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# MICROTUBULE ORGANIZATION IN GERMINATED POLLEN OF THE CONIFER *PICEA ABIES* (NORWAY SPRUCE, PINACEAE)[1]

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The organization of microtubules in germinated pollen of the conifer *Picea abies* (Norway spruce, Pinaceae) was examined using primarily confocal microscopy. Pollination in conifers differs from angiosperms in the number of mitotic divisions between the microspore and the sperm and in the growth rate of the pollen tube. These differences may be orchestrated by the cytoskeleton, and this study finds that there are important functional differences in microtubule organization within conifer pollen compared to the angiosperm model systems. Pollen from *P. abies* contains two degenerated prothallial cells, a body cell, a stalk cell, and a vegetative cell. The body cell produces the sperm. In the vegetative cell, microtubules form a continuous network from within the pollen grain, out through the aperture, and down the length of the tube to the elongating tip. Within the grain, this network extends from the pollen grain wall to the body and stalk cell complex. Microtubules within the body and stalk cells form a densely packed array that enmeshes amyloplasts and the nucleus. Microtubule bundles can be traced between the body and stalk cells from the cytoplasm of the body cell to the adjoining cell wall and into the cytoplasm of the stalk cell. Body and stalk cells are connected by plasmodesmata. The organization of microtubules and the presence of plasmodesmata suggest that microtubules form a path for intercellular communication by projecting from the cytoplasm to interconnecting plasmodesmata. Microtubules in the elongating tube form a net axial array that ensheathes the vegetative nucleus. Microtubules are enriched at the elongating tip, where they form an array beneath the plasma membrane that is perpendicular to the direction of tube growth. This enriched region 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 core of the elongating tip. The organization of microtubules in the tip differs from that seen in angiosperm pollen tubes.

**Key words:** conifer; cytoskeleton; microtubule; *Picea abies;* Pinaceae; pollen; pollen tube.

Pollination in conifers differs from angiosperms in the number of mitotic divisions between the microspore and the sperm, the growth rate of the pollen tube, the length of time between pollination and fertilization, and the absence of double fertilization (reviewed in Singh, 1978). Since these differences may be orchestrated by cytoskeletal organization, this study examines microtubule organization in germinated conifer pollen. In Norway spruce (*Picea abies*), pollen is released in the spring in a five-cell stage containing two degenerated prothallial cells and a body and stalk cell complex surrounded by the vegetative cell (Singh, 1978). Pollen lands in the pollination droplet and germinates in the micropyle. The pollen tube tip secretes proteases and hydrolases (Pettitt, 1985) as it slowly digests through integumentary tissue to reach the egg cell within the female gametophyte (Singh, 1978). After the tube tip reaches the archegonium, the body and stalk cells separate, migrate into the tube, and the body cell undergoes mitosis to produce two sperm nuclei, but cell division is incomplete since only a partial cell plate forms in vivo (Dawkins and Owens, 1993). One of these sperm nuclei fertilizes the egg. The gap between pollination and fertilization in *P. abies* is 2–3 wk (Singh, 1978).

Angiosperms are the primary model systems for investigating the cytoskeleton in germinated pollen and pollen tubes. In angiosperms, both microfilaments and microtubules form a network in the vegetative cell within germinated pollen grains (reviewed in Pierson and Cresti, 1992). Microfilaments are found throughout the pollen tube in a net axial array (reviewed in Pierson and Cresti, 1992; Li et al., 1997), but their presence in the elongating tip is controversial. Microfilaments are found in chemically fixed and cryofixed material, but recent evidence indicates that in living material microfilaments are excluded from the tip (reviewed in Taylor and Hepler, 1997). 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). Much less is understood about the cytoskeleton in the pollination of conifers, an economically important plant group. Since *P. abies* pollen survives long-term storage and is easily germinated in vitro, it is a useful model system for investigating the role of the cytoskeleton in conifer pollen tube growth. Actin microfilaments in *P. abies* pollen tubes form an array that is parallel with the direction of elongation, ensheathes the vegetative nucleus, and is enriched at the tip (Lazzaro, 1996). In the terminal 30 μm of the pollen tube, actin filaments form a 6-μm thick band beneath the plasma membrane, which ensheathes a microfilament depleted

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core (Lazzaro, 1996). Within the pollen grain, actin microfilaments form a network in the vegetative cell that ensheathes the body and stalk cell complex. In addition, the body and stalk cells both contain actin microfilaments. The presence of microfilaments in the spermatogenous body cell is a fundamental difference from the angiosperms (Lazzaro, 1998), where the spermatogenous generative cell reportedly lacks microfilaments but contains microtubules organized in bundles that ensheathe the nucleus (reviewed in Palevitz and Tiezzi, 1992). Although microtubules have been identified with epifluorescence microscopy as parallel bundles in *Pinus densiflora* pollen tubes (Terasaka and Niitsu, 1994), microtubule organization has not been thoroughly examined. Using primarily confocal microscopy, the organization of microtubules within the pollen grain, the body and stalk cells, the pollen tube, and the elongating pollen tube tip is described here.

## MATERIALS AND METHODS

Male cones from *Picea abies* (L.) Karch were collected from a seed orchard 10 km from Stockholm, Sweden, and kept in the laboratory at 20°C for 10 d while the scales opened to shed pollen, which was then stored at −20°C. Pollen grains were taken directly from −20°C and scattered on a layer of 1% agar containing 10% sucrose, 1 mmol/L calcium chloride, and 1 mmol/L boric acid (Pettitt, 1985). After 24 h in a humid chamber at 30°C (Frankis and Grayson, 1990), 95% of the pollen grains had germinated, with pollen tubes 200 μm long. The vegetative nucleus had moved into the elongating tube, while the body and stalk cells remained within the pollen grain. Germinated pollen grains were fixed directly on the agar surface for 1 h with 4% paraformaldehyde in PEM buffer (66 mmol/L PIPES, 1 mmol/L EGTA, 1 mmol/L $MgSO_4$) at pH 7.0. Fixed pollen was then transferred to microfuge tubes and pelleted by centrifuging at 1400 × *g* for 10 s. Cell walls were then permeabilized using either enzymatic digestion of freeze shattering.

For enzymatic digestion, pollen was then rinsed three times for 10 min each in PEM buffer at pH 5.0. Pollen tubes in the final pellet were fractured in a microfuge tube using a plastic pestle. This partially ruptured the tube cell wall and increased the enzymatic digestion of the body cell walls and the penetration of antibodies. The body cell wall was digested by agitating material for 1 h at 35°C in 1% cellulase (Onozuka R-10; Serva, Heidelberg, Federal Republic of Germany) and 1% macerase (Macerozyme R-10; Serva, Heidelberg, Federal Republic of Germany) in PEM buffer (pH 5.0) containing protease inhibitors (4 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 50 μg/mL aprotinin, 10 μg/mL pepstatin).

As an alternative to enzymatic digestion, cell walls were permeabilized with freeze shattering using a method developed by Wasteneys, Willingale-Theune, and Menzel (1997). Pollen tube pellets were sandwiched between two glass slides. These slides were plunged into liquid nitrogen until they reached −180°C. The slide sandwich was then placed on a precooled aluminum block (−180°C), and even downward pressure was applied with another precooled aluminum block (−180°C). The pressure created small fractures in the pollen tube walls. Slides were then separated and placed on a third aluminum block at +20°C and as the pollen tubes warmed they were bathed in extraction buffer (phosphate-buffered saline, pH 7.5 plus 1% Triton X-100) for 30 min. The freeze shattering method greatly improved the percentage of pollen tubes with optimal cell morphology and immunolabeling.

The resulting samples from enzymatic digestion or freeze shattering were rinsed three times for 5 min each in blocking buffer (phosphate-buffered saline (pH 7.5), 1% bovine serum albumin, 1% TX-100) (Wick, 1993). The pollen sample was then divided into three aliquots for parallel treatment with primary and secondary antibody, secondary antibody alone, or no antibody. The first aliquot was incubated for 16 h at 20°C in a mouse monoclonal antibody to alpha tubulin (Amersham N356; Amersham, Buckinghamshire, UK), diluted 1:100 in blocking buffer. Pollen tubes were then rinsed four times for 10 min each in blocking buffer and then incubated for 2 h at 35°C with a Cy3-conjugated goat secondary antibody to mouse immunoglobins (Sigma C2181; Sigma, St. Louis, Missouri, USA), diluted 1:200 in blocking buffer. The pollen tubes were then rinsed three times for 10 min each in blocking buffer and mounted in Moviol. As a control, the second aliquot was processed in parallel with the first, but incubated in blocking buffer without primary antibody followed by incubation with the Cy3-conjugated secondary at 1:200 to evaluate nonspecific binding of the secondary antibody. The third aliquot was processed in parallel with the first and second, but incubated in blocking buffer without primary or secondary antibodies to measure autofluorescence. Pollen grains were examined on either a BioRad MRC600 or a Zeiss LSM510 confocal laser scanning microscope. Nomarski DIC images were collected simultaneously through the confocal scan head. Optical serial sections were collected at a thickness of 400 or 500 nm. Confocal projections were generated by digitally combining adjacent serial sections into a single image with the public domain program NIH Image on a Macintosh computer. Identical confocal settings were always used for both treated pollen tubes and controls. Confocal projections were used for the measurement of pollen tube tips, and the measurements were calibrated with an image of a stage micrometer recorded through the confocal microscope.

For electron microscopy, germinated pollen was fixed in 5% glutaraldehyde in 66 mmol/L PIPES (Piperazine-N,N'-bis[2-ethane-sulfonic acid]) buffer (pH 7.0) for 1 h directly on germination slides, and then transferred to microfuge tubes. Pollen grains were subsequently rinsed in PIPES buffer, postfixed in 1% $OsO_4$ for 1.5 h, rinsed again in PIPES buffer, dehydrated through an acetone series, and embedded in Spurr's resin. Thin sections (90 nm) were stained with uranyl acetate and lead citrate and examined on a Zeiss EM 906.

## RESULTS

Microtubules are present throughout the pollen grain and elongate pollen tube, forming a continuous network from within the grain to the tip (Fig. 1). Within the grain they are organized in a meshwork from the pollen grain wall to the body/stalk cell complex (Figs. 1 and 2). Microtubules are more densely organized within the body and stalk cells compared to the vegetative cell (Figs. 1 and 2). The body and stalk cells sit within the vegetative cell, and the stalk cell is appressed against the pollen grain wall, adjacent to the two degraded prothallial initial cells (Fig. 3). In median confocal sections through the body and stalk cells, microtubule bundles traverse the cytoplasm from the plasma membrane to the nuclear envelope (Fig. 4). In 500-nm thick optical section, microtubule bundles can be traced from the body cell across to the stalk cell forming apparent connections between the two cells (Fig. 4). Microtubule bundles were not observed in continuity between the body or stalk cells and the surrounding vegetative cell. Plasmodesmata connected the body and stalk cells (Fig. 5) and connected the two cells to the surrounding vegetative cell (Fig. 6). Within the body cell, microtubules are associated with the nuclear envelope (Fig. 4) and bundles viewed using transmission electron microscopy extend to the nuclear envelope (Fig. 7). Microtubules are also associated with the amyloplasts in the body cell (Fig. 8).

The network of microtubules in the vegetative cell of the pollen grain forms a meshwork that extends through

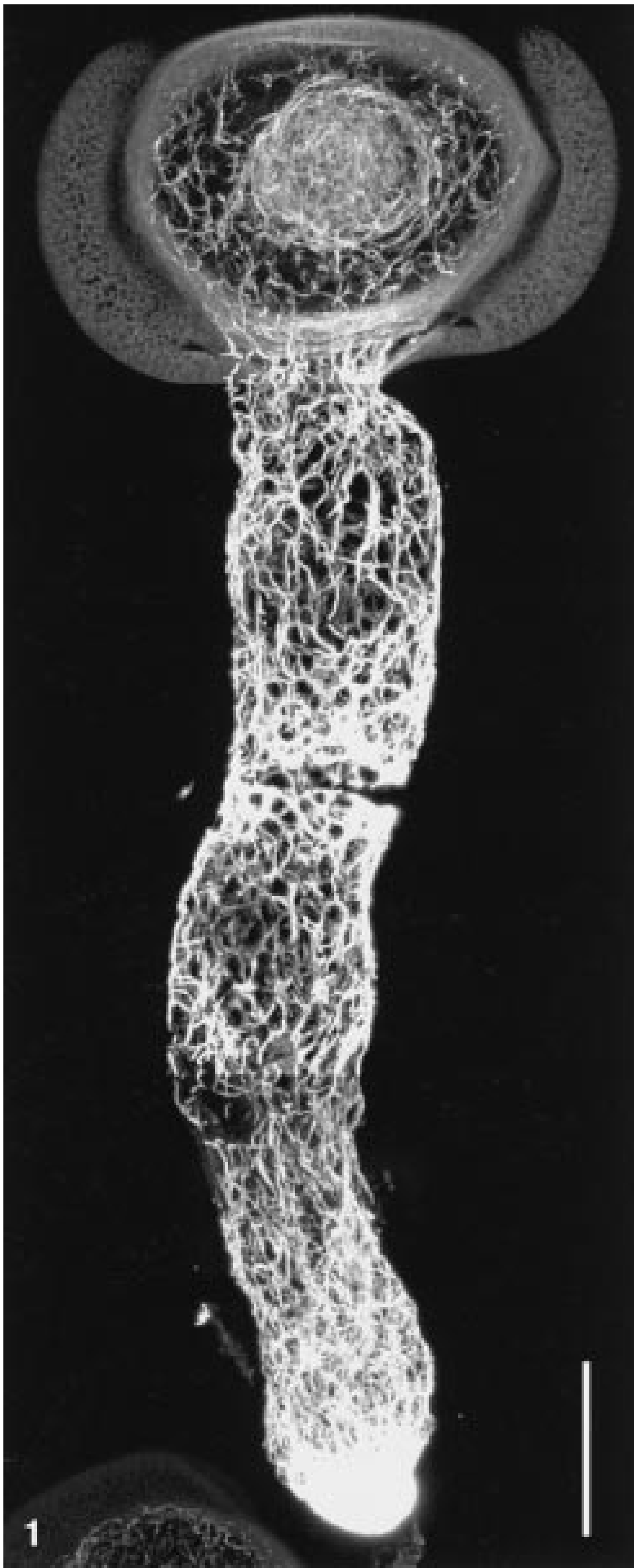


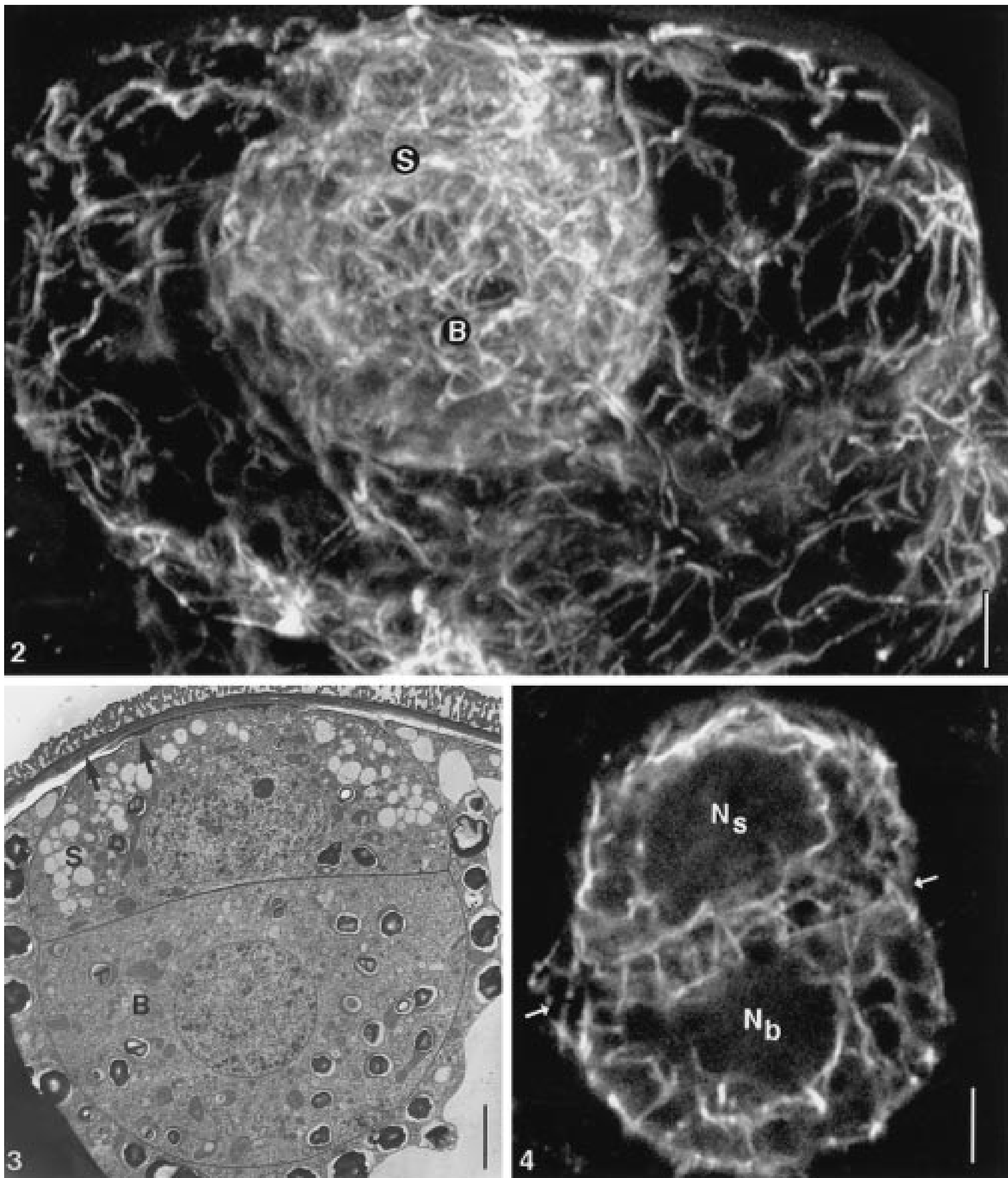

Fig. 1. Microtubules form a complex network, which extends from within the pollen grain, out the aperture, down the length of the tube, and is enriched at the pollen tube tip. The body and stalk cells remain within the grain in vitro and contain an enriched network of microtubules. This is a montage of four projections. Each projection is from 40 confocal optical sections through 20 μm of total thickness. Scale bar = 25 μm.

the pollen grain aperture and into the elongating pollen tube (Figs. 1 and 9). This network is concentrated around the vegetative nucleus and in single confocal section, microtubule bundles surround the nuclear envelope and extend along the envelope (Fig. 10). Microtubules extend down the entire pollen tube and are enriched at the tip (Fig. 1). The overall organization of microtubules is parallel to the direction of elongation (Fig. 11). However, microtubules in the cell cortex at the tip are organized perpendicular to the direction of elongation (Fig. 12). There is an abrupt transition to a perpendicular array at the border of the enriched tip region (Fig. 12). The enriched region of microtubules extends back from the tip 20.15 ± 4.03 μm (mean ± 1 SD, N = 17 pollen tubes). Microtubules are present throughout the pollen tube tip. In median confocal sections, microtubules are present but no longer have a net organization perpendicular to the direction of elongation (Fig. 13). There was no nonspecific binding of the secondary antibody, and only faint autofluorescence from the nuclei and from the pollen grain walls was detected with the confocal settings used (not shown).

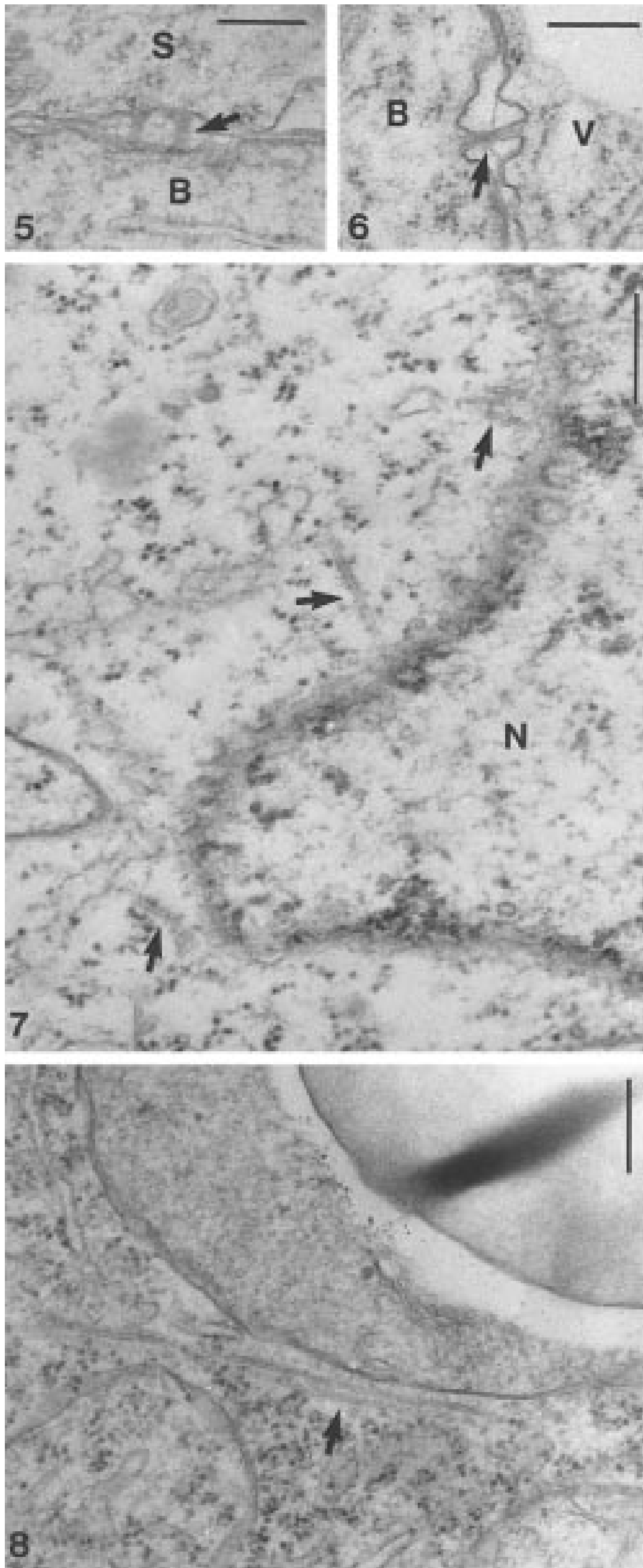
## DISCUSSION

Within the pollen grain, microtubules are found in the vegetative, body, and stalk cells. The network in the vegetative cell is a meshwork from the pollen grain wall to the body/stalk cell complex. Microtubules are more dense in the body and stalk cells, where they are found throughout the cytoplasm, extending from the plasma membrane to the nuclear envelope. Microfilaments also form a network throughout the vegetative cell and are found in both the body and stalk cells (Lazzaro, 1998) so both elements may coordinate to organize the cytoplasm within the pollen grain. However, the detailed organization of microtubules within the pollen grain differs from the organization of actin microfilaments, and these differences may reflect functional distinctions between the two cytoskeletal systems. Microfilaments form an enmeshing basket in the vegetative cell that surrounds the body and stalk cells (Lazzaro, 1998). Microfilaments are also present within the body and stalk cells and form an array throughout the cell cortex, which is enriched beneath the plasma membrane (Lazzaro, 1998). The microtubule array was not particularly enriched beneath the plasma membrane in either the body, stalk, or vegetative cells. In the angiosperms, microtubules form a network throughout the germinated pollen grain (Tiwari and Polito, 1988, 1990) and form helical or longitudinal arrays in the generative cell, which ensheathe the nucleus (Derksen, Pierson, and Traas, 1985; Pierson, Derksen, and Traas, 1986; Raudaskoski et al., 1987; Heslop-Harrison et al., 1988).

Microtubule bundles can be traced between the body and stalk cells from the cytoplasm of the body cell to the adjoining cell wall and into the cytoplasm of the stalk cell. These cells are mitotic sisters (Singh, 1978) and are connected by plasmodesmata. The organization of microtubules and the presence of plasmodesmata suggest that microtubules form a path for intercellular communication by projecting from the cytoplasm to interconnecting plasmodesmata. There is evidence that the cytoskeleton is




Figs. 2–4. Microtubules within the germinated pollen grain. Scale bars = 5 μm. **2.** Microtubules form a network from the germinated pollen grain wall to the body and stalk cells, where they are enriched. Projection of 25 optical sections (12.5 μm total thickness). **3.** The body and stalk cells are appressed against the pollen grain wall near the two degenerated prothallial initials (arrows). **4.** Microtubules form a network in the body and stalk cells. Discrete bundles can be traced from the body cell nuclear envelope ($N_b$), across the cell wall space, to the stalk cell nuclear envelope ($N_s$). The separating cell wall is marked by arrows. Single confocal optical section (500 nm thick).

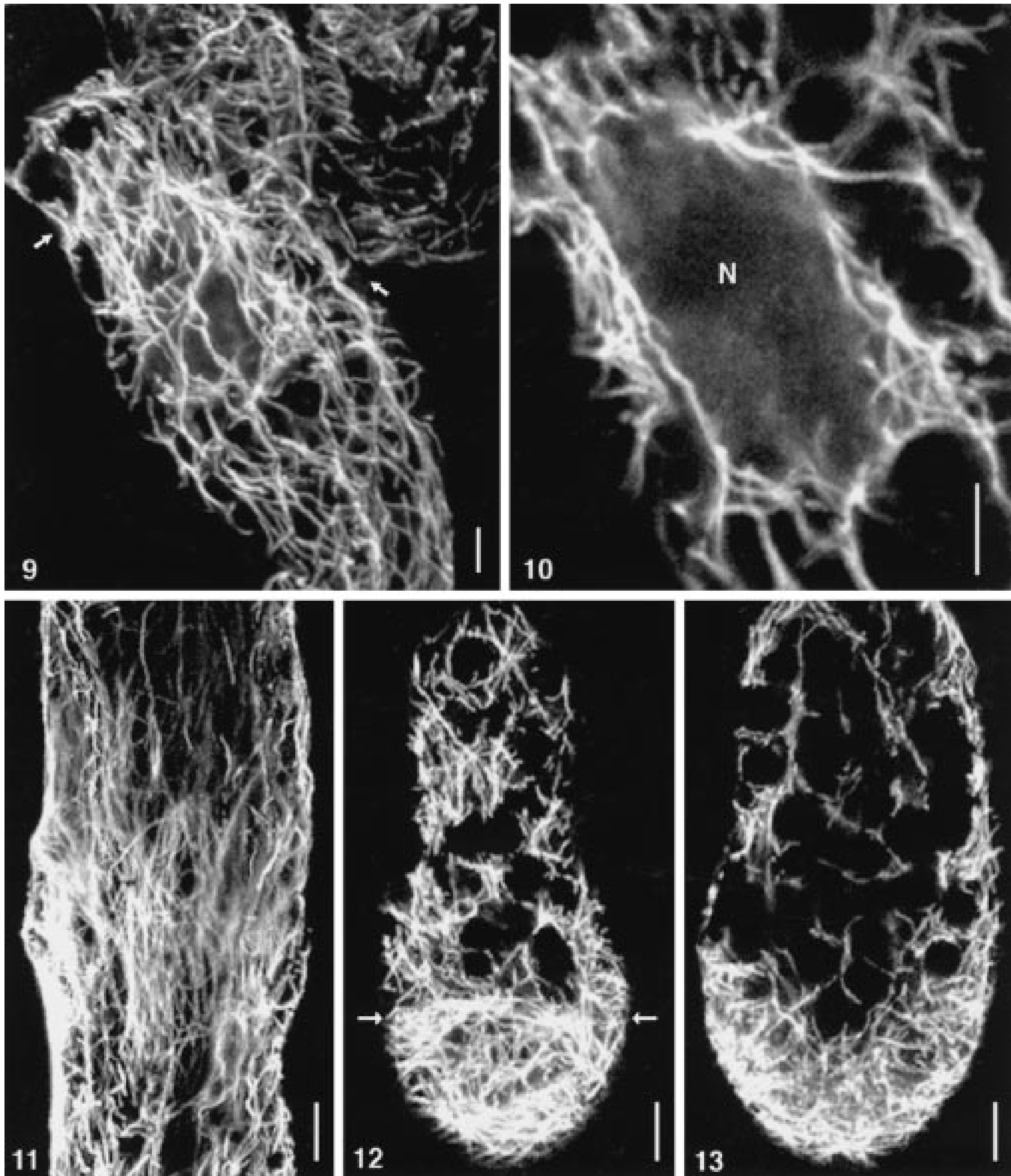



Figs. 5–8. Electron micrographs of the body cell within the germinated pollen grain. Scale bars = 300 nm. **5.** Plasmodesmata (arrow) connect the body and stalk cells. **6.** Plasmodesmata (arrow) also connect the body and vegetative cells. **7.** Microtubules (arrows) are closely associated with the body cell nucleus. **8.** Microtubule bundles are also closely associated with amyloplasts (arrow) in the body cell.

involved in intercellular traffic via plasmodesmata. The movement protein from tobacco mosaic virus colocalizes with regions of the cytoplasm containing microtubules and microfilaments suggesting that protein transport through plasmodesmata may use the cytoskeleton (Heinlein et al., 1995; McLean, Zupan, and Zambryski, 1995). Microfilaments may control the size exclusion limit of plasmodesmata since cytochalasins and profilin, which depolymerize microfilaments, increase the size exclusion limit and diameter of plasmodesmata (White et al., 1994; Ding, Kwon, and Warnberg, 1996). However, since microtubules were not specifically detected within plasmodesmata in electron micrographs, one can only conclude from the confocal micrographs that the organization of microtubules provides a direct path up to and away from the plasmodesmata between the body and stalk cells. Since there was no continuity between microtubules in the body and stalk cells and the surrounding vegetative cell, this pathway appears specific for the plasmodesmata between body and stalk cells and not vegetative cells. In *Picea glauca* pollen germinated in vitro, plasmodesmata were observed between all three cells, but in pollen germinated in vivo, they were only observed between the body and stalk cells and not to the surrounding vegetative cell (Dawkins and Owens, 1993). Although actin microfilaments have been detected in plasmodesmata in other plant material (White et al., 1994), they were not seen in continuity between the body and stalk cells in *Picea abies* pollen (Lazzaro, 1998).

Microtubule bundles extend through the pollen grain aperture and down the length of the tube in a generally axial orientation. This is similar to the overall organization of microtubules in *Pinus densiflora* (Terasaka and Niitsu, 1994), *Pinus sylvestris* (de Win et al., 1996), and in angiosperm pollen tubes (Derksen, Pierson, and Traas, 1985; Pierson, Derksen, and Traas, 1986; Raudaskoski et al., 1987). Microtubules are enriched around the vegetative nucleus and are closely associated with the nuclear envelope. Actin microfilaments also form a net axial organization in these pollen tubes and are enriched around the vegetative nucleus (Lazzaro, 1996). The possibility therefore exists that both microtubule and microfilament systems may be involved in the transport of the tube nucleus. In *Pinus sylvestris*, microtubules and microfilaments are closely associated in cryofixed material (de Win et al., 1996). In angiosperms, treatment with cytoskeletal inhibitors indicates that microfilaments (Franke et al., 1972; Heslop-Harrison et al., 1988) and microtubules (Joos, van Aken and Kristen, 1994; Åström, Sorri, and Raudaskoski, 1995; Heslop-Harrison and Heslop-Harrison, 1996) direct the movement of the vegetative nucleus. In *Pinus densiflora*, the microfilament motor protein myosin is found on the nuclear surface (Terasaka and Niitsu, 1994). Myosin is also detected on the surface of the vegetative nucleus in angiosperms (Tang, Hepler, and Scordilis, 1989; Heslop-Harrison and Heslop-Harrison, 1989; Miller, Scordilis, and Hepler, 1995), but the microtubule motor proteins kinesin and dynein are not (Cai et al., 1993; Liu and Palevitz, 1996; Moscatelli et al., 1998).

The most intriguing observation in this study is the organization of microtubules in the pollen tube tip, where they are enriched in the terminal 20 μm of the pollen




Figs. 9–13. Microtubules within the pollen tube. Scale bars = 5 μm. **9.** Microtubule bundles extend from the pollen grain, through the aperture (arrows), and into the pollen tube. Projection of 13 optical sections (6.5 μm total thickness). **10.** Microtubule bundles are associated with the nuclear surface and enmesh the tube nucleus. Projection of two optical sections (1 μm total thickness). **11.** Microtubules form a net axial array parallel to the direction of elongation. Projection of 25 optical sections (10 μm total thickness). **12.** At the pollen tube tip, microtubules beneath the plasma membrane are enriched in a net radial array. The abrupt transition from net radial to net axial is marked by arrows. Projection of six optical sections (3 μm total thickness). **13.** In median sections, the central core of the tube tip contains microtubules in a net axial array. Projection of six optical sections (3 μm total thickness).

tube, coincident with the enriched actin microfilament zone (Lazzaro, 1996). Microtubules in the cell cortex at the tip are oriented perpendicular to the growing axis. This orientation changes abruptly 20 μm from the tip to a net axial organization. The microtubules perpendicular to the elongating axis are coincident with the 6-μm thick microfilament band in the tip that ensheathes a microfilament-depleted core (Lazzaro, 1996). In medial sections through the tip, microtubules are present in a net axial orientation and in the same place as the microfilament-depleted core (Lazzaro, 1996), so this core region, which contains small organelles, does have a cytoskeletal (microtubule) network. The cytoskeletal organization of the tip is very different from that in angiosperm pollen tubes (reviewed in Pierson and Cresti, 1992; Li et al., 1997), where microtubules are either not observed in the tips or form short elements that are not organized in a radial array (Derksen, Pierson, and Traas, 1985; Raudaskoski et al., 1987; Del Casino et al., 1993). However, in fern protonemal cells, microtubules form a specific radial array at the growing tip, which shifts to a net axial array farther back in the tube (Kadota and Wada, 1992), similar to the organization seen in *Picea abies*.

Why are microtubules enriched in the growing tip and oriented perpendicular to the growing axis? Conifer pollen tube tips secrete proteases and hydrolases (Pettitt, 1985) as they slowly digest their way through integumentary tissue to reach the egg cell within the female gametophyte (Singh, 1978). Pollen tubes grow by the deposition of cell wall components at the tip (reviewed in Derksen et al., 1995; Taylor and Hepler, 1997). My working model is that the radial microtubules at the tip may orient the deposition of cellulose microfibrils at the tip region. The cell wall may develop in the terminal 20 μm but may be mature in older parts of the tube. Farther back in the tube where the microtubules are axial, their function may change to direct the migration of the nucleus and other organelles, rather than the growth of the cellulose microfibrils. There is an abrupt transition in microtubule organization from radial to axial in the pollen tube cortex 20 μm back from the tip. As the tube elongates and this transition region moves down the tube, microtubules are presumably probably broken down and reorganized. Microtubules are dynamic structures where tubulin dimers are continuously added and removed, and plant microtubules have a high degree of dynamic instability (Moore et al., 1997) and can rapidly reorganize from transverse to longitudinal in living cells (Yuan et al., 1994).

In conclusion, the organization of microtubules in the pollen grain and elongating tube of this conifer has some similarities to microtubule organization in angiosperm pollen, but there are important differences, especially in the elongating tip where they are enriched in a radial array. This work provides additional evidence that cytoskeletal organization in conifer pollen is unique.

## LITERATURE CITED


ÅSTRÖM, H., O. SORRI, AND M. RAUDASKOSKI. 1995. Role of microtubules in the movement of the vegetative nucleus and generative cell in tobacco pollen tubes. *Sexual Plant Reproduction* 8: 61–69.

CAI, G., A. BARTALESI, C. DEL CASINO, A. MOSCATELLI, A. TIEZZI, AND M. CRESTI. 1993. The kinesin immunoreactive homolog from *Nicotiana tabacum* pollen tubes: biochemical properties and subcellular localization. *Planta* 191: 496–506.

DAWKINS, M. D., AND J. N. OWENS. 1993. In vitro and in vivo pollen hydration, germination, and pollen tube growth in white spruce, *Picea glauca* (Moench) Voss. *International Journal of Plant Science.* 154: 506–521.

DEL CASINO, C., Y. LI, A. MOSCATELLI, M. SCALI, A. TIEZZI, AND M. CRESTI. 1993. Distribution of microtubules during the growth of tobacco pollen tubes. *Biology of the Cell* 79: 125–132.

DERKSEN, J., E. S. PIERSON, AND J. A. TRASS. 1985. Microtubules in vegetative and generative cells of pollen tubes. *European Journal of Cell Biology* 38: 142–148.

———, T. RUTTEN, T. VAN AMSTEL, A. DE WIN, F. DORIS, AND M. STEER. 1995. Regulation of pollen tube growth. *Acta Botanica Neerlandica* 44: 93–119.

DE WIN, A. H. N., B. KNUIMAN, E. S. PIERSON, H. GEURTS, H. M. P. KENGEN, AND J. DERKSEN. 1996. Development and cellular organization of *Pinus sylvestris* pollen tubes. *Sexual Plant Reproduction* 9: 93–101.

DING, B., M. O. KWON, AND L. WARNBERG. 1996. Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll. *Plant Journal* 10: 157–164.

FRANKE, W. W., W. HERTH, J. VAN DER WOUDE, AND D. J. MORRÉ. 1972. Tubular and filamentous structures in pollen tubes: possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. *Planta* 105: 317–341.

FRANKIS, R. C., AND G. K. GRAYSON. 1990. Heat-shock response in germinating pine pollen. *Sexual Plant Reproduction* 3: 195–199.

HEINLEIN, M., B. L. EPEL, H. S. PADGETT, AND R. N. BEACHY. 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. *Science* 270: 1983–1985.

HESLOP-HARRISON, J., AND Y. HESLOP-HARRISON. 1989. Myosin associated with the surfaces of organelles, vegetative nuclei and generative cells in angiosperm pollen grains and tubes. *Journal of Cell Science* 94: 319–325.

———, AND ———. 1996. Microtubules and the positioning of the vegetative nucleus and generative cell in the angiosperm pollen tube: a quantitative study. *Proceedings of the Royal Society of London* 263: 1299–1304.

———, ———, M. CRESTI, A. TIEZZI, AND A. MOSCATELLI. 1988. Cytoskeletal elements, cell shaping and movement in the angiosperm pollen tube. *Journal of Cell Science* 91: 49–60.

JOOS, U., J. VAN AKEN, AND U. KRISTEN. 1994. Microtubules are involved in maintaining the cellular polarity in pollen tubes of *Nicotiana sylvestris. Protoplasma* 179: 5–15.

KADOTA, A., AND M. WADA. 1992. Reorganization of the cortical cytoskeleton in tip growing fern protonemal cells during phytochrome mediated phototropism and blue light induced apical swelling. *Protoplasma* 166: 35–41.

LAZZARO, M. D. 1996. The actin microfilament network within elongating pollen tubes of the gymnosperm *Picea abies* (Norway spruce). *Protoplasma* 194: 186–194.

———. 1998. The spermatogenous body cell of the conifer *Picea abies* (Norway spruce) contains actin microfilaments. *Protoplasma* 201: 194–201.

LI, Y., MOSCATELLI, G. CAI, AND M. CRESTI. 1997. Functional interactions among cytoskeleton, membranes, and cell wall in the pollen tube of flowering plants. *International Review of Cytology* 176: 133–199.

LIU, B., AND B. A. PALEVITZ. 1996. Localization of a kinesin-like protein in generative cells of tobacco. *Protoplasma* 195: 78–89.

MCLEAN, B. G., J. ZUPAN, AND P. C. ZAMBRYSKI. 1995. Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco cells. *Plant Cell* 7: 2101–2114.

MILLER, D. D., S. P. SCORDILIS, AND P. K. HEPLER. 1995. Identification and localization of three classes of myosins in pollen tubes of *Lilium longiflorum* and *Nicotiana alata. Journal of Cell Science* 108: 2549–2653.

MOORE, R. C., M. ZHANG, L. CASSIMERIS, AND R. J. CYR. 1997. In vitro assembled plant microtubules exhibit a high state of dynamic instability. *Cell Motility and Cytoskeleton* 38: 278–286.

MOSCATELLI, A., G. CAI, F. CIAMPOLINI, AND M. CRESTI. 1998. Dynein heavy chain-related polypeptides are associated with organelles in

pollen tubes of *Nicotiana tabacum. Sexual Plant Reproduction* 11: 31–40.

PALEVITZ, B. A., AND A. TIEZZI. 1992. Organization, composition, and function of the generative cell and sperm cytoskeleton. *International Review of Cytology* 140: 149–185.

PETTITT, J. M. 1985. Pollen tube development and characteristics of the protein emission in conifers. *Annals of Botany* 56: 379–397.

PIERSON, E. S., AND M. CRESTI. 1992. Cytoskeleton and cytoplasmic organization of pollen and pollen tubes. *International Review of Cytology* 140: 73–125.

———, J. DERKSEN, AND J. A. TRAAS. 1986. Organization of microfilaments and microtubules in pollen tubes grown in vitro or in vivo in various angiosperms. *European Journal of Cell Biology* 41: 14–18.

RAUDASKOSKI, M., H. ÅSTRÖM, K. PERTILLÄ, I. VIRTANEN, AND J. LOUHELIAN. 1987. Role of the microtubule cytoskeleton in pollen tubes: an immunocytochemical and ultrastructural approach. *Biology of the Cell* 61: 177–188.

SINGH, H. 1978. Embryology of gymnosperms. *In* W. Zimmermann, S. Carlquist, P. Ozenda, and H. D. Wulff [eds.], Handbuch der Pflanzenanatomie, vol. 10, part 2. Gebrüder Borntraeger, Berlin.

TANG, X., P. K. HEPLER, AND S. P. SCORDILIS. 1989. Immunochemical and immunocytochemical identification of a myosin heavy chain polypeptide in *Nicotiana* pollen tubes. *Journal of Cell Science* 92: 569–574.

TAYLOR, L. P., AND P. K. HEPLER. 1997. Pollen germination and tube growth. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 461–491.

TERASAKA, O., AND T. NIITSU. 1994. Differential roles of microtubule and actin-myosin cytoskeleton in the growth of *Pinus* pollen tubes. *Sexual Plant Reproduction* 7: 264–272.

TIWARI, S. C., AND V. S. POLITO. 1988. Organization of the cytoskeleton in pollen tubes of *Pyrus communis*: a study employing conventional and freeze-substitution electron microscopy, immunofluorescence, and rhodamine-phalloidin. *Protoplasma* 147: 100–112.

———, AND ———. 1990. The initiation and organization of microtubules in germinating pear (*Pyris communis* L.) pollen. *European Journal of Cell Biology* 53: 384–389.

WASTENEYS, G. O., J. WILLINGALE-THEUNE, AND D. MENZEL. 1997. Freeze shattering: a simple and effective method for permeabilizing higher plant cell walls. *Journal of Microscopy* 188: 51–61.

WHITE, R. G., K. BADELT, R. L. OVERALL, AND M. VESK. 1994. Actin associated with plasmodesmata. *Protoplasma* 180: 169–184.

WICK, S. M. 1993. Immunolabeling of antigens in plant cells. *Methods in Cell Biology* 37: 171–200.

YUAN, M., P. J. SHAW, R. M. WARN, AND C. W. LLOYD. 1994. Dynamic reorientation of cortical microtubules, from transverse to longitudinal, in living plant cells. *Proceedings of the National Academy of Sciences, USA* 91: 6050–6053.