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Differential roles of microtubule and actin-myosin cytoskeleton in the growth of *Pinus* pollen tubes

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Abstract The behavior and role of the microtubule (MT) and actin-myosin components of the cytoskeleton during pollen tube growth in two species of *Pinus* were studied using anti-α-tubulin, rhodamine-phalloidin, anti-myosin, and the appropriate inhibitors. Within germinated pollen tubes MTs were arranged obliquely or transversely, but in elongated tubes they were arranged along the tube's long axis. MTs were localized in the tube tip region, excluding the basal part. Altered growth was found in pollen tubes treated with colchicine; the tips of many pollen tubes incubated in the liquid medium were branched and/or rounded, and those in the agar medium were divided into many branches. Both the branching and the rounding were considered to be caused by the disturbance of polarizing growth of the tube due to MT disorganization with colchicine treatment. Actin filaments (F-actin) were found in the major parts of many pollen tubes along their long axis, excluding the tip region. In a few tubes, however, F-actin was distributed throughout the tube. The areas in the pollen tube containing F-actin were filled with abundant cytoplasmic granules, but the areas without F-actin had very few granules. The tube nucleus, which migrated from the grain area into the tube, was closely associated with F-actin. Germination of pollen grains treated with cytochalasin B was little affected, but further tube elongation was inhibited. Myosin was identified on cytoplasmic granules and to a lesser extent on the tube nucleus in the pollen tubes. Several granules were attached to the nuclear envelope. Tube growth was completely inhibited by *N*-ethylmaleimide treatment. In generative cells that were retained in the pollen grain, both MT and F-actin networks were observed. Myosin was localized on the cytoplasmic granules but not on the cell surface. In conclusion, it was shown that actin-myosin and MTs were present in gymnospermous *Pinus* pollen tubes and it is suggested that the former contributed to outgrowth of the tubes and the latter contributed to polarized growth. Several differences in the behavior of cytoskeletal elements in generative cells compared to angiosperms were revealed and are discussed.

Key words Actin · Cytoskeleton · Microtubule Myosin · *Pinus densiflora* · *Pinus thunbergii* Pollen tube

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Introduction

It has been indicated that F-actin in angiospermous pollen tubes participates in cytoplasmic streaming or tube growth (Franke et al. 1972; Mascarenhas and Lafountain 1972; Perdue and Parthasarathy 1985; Terasaka and Niitsu 1992). Kohno and co-workers (1987, 1988, 1990, 1991) reported in demembranated cell model experiments and in vitro motility assays that the translocator generating a motive force in cytoplasmic streaming was myosin. Myosin was immunologically visualized on the surface of organelles, vegetative nuclei, and generative cells in pollen grains and tubes of some angiosperms (Tang et al. 1989; Heslop-Harrison and Heslop-Harrison 1989; Palevitz and Liu 1992). From these reports, it has been indicated that an actomyosin motility system for cytoplasmic streaming and tube growth are present in angiospermous pollen tubes.

Microtubules (MTs) were also found to exist and colocalize with F-actin in the pollen tubes of some species (Lancelle et al. 1987; Pierson et al. 1986, 1989; Tiezzi et al. 1987; Tiwari and Polito 1988) and, with the colchicine treatment, to affect pollen tube growth (Derksen and Traas 1984). Åström et al. (1991) reported that in *Nicotiana* pollen, the cessation and recovery of generative cell and vegetative nucleus movements by cold treatments correlated with the de- and reassembly of MTs, which indicated that MTs, in addition to microfilaments, may be necessary for their movements. The

presence of kinesin, which is a force-generating protein that cooperates with MTs in the movement of vesicles, was shown in the *Nicotiana* pollen tube (Moscatelli et al. 1988; Cai et al. 1992). On the other hand, it was reported that colchicine in a moderate concentration that leads to the disassembly of MTs in pollen tubes did not affect pollen tube shape, cytoplasmic streaming, tube growth, or the movements of vegetative nucleus and generative cell throughout the tube (Franke et al. 1972; Heslop-Harrison et al. 1988; our unpublished data). A definite role for MTs in angiospermous pollen tubes is still unknown.

Not much is known about the cytoskeleton or tube growth in pollen of gymnosperms. The characteristic features that differentiate gymnospermous pollen tubes from those in angiosperms include their growth morphology and their slow growth rate. In addition, tube branching indicates primitive haustorial functions in nucellar tissue.

The present study was undertaken to examine the presence or absence of MT and actin-myosin and their roles in the pollen tubes of two species of *Pinus*. The results were compared and discussed with those of angiosperms reported previously.

Materials and methods

In the present study, pollen grains from *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parlatore were used. Mature pollen grains were cultured in the liquid medium of Brewbaker and Kwack (1963) for 5 days and in a solid medium with 3% agar for 35 days at room temperature (18–23° C). Some of these pollen grains were incubated in those media with 0.25, 0.5 or 1.0 mM colchicine (an inhibitor of MT organization), 10, 20 or 50 μg/ml cytochalasin B (an inhibitor of actin polymerization) or 5 mM *N*-ethylmaleimide (an inhibitor of actomyosin ATPase activity). MTs were detected using α-tubulin immunofluorescence methods given in Terasaka and Niitsu (1990). Pollen tubes were fixed for 1 h with 3.7% paraformaldehyde in 50 mM potassium phosphate buffer solution with 5 mM EGTA, pH 6.8. The materials were rinsed in the buffer solution for 10–30 min after each treatment. The mouse monoclonal anti-α-tubulin antibody (Amersham International, Amersham, Bucks., UK) and goat anti-mouse IgG-FITC (Tago, Burlingen, Calif., USA) were used as the first and second antibodies, respectively. For actin visualization, rhodamine-phalloidin diluted 1:5 with PBS (Sigma St. Louis, Mo., USA), 4% formaldehyde (pH 6.9), and PBS with 1% Triton X-100 were prepared and mixed in a 2:1:1 ratio. Pollen tubes were incubated in the mixed solution for 1 h at room temperature and were rinsed with PBS for 30 min. Myosin was detected using immunofluorescence with anti-myosin IgG. Pollen tubes were fixed for 10 min at 37° C with 3% paraformaldehyde buffered with PBS. The fixed pollen tubes were rinsed in PBS for 30 min and dehydrated with absolute acetone cooled to −20° C for 1 min. After rinsing in PBS for 10 min, they were incubated with the first antibody, rabbit anti-myosin IgG (Paesel + Lorei, Frankfurt, Germany) diluted 1:50 with PBS for 1 h. The antibody specifically binds only myosin heavy chain from smooth muscle or non-muscle sources, but does not bind any of the following: myosin from skeletal or cardiac muscle, actin, tropomyosin, and tubulin. They were rinsed again in PBS for 30 min and incubated with the second antibody, goat anti-rabbit IgG fluorescein conjugated (Organon Teknika, West-Chester, Pa., USA) diluted 1:20 with PBS for 1 h. Pollen in some developmental stages was observed using 1% aceto-carmine staining. The exact positions of tube nucleus and generative cell in a pollen grain and tube were examined with a phase contrast microscope or fluorescence microscopy using 1 μg/ml 4'6-diamidino-2-phenylindole (DAPI). Fluorescence was observed with a Nikon Microphoto-FX microscope and photographed on Kodak Tri-X pan film.

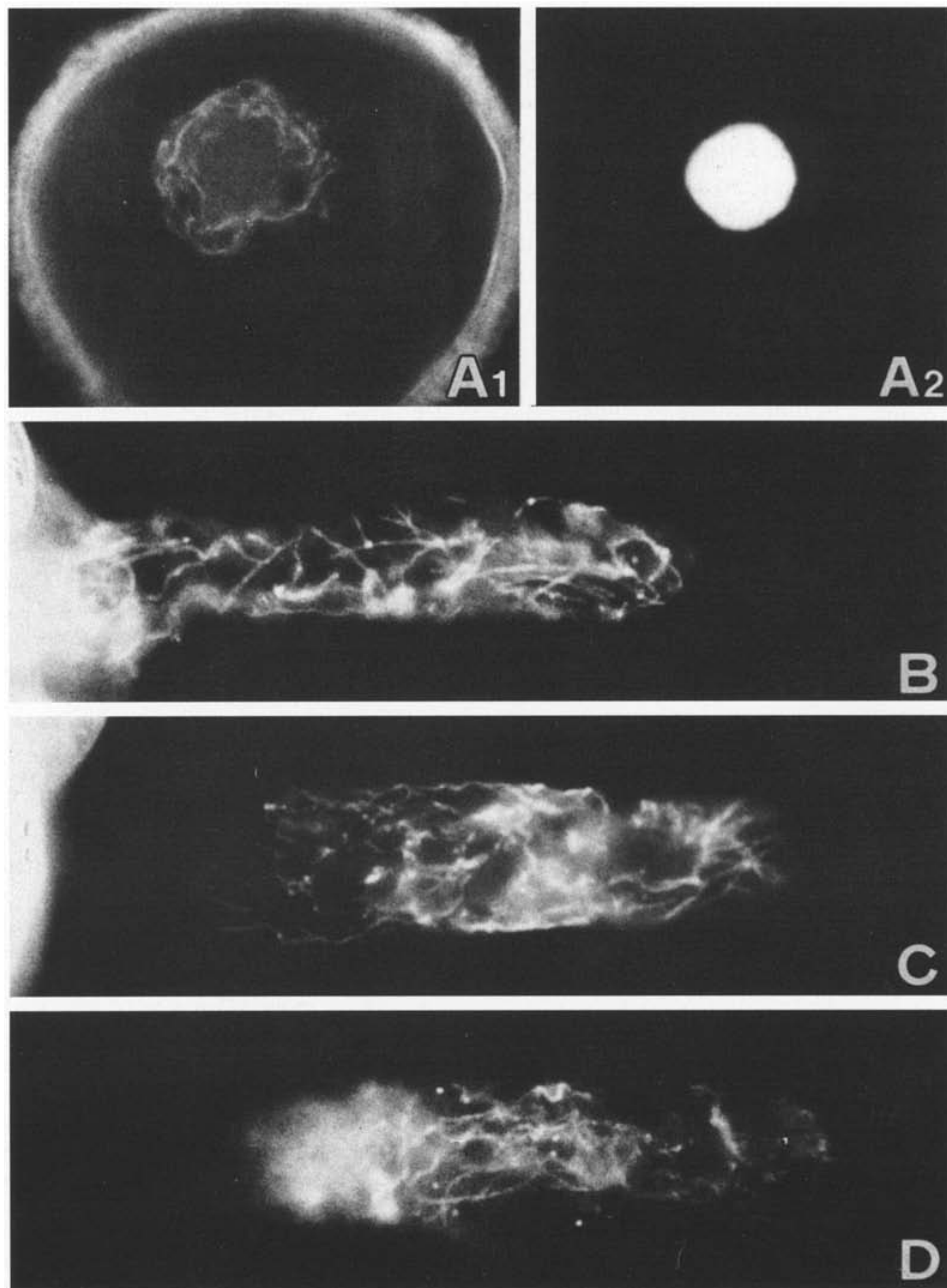
Results

Mature pollen grains of the two species were composed of two degenerated prothallial cells, a small generative cell, and a large tube cell. The pollen grains incubated in the liquid medium germinated at a rate of 94.0% within 1 day in culture. After 2–3 days, the tube nucleus had migrated into the elongating pollen tube, but the generative cell remained in the pollen grain for the duration of the culture, 1–5 days (Fig. 2A). Almost all pollen grains in the agar medium took more than 2 days to germinate. The tube nucleus migrated into the tube after 3–4 days and the generative cell migration was rarely observed after 30 days. Under these culture conditions, a few pollen tubes developed into two or more branches, and the tips of a few tubes were slightly rounded, or swollen. This branching and rounding increased with the duration of culture (Fig. 3).

Microtubules

At the germination of pollen tubes, MTs were found in both the grain and tube areas. Fig. 1A1 shows the fluorescence originating from α-tubulin of the grain area. MTs were observed to be arranged randomly around the nucleus of the generative cell and a few MTs existed in the tube cell cytoplasm. A tube nucleus was not found in the grain, since it migrated into the tube. In the germinated pollen tubes, MTs were arranged obliquely or transversely (Fig. 1B). In the elongated pollen tubes, however, MTs were rearranged along the long axis of the tube and localized in the tip region. In most pollen tubes, no MTs were observed in the basal part near the grain (Fig. 1C, D). MT fluorescence was not always found in all pollen. The mature pollen grains were incubated in the liquid medium with 0.25, 0.5, 1.0 mM colchicine or in the agar medium with 0.5 mM colchicine. After 1 day in the liquid medium, the pollen grains germinated at a rate of 96.9% and the tube nucleus migrated into the tube normally, although MTs degenerated completely in the grain and the tube. After 2 days in the culture, the tubes continued to grow, but many of their tips were abnormally rounded or divided into two or three branches (Fig. 2B). After 3 days, the tips of these branched tubes were also rounded (Fig. 2C, D). The branched and/or rounded pollen tubes increased in number with the concentration of colchicine or the duration of the culture (Fig. 3). After 10 days in culture under the agar medium with colchicine, many pollen tubes were divided into two or three branches, and after 20 days, the tips of these branches were further divided

Fig. 1A–D MT distribution in *P. densiflora* pollen. **A1** MTs in a generative cell within the grain area of the germinated pollen. **A2** DAPI fluorescence of the nucleus of the same cell shown in **A1**. A tube nucleus was not found in the grain as it migrated into the tube. **B–D** MTs localized in the tip regions of pollen tubes. In germinated tubes, MTs were arranged obliquely or transversely (**B**), but in elongated tubes, they were rearranged along the tube long axis (**C, D**). ×1250



into several branches (Fig. 2E, F). Tube tip rounding was, however, not observed in any pollen tubes within the agar medium.

Actin

In the pollen grains just after germination, F-actin and its bundles formed cage-like structures within the generative cell cytoplasm, but amorphous actin filled the tube cell cytoplasm (Fig. 4A). When pollen tubes were elongated in some degree, after 3 days, amorphous actin was reconstructed into F-actin or F-actin bundles in the whole pollen. They were arranged generally along the tube. In many pollen tubes, after 5 days, F-actin in the grain area was translocated to the tube and disappeared from the basal region, including the grain. F-actin was found in a major part of the tube, excluding the tip or apical region (Fig. 4B, C). In a few tubes, however, F-actin developed throughout the tube, including the tip

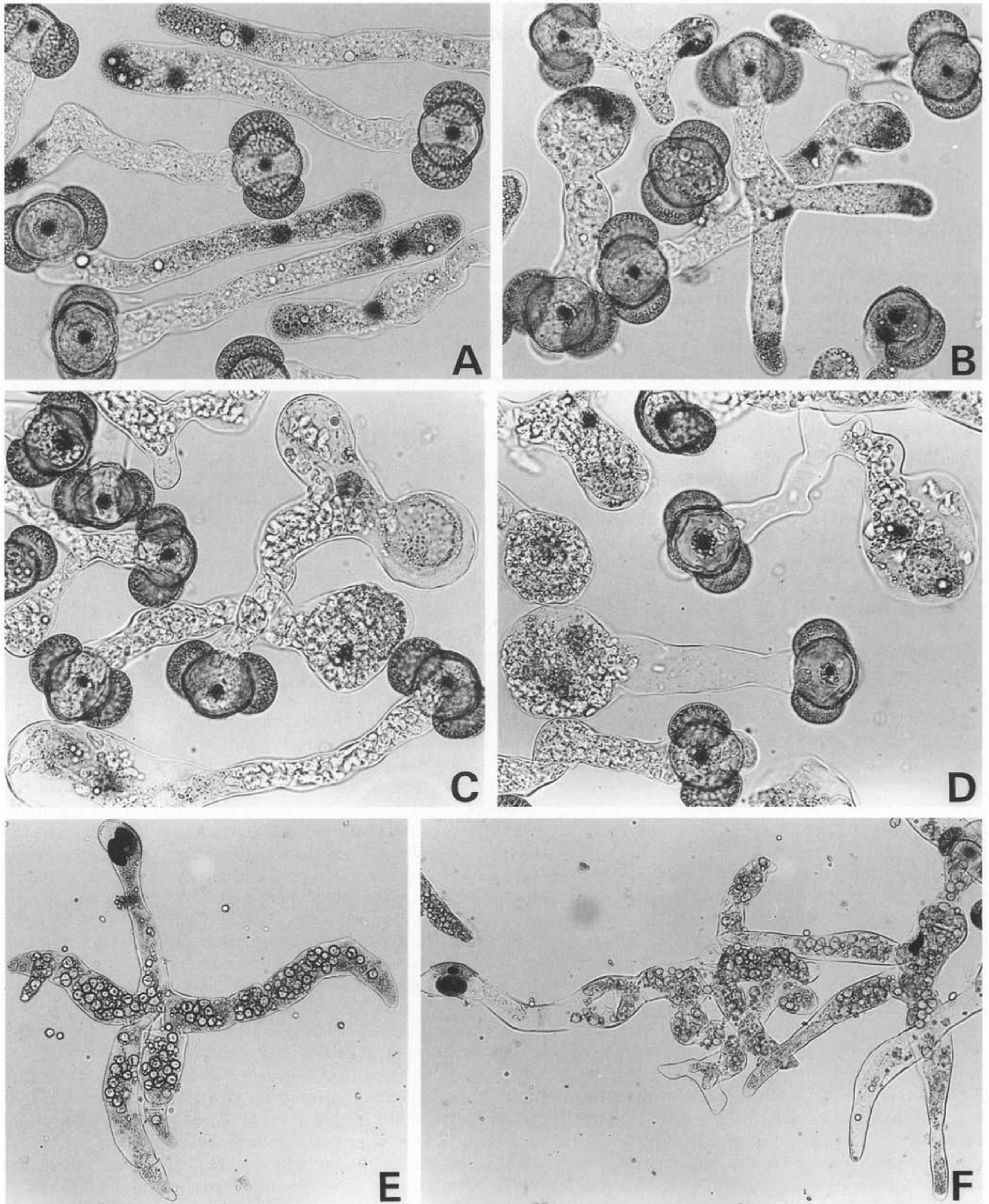


Fig. 2 Pollen of *P. densiflora* cultured in liquid medium (**A**, **B** after 3 days in the culture; **C**, **D** 5 days) and *P. thunbergii* cultured in agar medium (**E**, **F** 25 days). **A** Control. **B–F** Pollen induced by 0.5 mM colchicine treatments: **B** pollen with two or three branched tubes; **C**, **D** pollen with branched and/or rounded tubes; **E**, **F** pollen with numerous branches. **A–D** ×275, **E–F** ×135

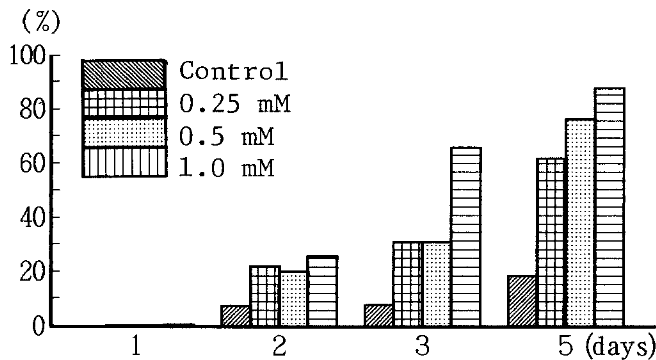


Fig. 3 The proportion of branched and/or rounded pollen tubes of *P. densiflora* induced by different concentrations of colchicine and the duration of the culture

region (Fig. 5A). The length of the tip region that did not include F-actin varied among tubes. The areas in the pollen tube containing F-actin were filled with abundant cytoplasmic granules, but in the areas without F-actin almost no granules existed (Fig. 4B). The tube nucleus, having migrated from the grain into the tube and located near the tip, was closely associated with F-actin (Fig. 4C). Pollen grains treated with 10 μg/ml cytochalasin B for 3 days were able to germinate at almost the normal rate of 95.3%, but further tube elongation was inhibited (Fig. 5B). Many tube nuclei remained in the pollen grains but some others migrated into the tubes. Almost all F-actin in treated tubes and generative cells within grains was depolymerized, but thick bundles of F-actin associated with the tube nucleus survived, even though they were treated by higher concentrations of cytochalasin B, such as 20–50 μg/ml (Fig. 5C).

Myosin

In mature pollen grains, fluorescence originating from myosin was observed to be localized on the surface of cytoplasmic granules within the tube and generative cells. At the beginning of germination, many fluorescent spots migrated into the tube and aggregated in the pollen tube apex (Fig. 6A). In elongated pollen tubes, various sized granules marked by myosin appeared in major part of the tube (Fig. 6B). Some of these granules were attached to the envelope of the tube nucleus or assembled into clumps. The surface of the tube nucleus was weakly stained (Fig. 6B, C). When almost all tube cell cytoplasm had migrated into the tube, myosin fluorescence was clearly observed on the granules included in the generative cell cytoplasm, although the generative cell surface was hardly stained (Fig. 6D). No fluorescence was detected in the pollen grains and tubes with the staining of only second antibody fluorescein conjugated without anti-myosin IgG as the first antibody (data was not shown). Mature pollen grains were cultured in a liquid medium containing 5 mM *N*-ethylmaleimide, an inhibitor of actomyosin ATPase activity, for 5 days. Germination was completely inhibited in all pollen grains treated (Fig. 6E), although in the control medium, many pollen tubes grew more than 300 μm in length.

Discussion

In the present study, the behavior and role of MTs and actin-myosin during growth of *Pinus* pollen tubes were examined and compared with those of angiosperms previously reported. In *Pinus* pollen tubes, MTs were localized in the tube tip region but not detectable in the basal part of the tube near the grain. Colchicine treatment of MTs induced pollen tube branching and rounding more frequently than non-treated tubes. Branching was induced preferentially in the agar medium and rounding in the liquid medium. It was presumed that colchicine affected pollen tubes in the liquid medium more effectively than those in the agar medium, or that the liquid and agar media offered physically different conditions for the colchicine-affected pollen tubes to grow. Branching and rounding were considered to be caused by depolarized tube tip extension due to MT disorganization with the colchicine treatment. The rounding seemed to be an extremely depolarized extension. It is well known that tip extension of angiospermous pollen tubes is promoted by the fusion of vesicles derived from dictyosomes with the apical plasma membrane (Rosen et al. 1964; Picton and Steer 1983). In colchicine-treated *Pinus* pollen tubes, presumably vesicles cannot precisely fuse with the plasma membrane at the apex but fuse elsewhere as well. Thus, depolarized growth of the tube is induced. It is suggested that MTs localized in the tip of *Pinus* pollen tubes might guide membrane materials toward the tube apex and ensure polarized tube growth.

F-actin arrays and myosin coated cytoplasmic granules including tube nuclei were found in *Pinus* pollen tubes. Under cytochalasin B and *N*-ethylmaleimide treatments, cytoplasmic streaming and tube growth were inhibited. From these results, we consider that the actin-myosin system participated in cytoplasmic streaming or in pollen tube growth of the present gymnospermous species, as well as angiospermous pollen tubes. In moss (*Physcomitrella*) caulonemal apical cells, Doonan et al. (1988) reported that the herbicide cremart, a MT-disrupting drug, caused tip swelling and cytochalasin D arrested tip growth. From these results, they concluded that F-actin is necessary for outgrowth and MTs are essential to impose tubular shape and directionality upon expansion. This interpretation of the roles of MTs and F-actin in moss caulonemal apical cells is generally in accord with our own results. In much of the *Pinus* pollen, F-actin was found throughout the tube except for the tip region, but MTs were localized in the tube tip region. The segregative distribution of F-actin and MTs in the tube indicated dual roles in the

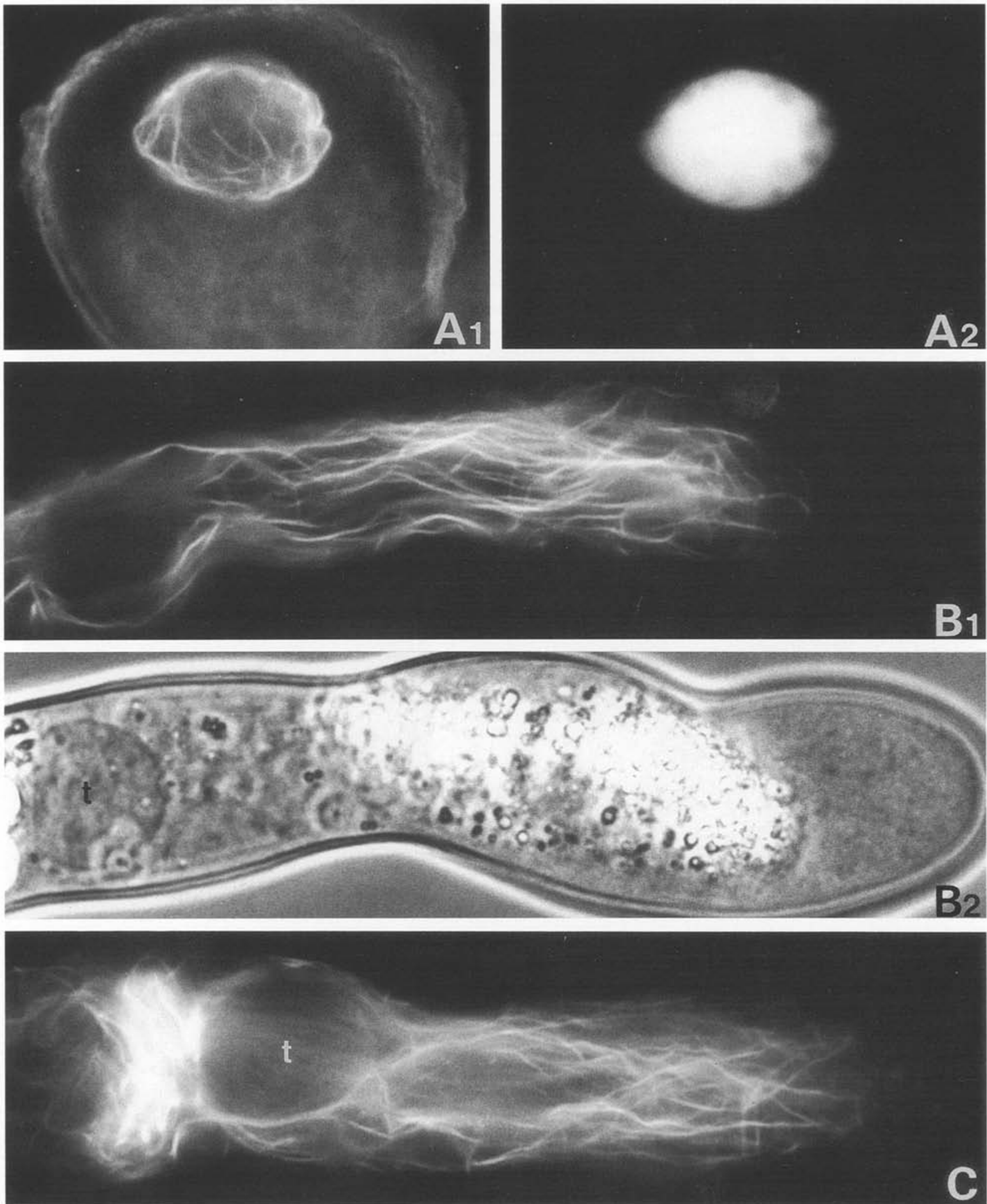


Fig. 4A–C Actin distribution in *P. densiflora* pollen. **A1** F-actin network in a generative cell within the germinated pollen grain; **A2** DAPI fluorescence of the nucleus of the same cell. **B1**, **C** F-actin distributed throughout a major part of the pollen tube, excluding the tip region; **B2** phase contrast microscopic figure of the same tube as **B1**. The F-actin distributed area in the pollen tube was filled with cytoplasmic granules, although in the apical region, which had no F-actin, almost no granules appeared. *t* Tube nucleus. ×1500

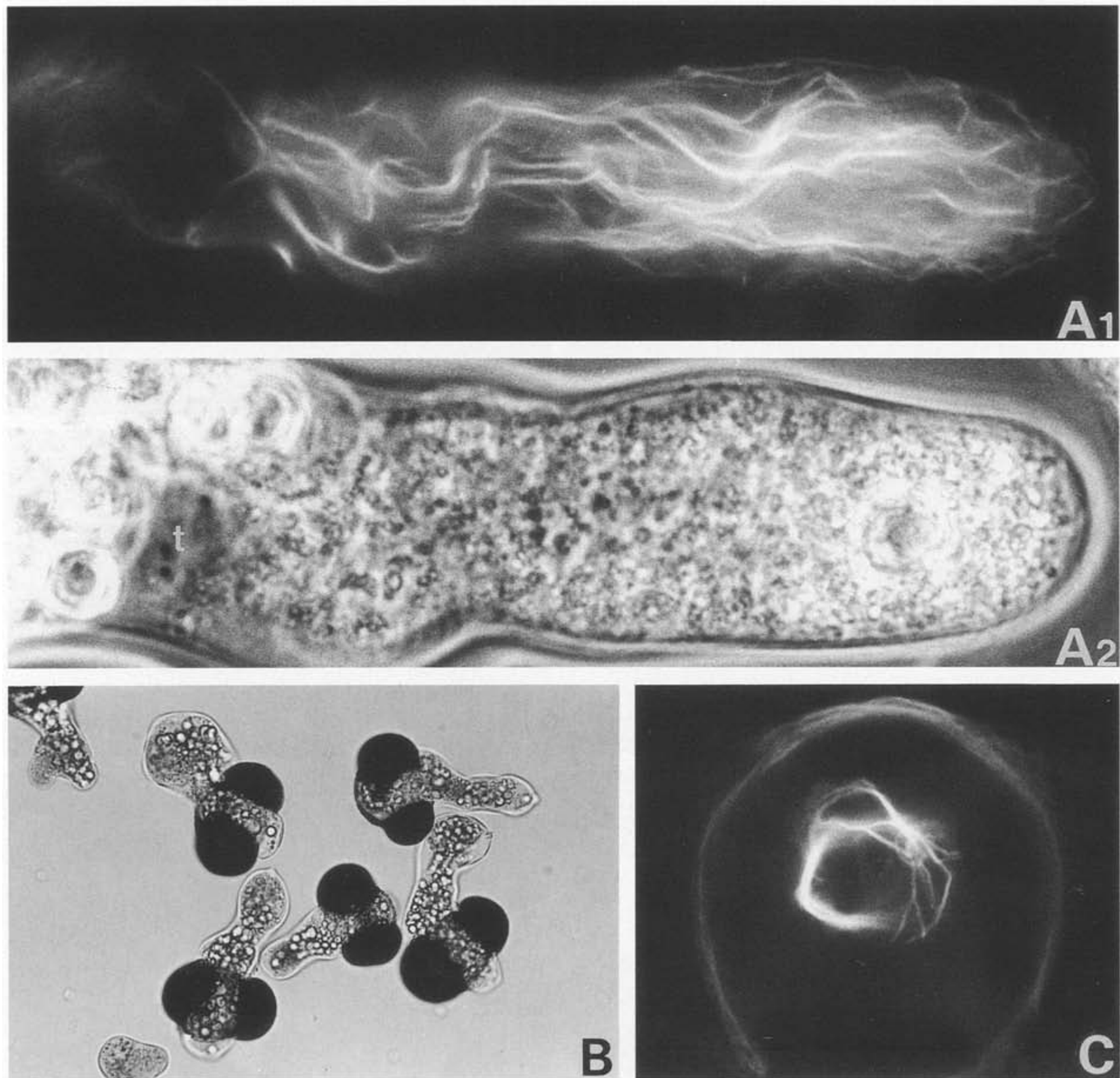


Fig. 5A–C Actin distribution in *P. densiflora* pollen. **A1** F-actin distributed throughout the tube; **A2** Phase contrast microscopic figure of the same tube: cytoplasmic granules filled the entire tube. **B** Pollen treated with 10 μg/ml cytochalasin B for 5 days. Compare with Fig. 2A. **C** F-actin bundles around the tube nucleus which remained in the grain survived the 20 μg/ml cytochalasin B treatment for 5 days. **A** ×1500, **B** ×250, **C** ×1250

transport of tube cell contents. In some angiosperms, it has been reported that MTs and F-actin were co-distributed through the pollen tube, suggesting a functional association between them (Lancelle et al. 1987; Pierson et al. 1986, 1989; Tiezzi et al. 1987; Tiwari and Polito 1988). However, no definitive evidence of their functional association was offered.

Generative cells of the present species generally remained in the pollen grain during the tube growth. Both F-actin and MT networks developed in the generative cells and myosin was localized on the cytoplasmic granules, but not on the cell surface. From these observations, the actin-myosin system was considered to mediate granular movement within generative cells. On the contrary, it was reported that F-actin was absent in angiospermous generative cells which migrated from the grain into the tube after dozens of minutes in the culture (Lancelle et al. 1987; Palevitz and Cresti 1989; Palevitz and Liu 1992; Terasaka and Niitsu 1992) and myosin was localized upon those cell surfaces, but not in the cytoplasm. That is, it was indicated that for angiospermous generative cells, the vegetative actin-myosin system contributed to cell migration. The presence or absence of myosin on the generative cell surfaces of angiospermous plants and *Pinus* correlated well with the occurrence or absence, respectively, of cell migrations from the grain to the tube.

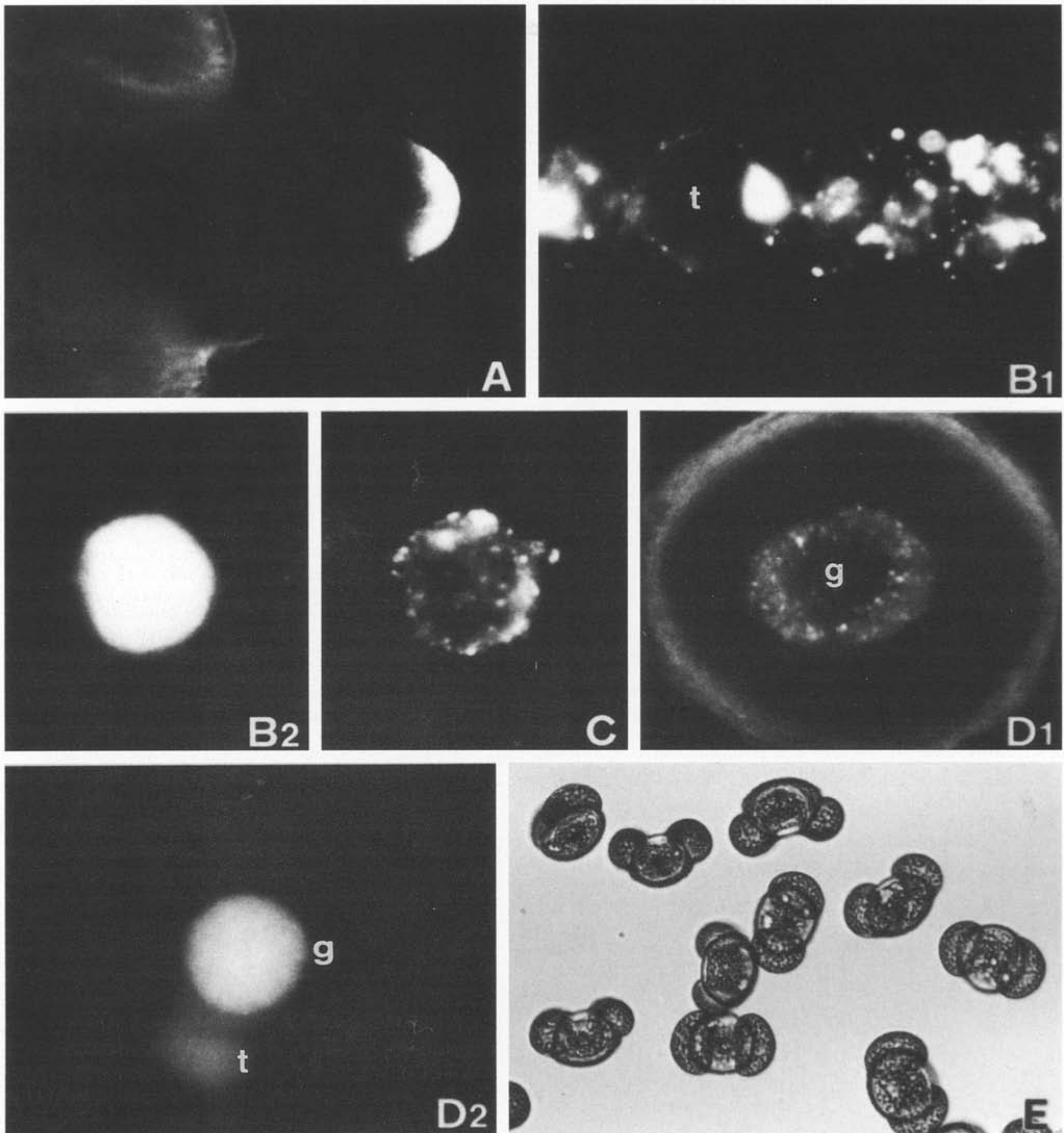


Fig. 6A–E Myosin distribution in *P. densiflora* pollen. **A** Pollen tube just after germination. Many fluorescent spots were aggregated in the tube apex. **B1** Elongated pollen tube: Myosin was distributed densely on granules and lightly on the tube nucleus (*t*), and in some places granules had assembled into clumps; **B2** DAPI fluorescence of the tube nucleus in the same tube. **C** The tube nucleus in the tube. A lot of granules attached to the nuclear envelope. **D1** Generative cell within a pollen grain. The camera was focused on the inside of the cell. Myosin was observed distinctly on cytoplasmic granules but hardly on the cell surface. **D2** DAPI fluorescence of the generative (g) and tube (t) nuclei in the same pollen grain shown in **D1**. **D1** and **D2** are in same focus. **E** Pollen grains inhibited from germinating by the 5 mM *N*-ethylmaleimide treatment for 5 days. **A–D**, ×1250, **E** ×250

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