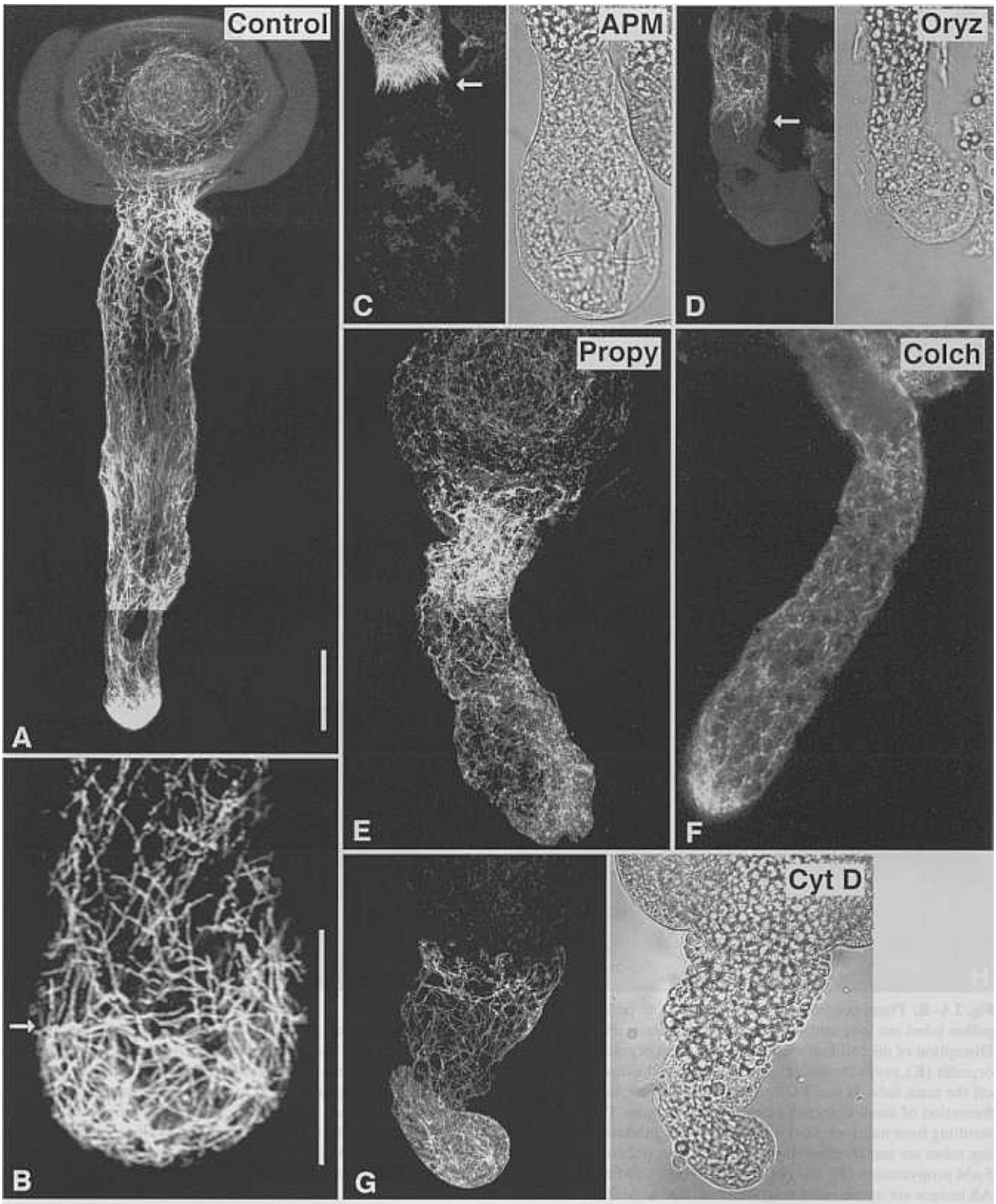


Fig. 2A–K. Phase contrast micrographs of living pollen tubes illustrating the morphology of tip swelling and tube branching. **A** Control pollen tubes are long and straight with amyloplasts distributed throughout the tube but excluded from the terminal 25 μm at the tip. **B–G** Disruption of microtubules with APM (**B**) and oryzalin (**D**) causes significant tip swelling, and disruption of microtubules with APM (**C**), oryzalin (**E**), propyzamide (**F**), or colchicine (**G**) causes a significant increase in branch formation. These branches look like side branches off the main tube. **H** and **I** Disruption of microfilaments with cytochalasin B (**H**) or cytochalasin D (**I**) causes a significant increase in the formation of small branches along the pollen tube. These branches do not elongate and are morphologically distinct from the branches resulting from microtubule disruption. **J** and **K** Inhibition of myosin with NEM (**J**) or BDM (**K**) has no effect on tip swelling or tube branching, tubes are just shorter. These representative pollen tubes were treated with 50 (**B**) or 100 μM APM (**C**), 50 (**E**) or 100 μM oryzalin (**D**), 5 μM propyzamide (**F**), 100 μM colchicine (**G**), 1 μM cytochalasin B (**H**), 0.5 μM cytochalasin D (**I**), 50 μM NEM (**J**), or 10 mM BDM (**K**). All images are at the same magnification. Bar in A: 50 μm


Control
A
B
APM
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inhibited by 500 μM NEM and 50 mM BDM. All four compounds caused an exponential decline in pollen tube elongation once the threshold concentration was reached (Fig. 1 E–H). These threshold concentrations were 0.1 μM for cytochalasins B and D, 50 μM for NEM, and 10 mM for BDM (Table 2). Pollen tube length was more severely inhibited by disruption of the actin-myosin system compared to disruption of microtubules, since the tube length declined precipitously to zero as germination was completely inhibited (Fig. 1 E–H).

Disruption of microfilaments had no effect on the frequency of pollen tubes with swollen tips. However, microfilament disruption with cytochalasins B and D significantly increased the frequency of pollen tube branching. The threshold concentrations for branch induction were 0.5 μM for cytochalasin B and 0.01 μM for cytochalasin D (Table 2). In addition to having a lower threshold concentration, cytochalasin D was also extremely effective at inducing branch formation, with 46% of pollen tubes branching in 0.5 and 1 μM cytochalasin D. We were unable to evaluate branching and swelling at the higher concentrations of cytochalasins B or D because the tubes were just too short. Pollen tube diameter is typically 30 μm, and at 10–50 μM cytochalasin B or 5 μM cytochalasin D tubes were only 40–50 μm long (Table 2). Myosin inhibition with NEM and BDM had no effect on pollen tube tip swelling or on tube branching.

Pollen tube branching occurred in a subset of the cytochalasin-treated pollen tubes, while the decrease in tube length occurred throughout the population treated with cytochalasins B or D, NEM, or BDM. Qualitatively, plastid migration was inhibited by microfilament disruption with cytochalasins, and the plastids remained inside the pollen grains (Fig. 2 H, I). The morphology of branches induced by microfilament disruption was completely different from the branching induced by microtubule disruption. Microfilament disruption caused the formation of numerous short branches along the length of the pollen tubes (Fig. 2 H, I). These branches look like pollen tube tips that were ineffective in driving elongation and were pushed aside as the forces driving tube expansion pushed a new tip forward. Branch formation was significant, even as tube length was severely diminished. In 1 μM cytochalasin D, pollen tubes were only 108 μm long but 46% of them had numerous small branches. The only morphological change caused by myosin inhibition with NEM or BDM was that the tubes were shorter. They looked like short controls (Fig. 2 J, K).

Microfilament organization was also examined with confocal microscopy. Pollen tubes were treated with inhibitor concentrations that were above the threshold values for decreasing tube length. These tubes were then immunolabeled with an actin antibody. In controls, microfilaments form a contiguous network of bundles throughout the tube mainly parallel with the direction of elongation (Fig. 4 A) (Lazzaro 1996). Treatment with intermediate concentrations of cytochalasins B and D severely disrupt microfilaments (Fig. 4 B, C). Treatment with the myosin inhibitor NEM did not significantly alter microfilament organization (Fig. 4 D).

Microfilament dynamics in growing pollen tubes

There are clear changes in pollen tube growth and morphology following disruption of the actin-myosin network. However, previous descriptions of the organization of actin microfilaments in *P. abies* pollen tubes were based solely on fixed material (Lazzaro 1996) and there is abundant evidence that fixation alters the organization of microfilaments in tip-growing systems (Taylor and Hepler 1997). Therefore, we injected growing pollen tubes with fluorescently labeled

Fig. 3. **A** Microtubules form a contiguous network of bundles parallel to the direction of elongation from the pollen grain to the tip. **B** At the tip, microtubules beneath the plasma membrane are oriented perpendicular to the direction of elongation. This orientation abruptly changes about 15 μm back from the tip to a net parallel organization (arrow). **C** and **D** Treatment with APM (**C**) and oryzalin (**D**) specifically disrupts this radial array of perpendicular microtubules (arrows) and this is coincident with the induction of tip swelling by these inhibitors. Microtubules further back in the tube are relatively intact. **E** and **F** Treatment with propyzamide (**E**) or colchicine (**F**) disrupts microtubules throughout the pollen tube, causing the formation of short, fragmented microtubule bundles. **G** Treatment with microfilament disruptors does not affect the organization of microtubules, as illustrated here by a cytochalasin D-treated pollen tube. These representative pollen tubes were treated with 100 μM APM (**C**), 50 μM oryzalin (**D**), 100 μM propyzamide (**E**), 100 μM colchicine (**F**), or 1 μM cytochalasin D (**G**). Images are confocal projections of pollen tubes immunolabeled with an α-tubulin antibody. **B** Projection through 400 nm of the cell cortex beneath the plasma membrane; **A** and **C–G** projections through the entire tube. Bars: in A, for A and C–G, 25 μm; B, 25 μm

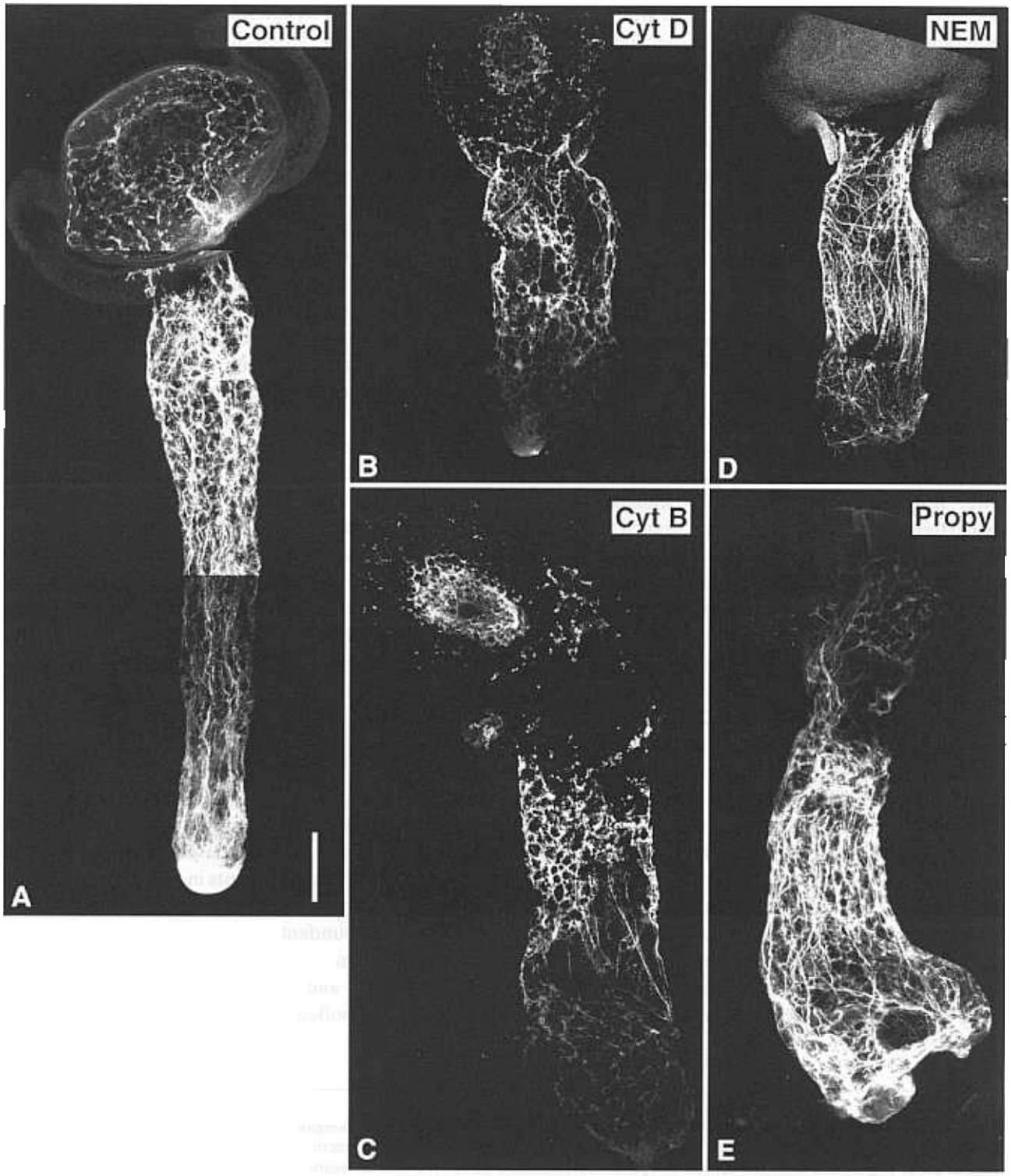

Fig. 4. **A** Microfilaments form a contiguous network of bundles mainly parallel with the direction of elongation from within the pollen grain towards the tip. Note that the apparent concentration of microfilaments in the tip is a fixation artifact (see Fig. 5). **B** and **C** Treatment with intermediate concentrations of cytochalasins B and D severely disrupt microfilaments. **D** Treatment with NEM does not significantly alter microfilament organization. **E** Treatment with microtubule disruptors does not affect microfilament organization, as illustrated by this propyzamide-treated pollen tube. These representative pollen tubes were treated with 0.5 μM cytochalasin D (**B**), 1 μM cytochalasin B (**C**), 100 μM NEM (**D**), or 1000 μM propyzamide (**E**). Images are confocal projections of pollen tubes immunolabeled with an actin antibody and are all at the same magnification. Bar in A: 25 μm

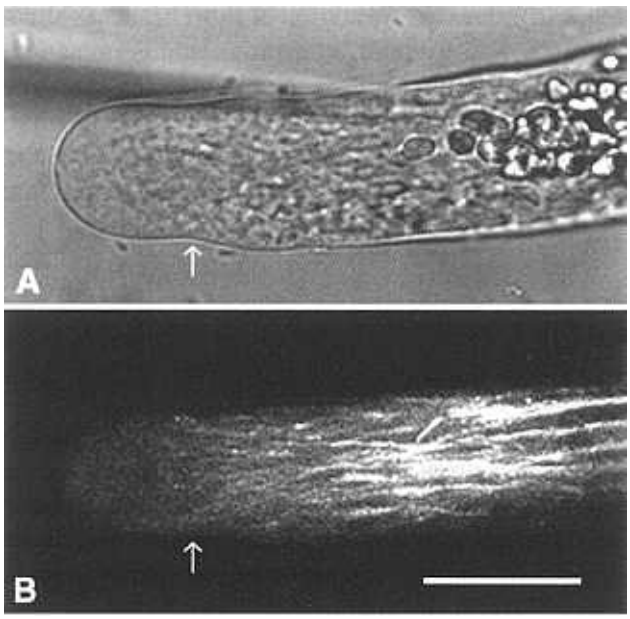

Fig. 5 A, B. Pollen tubes which resumed growth after transfer to culture slides were injected with fluorescein-phalloidin. The DIC (**A**) and confocal (**B**) images are exactly aligned and the arrows mark where new growth occurred after transfer to culture slides. Microfilament bundles extend down the tube well into the plastid-free zone but do not enter the region of new growth. The confocal image is a projection of six optical sections beginning at the tube edge and extending 10 μm into the tube. Bar: 25 μm

phalloidin to get a more accurate picture of microfilament organization.

Pollen tubes undergo a shock when transferred from thick agar germination media to a thin layer of the same media on a culture slide for microinjection. This shock interrupts growth and tips swell slightly. Pollen tubes that recover from this shock have a new tip zone which extends out from the swelling (Fig. 5 A). Recovered pollen tubes are easily identified about 30–60 min after transfer to culture slides. Time lapse DIC images of transferred pollen tubes were collected at 3–5 min intervals to insure that only actively growing tubes were injected. Time lapse images were also collected following both injection and confocal scanning to insure that pollen tubes survived injection and laser scanning. Only tubes that were clearly growing after transfer to culture slides were injected, and only tubes that were clearly growing after injection and laser scanning were analyzed. Confocal images from nongrowing tubes were discarded. Six tubes were successfully transferred, injected, and scanned. The average growth rate was 18.8 μm/h before injection and 18.1 μm/h after injection and scanning. Actin microfilaments are immediately labeled after microinjection (Fig. 5 B). Microfilament bundles parallel with the direction of elongation are found throughout the tube, and these bundles extend well into the plastid-free zone at the tip. However, they do not extend into the new, actively growing tips. This contradicts the description of microfilament organization in the tips of *P. abies* pollen tubes on the basis of formaldehyde-fixed material (Lazzaro 1996).

Experimental controls

Additional immunolabeling experiments were carried out to test whether microfilament disruptors were affecting microtubule organization and whether microtubule disruptors were affecting microfilament organization. Treatment with cytochalasins B or D or NEM had no effect on microtubule organization, as illustrated by the cytochalasin D-treated tube in Fig. 3 G. Treatment with APM, colchicine, oryzalin, or propyzamide did not affect microfilament organization, as illustrated by the propyzamide-treated pollen tube in Fig. 4 E.

We were concerned that our threshold values for significant inhibition of tube length (Table 2) might be inflated due to nonspecific interactions of the inhibitors with the agar matrix. To resolve this, we conducted additional experiments in liquid culture media with more dilute concentration series for all 8 compounds. The threshold concentrations for a significant inhibition of tube length by microtubule disruption were either the same or actually higher than those measured in experiments with agar media (Table 3). Threshold concentrations were 50 μM for APM (5 μM in agar), 10 μM for oryzalin (same as agar), not significant up to 50 μM for propyzamide (significant at 50 μM in agar), and not significant up to 100 μM for colchicine (significant at 1000 μM in agar). The threshold concentrations for a significant inhibition of tube length by microfilament disruption or myosin inhibition were lower in liquid media. Threshold concentrations were 0.001 μM for cytochalasin B (0.1 μM in agar), 0.01 μM for cytochalasin D (0.1 μM in agar), and 5 mM for BDM (10 mM in agar). For NEM, the threshold concentration was essentially the same, being 100 μM in liquid media (50 μM in agar, although 50 μM was not tested in liquid media).

In conclusion, cytochalasins B and D disrupt microfilaments throughout the pollen tube, inhibit pollen tube elongation, amyloplast migration, and germina-

Table 3. Pollen tube length after growth in liquid media[a]

Inhibitor and concentration (μM)	Tube length (μm)	Nr. of tubes
APM		
	207	52
	194	56
0.5	200	42
	194	
10	192	45
50	141*	54
Oryzalin		
0	186	51
0.1	212	37
0.5	191	35
	175	40
	118*	59
50	145*	37
Propyzamide		
0	207	43
0.1	204	38
0.5	186	32
	218	30
10	206	34
50	202	35
Colchicine		
0	203	38
1	237	33
5	190	37
10	188	28
50	228	28
100	206	32
Cytochalasin B		
0	219	44
0.001	181*	51
0.01	207	44
0.1	185*	48
0.5	157*	47
Cytochalasin D		
0	216	30
0.001	195	33
0.01	174*	35
0.1	182	41
0.5	128*	36
NEM		
0	236	48
0.1	216	47
	232	41
10	224	46
100	168*	54
BDM		
0	286	32
1000	295	50
5000	227*	49
10000	161*	58

[a] Values marked with asterisks are significantly less than controls (no inhibitor) on the basis of Tukey's multiple comparison at $P < 0.05$

tion, and induce the formation of short branches along the tubes. NEM inhibits elongation and germination. APM and oryzalin disrupt microtubules preferentially at the tip and inhibit elongation, reduce germination, and induce tip swelling and branching. Colchicine and propyzamide fragment microtubules throughout the tube, inhibit elongation, and induce branching.

Discussion

Sperm delivery in members of the family Pinaceae is different from angiosperms, so it makes sense that cytoskeletal organization and function also differ. Pollen tubes grow slowly through the ovular tissue to reach the egg, taking 1–2 weeks in *P. abies*. Since the reproductive cells remain within the grain during tube elongation, callose plugs do not form. The reproductive cells eventually migrate into the elongate tube and divide to produce the sperm (Singh 1978). Our results indicate that the actin-myosin system is primarily responsible for tip extension initiating germination and for continued tube elongation, but microtubules are also an important component in continued elongation. Microtubule stability mediates branch formation and the radial array of microtubules at the tip prevents tip swelling. We used eight cytoskeletal inhibitors in this study and the working concentrations were within the ranges of those used in other systems (Perdue and Parthasarathy 1985, Kohno and Shimmen 1988, Hoffman and Vaughn 1994, Joos et al. 1994, Åström et al. 1995, Baskin and Bivens 1995).

Disruption of microfilaments and inhibition of myosin will both completely inhibit germination and profoundly inhibit pollen tube elongation. This is similar to qualitative evidence in *Pinus densiflora*, where cytochalasin D inhibited elongation and NEM inhibited germination (Terasaka and Niitsu 1994). The data for conifers also agree with the predominant model for angiosperm pollen tubes, where the actin-myosin system is responsible for tip extension (reviewed in Pierson and Cresti 1992, Derksen et al. 1995b, Taylor and Hepler 1997, Li et al. 1997). Microfilaments form a net axial array throughout angiosperm pollen tubes (Pierson and Cresti 1992), but the presence of microfilaments in the growing tip is controversial. They are found at the tip in chemically fixed material (reviewed in Pierson and Cresti 1992) but are excluded from the extreme tip when visualized by microinjection of fluorescently conjugated phalloidin in living pollen tubes (Miller et al. 1996). An

actin-depleted zone at the extreme tip is also observed when microfilaments are detected in living tubes by the binding of green-fluorescent-protein-labeled mouse talin (Kost et al. 1998). Myosin inhibition with NEM stops organelle motility in *Lilium longiflorum* pollen tubes (Kohno and Shimmen 1988), and myosin is detected throughout angiosperm pollen tubes (Tang et al. 1989, Miller et al. 1995, Yokota et al. 1995). In conifers, microfilaments form an axial array in the pollen tube (Terasaka and Niitsu 1994, de Win et al. 1996, Lazzaro 1996). In chemically fixed material labeled with rhodamine-phalloidin (Lazzaro 1996) or immunolabeled with actin antibodies (Fig. 4) this array extends into the tip where microfilaments form a 6 μm thick band that ensheathes an actin-depleted core (Lazzaro 1996). However, the present study indicates that while microfilaments do extend well into the plastid-free zone near the tip of growing tubes, they are not found within the actively growing tip.

The results of fluorescein-phalloidin injections must be interpreted carefully. Since phalloidin is a toxin that binds to and stabilizes filamentous actin, injection of high concentrations may ensure that most microfilaments are labeled but may also damage the cells. In this study, we injected lower concentrations of fluorescein-phalloidin and found that there was no difference in growth rates before and after injection, indicating that phalloidin did not inhibit tip growth (see also Miller et al. 1996). It is possible that some microfilaments were not labeled by this nontoxic concentration of fluorescein-phalloidin, although we found that labeling occurred within seconds after injection, and the pattern of labeling did not change over several minutes (data not shown). It is perhaps most compelling that the actin-depleted zone at the extreme tip is also observed in living pollen tubes expressing green-fluorescent-protein-labeled mouse talin (Kost et al. 1998).

Microfilament disruption had no effect on tip swelling in *P. abies*. This contrasts with the evidence in angiosperm pollen tubes where cytochalasin treatment causes tip swelling as it stops growth and leads to the accumulation of vesicles at the tip (Picton and Steer 1981, Steer and Steer 1989, Heslop-Harrison and Heslop-Harrison 1989). The formation of numerous branches following cytochalasin treatment is a unique feature of conifer pollen tubes since this is not a common response to microfilament disruption in angiosperms, although side branches are occasionally observed at the tips of *Iris* sp. pollen tubes following cytochalasin treatment (Heslop-Harrison and Heslop-Harrison 1989). Myosin inhibition had no effect on the formation of branches nor on tip swelling in *P. abies* and only inhibited elongation and germination.

Microtubule disruption clearly inhibits pollen tube elongation in *P. abies*. This was true with all four tested inhibitors where increasing concentrations correlated with a significant, linear decline in elongation. In addition, high concentrations of oryzalin and APM reduced germination. Disruption of microtubules also alters angiosperm pollen tube morphology, but the role of microtubules in pollen tube growth remains unclear. Microtubule disruption can inhibit germination (He et al. 1996) and inhibits elongation in some in vitro studies (Heslop-Harrison et al. 1988, Joos et al. 1994, He et al. 1996), while having no effect in others (Franke et al. 1972, Åström et al. 1995). Microtubule disruption does stop growth of *Nicotiana sylvestris* pollen tubes in vivo (Joos et al. 1995). Microtubules in angiosperm pollen tubes are localized primarily in the cell cortex, forming either radial or axial arrays, and are usually excluded from the growing tip (reviewed in Pierson and Cresti 1992, Li et al. 1997). Kinesin-like molecules are localized in the tip (Tiezzi et al. 1992, Cai et al. 1993) and dynein-like molecules throughout the tube (Moscatelli et al. 1998), but the function of these putative microtubule motor proteins in elongation is unknown. In conifers, microtubules form a net axial array (Terasaka and Niitsu 1994, Lazzaro 1999) and this array extends throughout the pollen tube and is not concentrated in the cell cortex (Lazzaro 1999). Microtubules also extend to the tip, where they are enriched in a radial array beneath the plasma membrane. This array extends back 20 μm from the tip. Within the tip center, microtubules are in a net axial array (Lazzaro 1999).

Microtubule disruption caused the formation of branches that are common features in conifer pollen tubes grown in vivo (Singh 1978). The formation of short branches just behind the growing tip occurs in *Picea glauca* pollen tubes growing in vivo but not in vitro (Dawkins and Owens 1993). In *P. abies*, the frequency of in vitro tube branching was only 5.1% in controls, but this branching increased significantly following microtubule disruption and the branches were morphologically similar to branches seen in vivo in *Picea glauca* (Dawkins and Owens 1993). Pinus pollen tubes are extremely branched both in vivo and in vitro (Singh 1978). This branching occurs by bifurcation of the tip (de Win et al. 1996) and is increased by

colchicine treatment (Terasaka and Niitsu 1994). The threshold concentrations for significant branching in *P. abies* were similar to the thresholds for inhibition of tube elongation caused by APM, oryzalin, and propyzamide. However, significant branching was induced at much lower concentrations of colchicine (10–100 μM) compared to the 1000 μM threshold for inhibiting elongation. APM, oryzalin, and colchicine all caused branching at a distinct subset of intermediate concentrations, but at higher levels branching dropped off to control values. Our explanation for this is that microtubules are necessary for branch formation, but the dynamic state of the microtubules may be an important factor. Plant microtubules have a high degree of dynamic instability (Hush et al. 1994, Moore et al. 1997). When the stability of microtubules first decays at threshold concentrations, branching occurs as elongation of the tube is inhibited. At higher concentrations, when microtubules are more significantly disrupted, branching presumably diminishes because the microtubules are now too unstable to initiate branching or support elongation. This phenomenon was not seen in propyzamide-treated tubes, but this may be because the threshold for increasing branching and inhibiting elongation was 50 μM and we only tested propyzamide up to 100 μM. Branching does occur in angiosperm pollen tubes (reviewed in Derksen et al. 1995b), but to our knowledge there is no information on the role of the cytoskeleton in branch formation.

Our working model for tip extension is that Golgi bodies produce secretory vesicles within the core of the tip. These vesicles then move out to the tip plasma membrane. The core region contains microtubules (Lazzaro 1999) and we hypothesize that these microtubules position the Golgi bodies near the growing tip. Disruption of these microtubules may lead to the inhibition of tube elongation as Golgi placement is disorganized. The growing tip lacks microfilaments, but microfilament bundles extend into the plastid-free zone at the tip. Since disruption of these microfilament bundles strongly inhibits elongation, we hypothesize that these microfilaments coordinate tip extension. At intermediate cytochalasin concentrations, the formation of numerous short branches along tubes may result from damaged microfilament bundles that are ineffective in directing tip extension so growth driven supposedly by turgor pressure continues in a new direction. Organelle zonation differs between cryofixed conifer and angiosperm pollen tube tips. In *Pinus sylvestris* (de Win et al. 1996), amyloplasts are excluded from the growing tip, but mitochondria are concentrated in the cell cortex around the tip, coincident with the radial array of microtubules in *P. abies*. Growing tips contained abundant secretory vesicles, but they were not organized in a growth cone and dictyosomes did not have any preferential localization. In angiosperms, there is a clear organization of organelles at the tip. This has been quantitatively measured in cryofixed *Nicotiana tabacum* pollen tubes, where secretory vesicles form a dense growth cone from 0 to 10 μm back from the tip, while dictyosomes accumulate 25–30 μm behind the tip (Derksen et al. 1995a).

Our working model differs from angiosperms in several respects. The clear presence of microtubules in the tip (Lazzaro 1999) and the obvious swelling induced by their absence is completely different from angiosperms, where there is no evidence that microtubules form a radial array in the tip. It is intriguing that tip swelling, branching, and cytoskeletal organization in *P. abies* is similar to what is seen in tip-growing protonema cells of mosses and ferns. In moss protonema, microtubule disruption induces branching (Schwuchow et al. 1990, Meske et al. 1996) and causes dramatic tip swelling, while microfilament disruption has little to no effect on tip swelling (Doonan et al. 1988, Schwuchow and Sack 1994). In fern protonema, microtubules and microfilaments are both concentrated in the tips and microtubules are organized in a radial array at the tip which shifts to a net axial array further back (Kadota and Wada 1992).

Why do APM and oryzalin preferentially disrupt the radial array of microtubules at the tip? These microtubules may be more dynamic, with a more rapid turnover rate of tubulin dimers, than those farther back in the tube. Microtubules can be made more stable by detyrosination or acetylation of alpha tubulin (Gelfand and Bershadsky 1991). In *Nicotiana tabacum* pollen tubes, tyrosinated tubulin is detected throughout the tube and acetylated tubulin is detected further back in the more mature regions of the tube (Åström 1992). Acetylated microtubule arrays in the flagella of pteridophytes are also resistant to microtubule disruptors including oryzalin and APM (Hoffman and Vaughn 1996). It is possible that the axial microtubules in the tube are acetylated and thus resistant to oryzalin and APM, while those in the radial array at the tip are more dynamic.

Our working model for the radial array of microtubules at the tip is that they organize the cortical cyto-

plasm and also direct the deposition of cellulose. The prevailing model for cell wall deposition is that cortical microtubules direct the growth of cellulose microfibrils (Cyr 1994), but in angiosperm pollen tubes, there is no radial array for this to occur. However, there is one in *P. abies*, and these radial microtubules may dynamically control tip extension, prevent tip swelling, and mediate tube branching by orchestrating cellulose microfibril deposition. Recent evidence that the inhibition of cellulose synthesis can disrupt microtubules suggests that there is bidirectional communication between microfibrils and microtubules (Fisher and Cyr 1998). The encircling layer of perpendicular microtubules at the tip (Lazzaro 1999) maintains tip integrity since its loss leads to tip swelling. Pinaceous tips secrete hydrolases, esterases, and acid phosphatases (Pettitt 1985), and *Picea glauca* tubes digest their way through ovular tissue to reach the egg cell (Dawkins and Owens 1993). The perpendicular microtubules may contribute to tip integrity in vivo as the tubes elongate through a harsh environment of hydrolytic enzymes and degraded cells.

Microtubules (Lazzaro 1999) and microfilaments (Lazzaro 1996) colocalize in the axial array throughout *P. abies* pollen tubes. In angiosperms, there is also evidence that microtubules and microfilaments colocalize in the tube (Franke et al. 1972, Lancelle et al. 1987, Raudaskoski et al. 1987, Tiwari and Polito 1988, Lancelle and Hepler 1991). However, comparative studies evaluating whether microtubule disruptors affect microfilaments, and vice versa, have not been reported. Our results indicate that microtubule disruptors did not affect microfilament organization and that microfilament disruption did not affect microtubule organization. All our experiments tested these inhibitors individually, we did not do any paired, simultaneous tests. In nonpollen systems such tests indicate that microtubules and microfilaments interact. In *Hydrocharis dubia* root hairs, cytochalasin B reversibly inhibits streaming, while propyzamide has no effect. However, when cells are treated with both cytochalasin B and propyzamide, streaming does not recover when cytochalasin B is removed, but only after propyzamide is removed as well. Dual treatment also causes the reorganization of microfilaments into a meshwork that is not reorganized into long filaments until propyzamide is removed (Tominaga et al. 1997). Microtubule disruption in *Nitella pseudoflabellata* lowers the cell's sensitivity to the inhibition of streaming by cytochalasin (Collings et al. 1996). In protonemal cells of the moss *Ceratodon purpureus*, cytochalasin D alone has no effect on tip swelling, while APM dramatically increases tip swelling. This increase is moderated when cells are treated simultaneously with cytochalasin D and APM (Schwuchow and Sack 1994).

In conclusion, microfilaments and microtubules coordinate to orchestrate tip extension and branching in conifer pollen tubes in a mechanism which is different from angiosperm pollen tubes but has functional analogies to tip growth in mosses and ferns. Conifer pollen tubes are an exciting model to study tip extension in a system where the dynamics of growth are much slower than in angiosperm pollen tubes.

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