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Development and cellular organization of *Pinus sylvestris* pollen tubes

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Abstract The organization of *Pinus sylvestris* pollen tubes during growth was studied by video microscopy of living cells and by electron microscopy after freeze-fixation and freeze-substitution (FF-FS). Pollen germinated and the tubes grew slowly for a total period of about 7 days. Some of the grains formed two tubes, while 10–50% of the tubes ramified. These features are in accordance with development in vivo. The cytoplasmic hyaline cap at the tip disappeared during the 2nd or 3rd day of culture. Aggregates of starch grains progressively migrated from the grain into the tube and later into the branches. Vacuoles first appeared at day 2 and eventually filled large parts of the tube. The tube nucleus was located at variable distances from the tip. Some of the organelles showed linear movements in a mostly circulatory pattern, but the majority of the organelles showed brownian-like movements. Rhodamine-phalloidin-stained actin filaments had a gross axial orientation and were found throughout the tube including at the tip. The ultrastructure of pollen tubes was well preserved after FF-FS, but signs of shrinkage were visible. The secretory vesicles in growing tips were not organized in a vesicle cone, and coated pits had a low density with only local accumulations, which is in accordance with slow growth. The mitochondria contained small cristae and a darkly stained matrix and were located more towards the periphery of the tube, indicating low respiratory activity and low oxygen levels. The dictyosomes carried typical trans-Golgi networks, but some contained less than the normal number of cisternae. Other elements of the cytoplasm were irregularly spaced rough endoplasmic reticulum, many multivesicular bodies, lipid droplets and two types of vacuoles. The typical organization associated with tip growth in angiosperm pollen tubes, e.g. *Nicotiana tabacum*, was not present in *P. sylvestris* pollen tubes. The different morphology may relate to the growth rate and not to the type of growth.

Key words Cytoskeleton · Microscopy · *Pinus sylvestris* · Pollen · Ultrastructure

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Introduction

Pollen tubes of gymnosperms exhibit some evolutionarily primitive characteristics, i.e. slow germination and growth, branching and longevity (Singh 1978). In *Pinus sylvestris* L., the pollen tubes penetrate the nucellus intercellularly and anchor themselves in the nucellar tissue by ramification (Willemse 1968; Willemse and Linskens 1969). Growth stops after some weeks. The antheridial cell hibernates in the pollen grain, moves into the tube after resumption of growth the next spring and divides just before fertilization (Chamberlain 1966). Early works by Tanaka (1955, 1956) report on the effects of physiological conditions on the pollen tube morphology of *P. densiflora*. The ultrastructure of developing microspores and germinating pollen of *P. sylvestris* has been studied by Willemse (1971a,b,c,d). Recently, Terasaka and Niitsu (1994) observed microtubules (MT) and actin filaments (AF) along the axis of the tube in *P. densiflora* and *P. thunbergii* and found MT in the very tip of the tube; however, AF have been reported to be absent from this zone.

The characteristics of tip growth in angiosperm pollen have been studied extensively (see reviews Heath 1990; Derksen et al. 1995b). For a long time, tip growth in angiosperm pollen tubes has been believed to be indissolubly connected to a particular type of cytoplasmic organization, i.e. specific accumulation of secretory vesicles (SV) in the tip, apical zonation of endoplasmic reticulum, dictyosomes and coated pits and no vacuoles between tip and nuclei (Joos et al. 1994; Derksen et al. 1995a). Further, the integrity of the cytoskeleton, with an axial orientation of MT and AF, seems to be needed for growth (Pierson and Cresti 1992; Derksen et al. 1995b).

Recently, the prerequisite of an intact tip zonation and microtubular network for tip expansion has been questioned (Joos et al. 1994).

Slow growth, branching and longevity of gymnosperm pollen tubes may be reflected in another type of cytoplasmic organization than that known from angiosperm pollen tubes. The present study examines the cytoplasmic organization of (living) pollen tubes of *P. sylvestris* grown in vitro and their ultrastructure after freeze-fixation and freeze-substitution (FF-FS). It also provides morphometric data on cells and organelles.

Materials and methods

Plant material

Cones with ripe pollen were collected from trees of *P. sylvestris* L. in the park of the University of Nijmegen prior to the beginning of the pollination season (Driessen et al. 1988), and stored in a desiccator at room temperature.

Pollen were germinated in a suspension culture (30 mg pollen per 2 ml medium containing 10% sucrose and 0.01% boric acid) in a shaker at 100 rpm (Kroh and Knuiman 1982). Alternatively, they were sown on 3-mm punches of Visking dialysis tubing placed on a 2% agar surface supplied with medium. Germination took place in the dark at 28°C. Differences were not observed in germination and growth between pollen in suspension culture or on solid surfaces, or between pollen grown on water or other media (Linskens 1967) (data not shown).

Real time observations

Observations of living pollen tubes were made with bright-field, phase-contrast or differential interference contrast (DIC) illumination on Leitz microscopes equipped with a monochromatic green light filter. Samples were additionally protected from heat by a 0.1% $CuSO_4$ solution placed between the light source and condenser.

Real-time images were recorded on S-VHS tape with a CCD video camera (SSC-M370 CE, Sony Europe, The Netherlands) in combination with a Panasonic AG-7355 video recorder (Matsushita Electric, Japan). Detailed analyses of the trajectories of 90 organelles in three different cells were made using a computer-based image analysis system (VIDAS version 2.1, Kontron, Germany). Recordings of the organelles were displayed through the frame grabber of the VIDAS, and 36 single-frame images (320×256 pixels) were collected at intervals of 0.24 s during 8.4 s. The trajectory of each discernable organelle was stored as a series of x- and y-coordinates of the centre of the particle, indicated interactively with the cursor. The trajectories were printed using SAS-GRAPH procedures, version 6.07 (SAS Institute, USA).

Fluorescence staining and microscopy

AF were stained with 0.3 µM rhodamine-phalloidin (Sigma and Molecular Probes) according to Traas et al. (1987), but with 1% instead of 5% dimethyl sulphoxide (DMSO). Shrinkage of the pollen tubes was prevented by adding 8% sucrose. Nuclei were stained with 5 µg/ml 4,6-diamino-2-phenylindole (DAPI). Mitochondria were stained with 2.5 µg/ml of the vital dye 3,3′-dihexylcarbocyanin iodide [$DiOC_6(3)$; Eastman, USA]. Images were made either with a Leitz fluorescence microscope or with a Bio-Rad MRC-600 confocal laser scanning microscope (CLSM) equipped with an argon laser.

Electron microscopy

Pollen tubes were fixed in 2% glutaraldehyde in 50 µM phosphate buffer, pH 6.8, for 2 h at room temperature, post-fixed with 1–2% OsO_4 in distilled water and dehydrated in a water-ethanol series. FF and FS were carried out as described by Derksen et al. (1995a).

Specimens from chemically fixed or FF-FS preparations were flat-embedded in Spurr's resin on Cylon CT-coated slides. Single and serial, longitudinal or oblique sections (100 nm) were made on a Sorvall MT 5000 microtome and collected on Formvar- and carbon-coated single slot grids. The preparations were post-stained with lead citrate for 1–3 min and examined in a Jeol 100 CX II electron microscope. Tubes without signs of ice crystal formation were analysed. Morphometric measurements were carried out on at least 20 different organelles from ten different pollen tubes.

Results

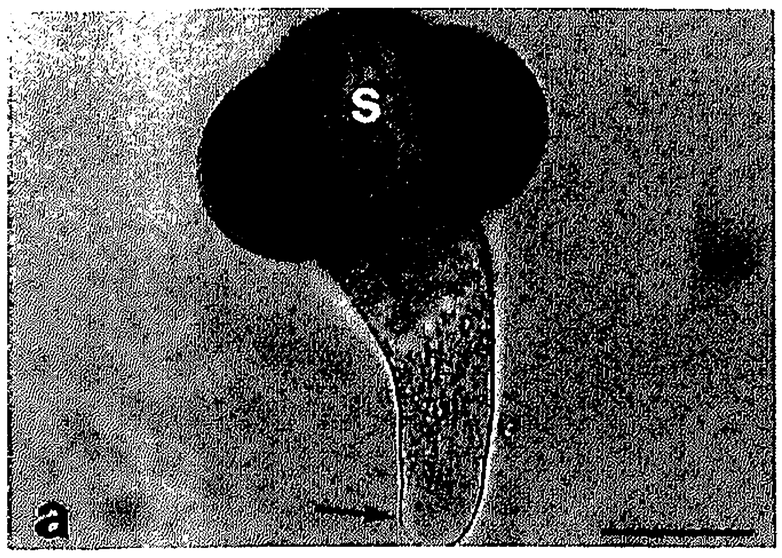
Germination, growth and ramification

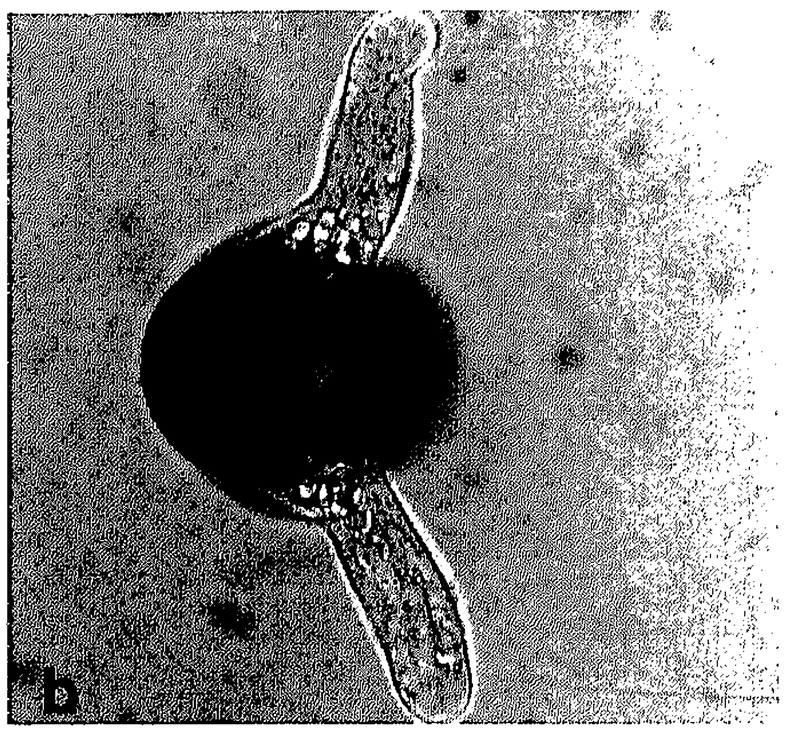
The germination percentage of collected *P. sylvestris* pollen was 90–95% but dramatically dropped to zero after 8 weeks of storage. Pollen germination started approximately 16 h after the onset of culture. A cylindrical tube was formed (Table 1, Fig. 1a) which grew at a rate

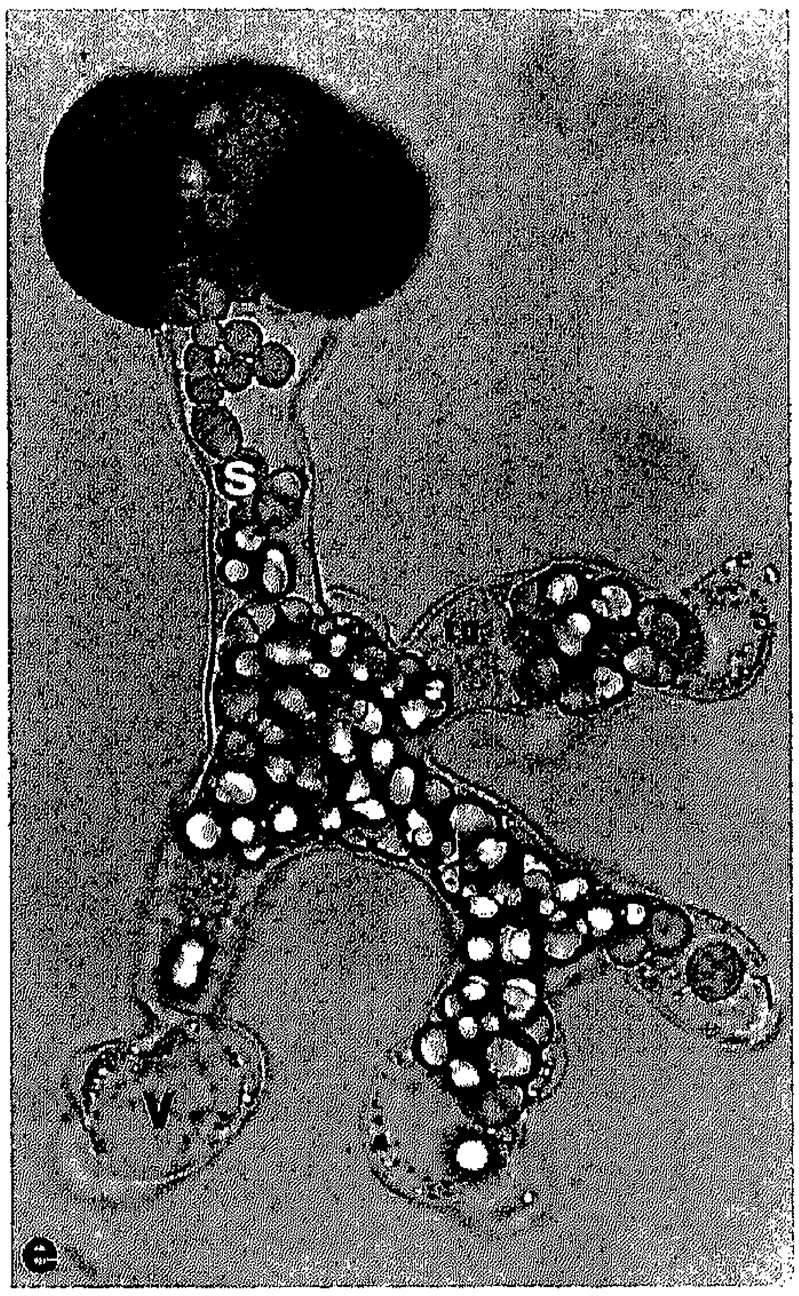
Table 1 Morphometric characteristics of pollen tubes of *Pinus sylvestris* grown in vitro

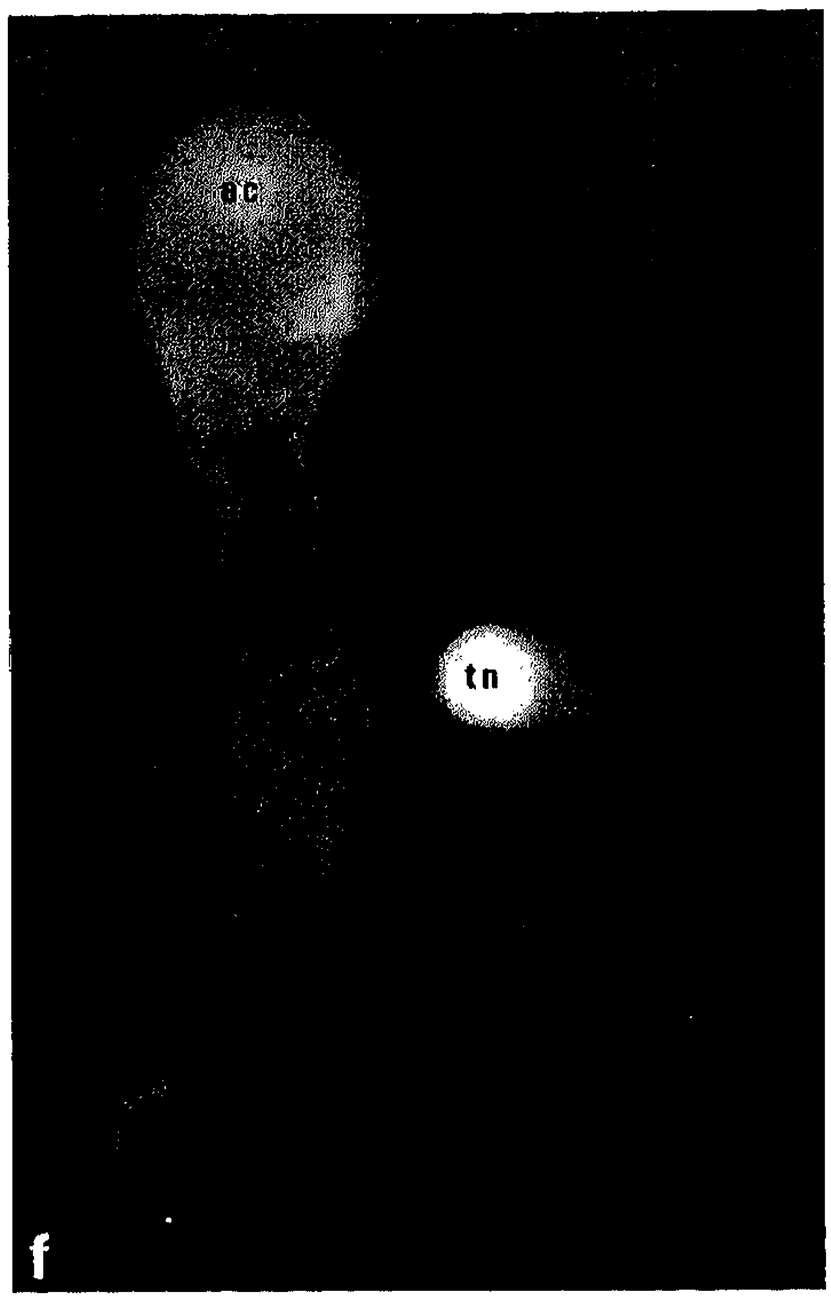
Trait	Type of measurement (µm)	Number of days in culture				
		1 day	2 days	3 days	4 days	5–7 days
Length of unbranched tube	Average (n)	17 (23)	48 (33)	66 (19)		
	Maximum	32	116	127		
Length of ramified tube	Average (n)	–[a]	82 (4)	111 (19)	152 (11)	179 (18)
	Maximum	–[a]	102	196	214	268
Distance from grain to first branch	Average (n)	–[a]	50 (4)	47 (20)	64 (11)	56 (17)
Length of final branch	Average (n)	–[a]	19 (4)	30 (23)	42 (55)	42 (90)
	Maximum	–[a]	29	71	89	177
Diameter of tube/branch	Range	14–21	13–23	11–25	9–27	9–75[b]

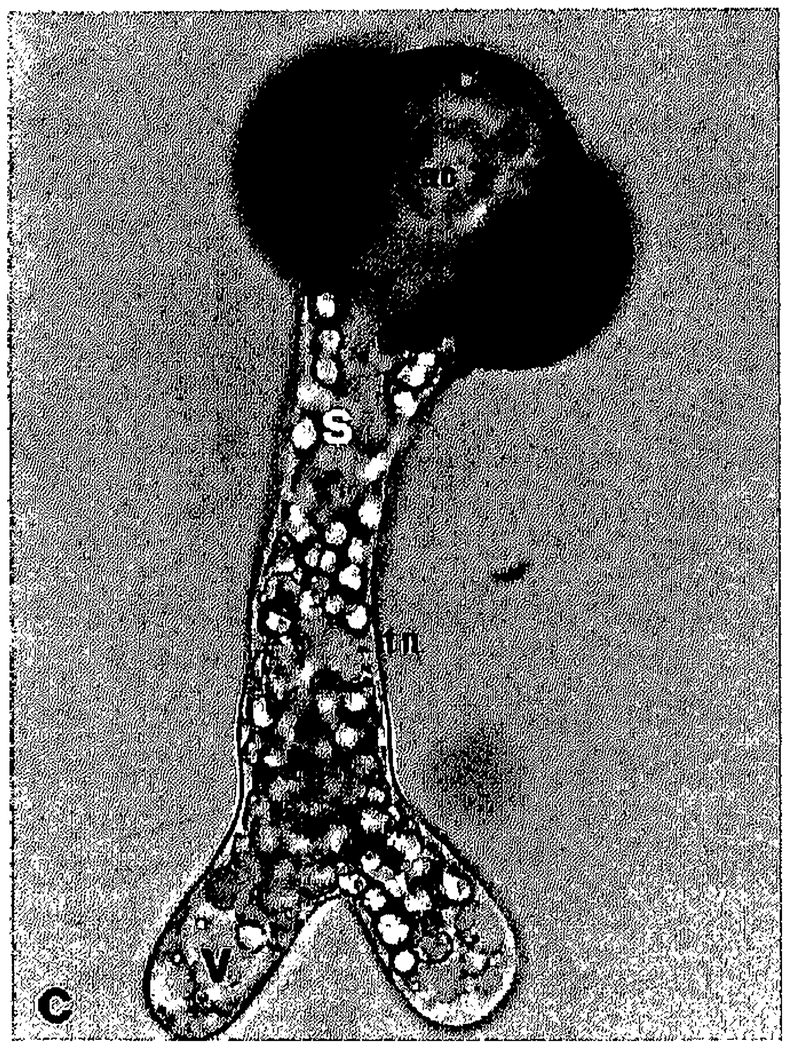
[a] Trait not relevant
[b] Extreme maximal value due to extensive swelling of tips

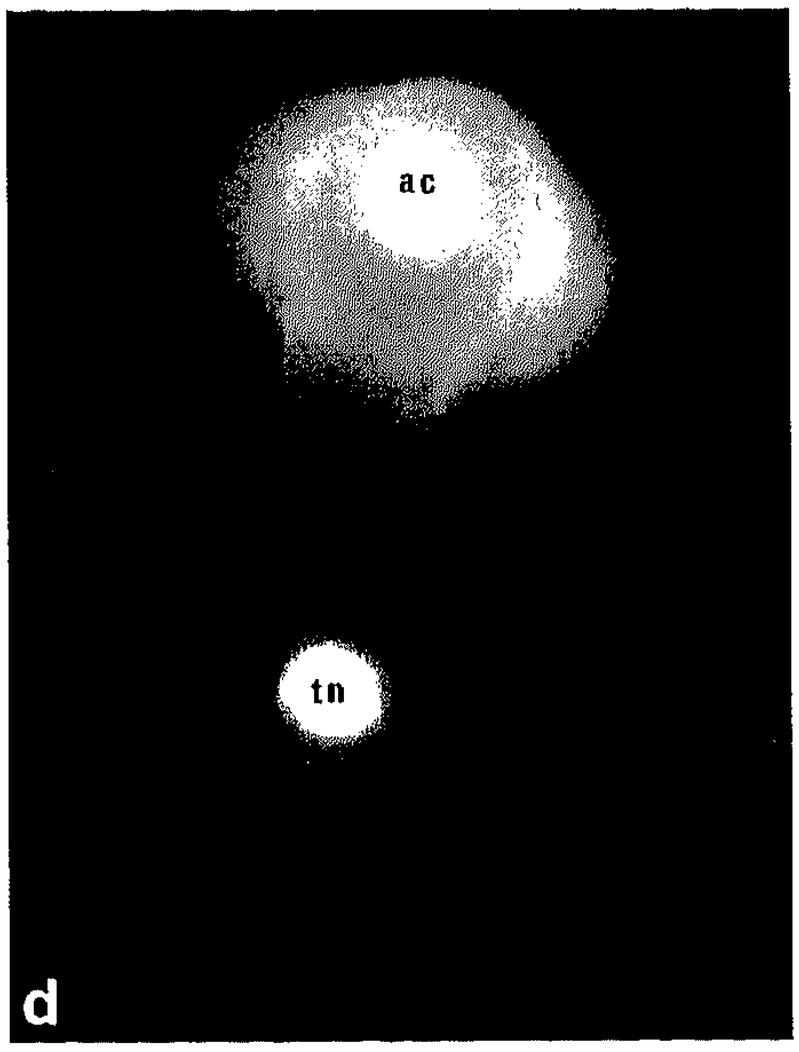












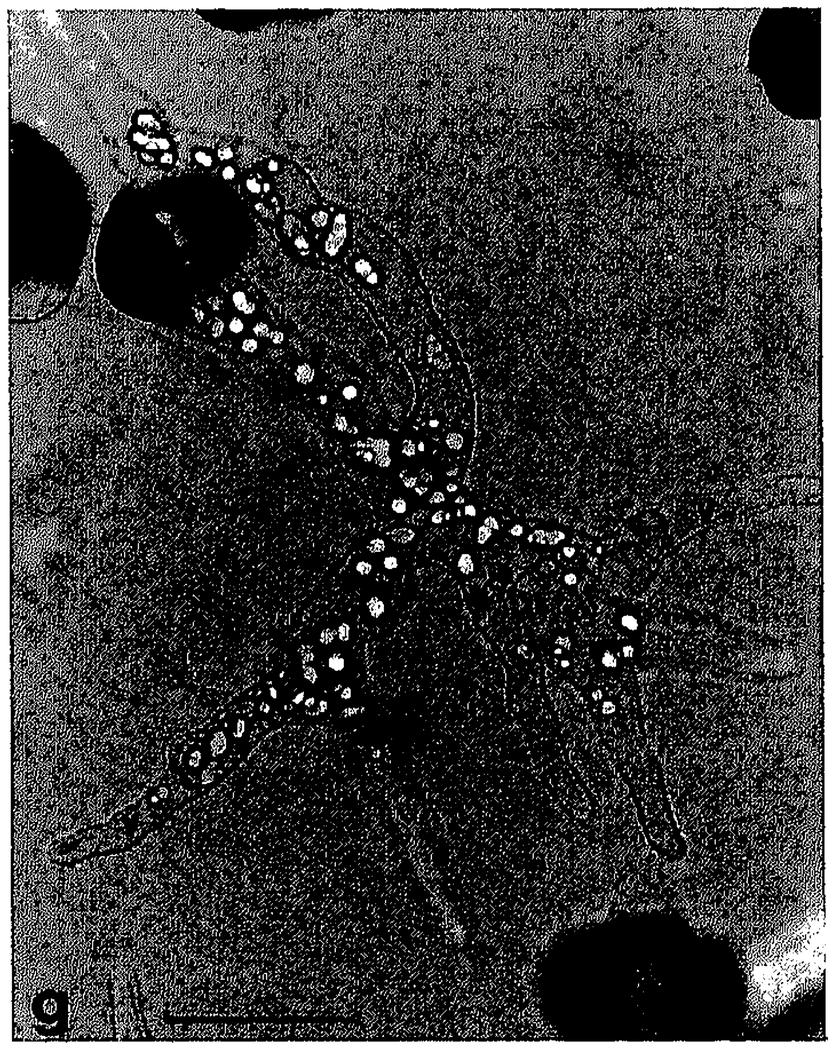


Fig. 1a–g Light microscopy of the development and morphology of *Pinus sylvestris* pollen grains and pollen tubes grown in vitro. **a** One-day-old culture. Starch grains (*s*) are located in the pollen grain. The tip of the unbranched tube shows a hyaline cap (*arrow*) without discernable vacuoles. **b** Two-day-old culture. Pollen grain with two tubes. **c,d** Two-day-old culture. Grain and bifurcated tube, imaged in (**c**) bright-field and (**d**) fluorescence microscopy after DAPI staining, show numerous starch grains (*s*) behind the tips, small vacuoles (*v*), the antheridial cell (*ac*) in the grain and the tube nucleus (*tn*) among an aggregate of starch grains. The hyaline cap has disappeared. **e,f** Four-day-old culture. Tube branched by trifurcation and subsequent bifurcation imaged in (**e**) bright-field and (**f**) fluorescence microscopy after DAPI staining. The micrograph shows vacuoles (*v*) in the apex, numerous starch grains (*s*) in all branches, the tube nucleus (*tn*) in one of the first branches and the antheridial cell (*ac*) in the grain. **g** Seven-day-old culture. Extensively ramified tube with highly vacuolized (*v*) cytoplasm. The starch grains are no longer organized in aggregates. *Bar* **a–f** 25 μm, **g** 50 μm

of about 1 μm/h. Depending on the lot, about 10–25% of the pollen grains developed a second tube, which grew independently of the first tube (Fig. 1b).

During the 2nd or 3rd day the shape of the pollen tubes became irregular, and some tubes started branching (Table 1, Fig. 1c,e,g). This process continued until about day 6. The proportion of branching tubes and the extent of branching varied between lots and ranged, at day 4, from more than 50% to less than 10%. Cells generally developed four to six branches (Fig. 1g), but tubes with up to 16 branches were observed. Most branches were formed by bifurcation (Figs. 1c,g, 2a) or, in a few cases, by trifurcation of the tip (Fig. 1e,g). The branches were initially isomorphic, but gradually became unequal in length (Table 1) and organelle content. Small (<20 μm)

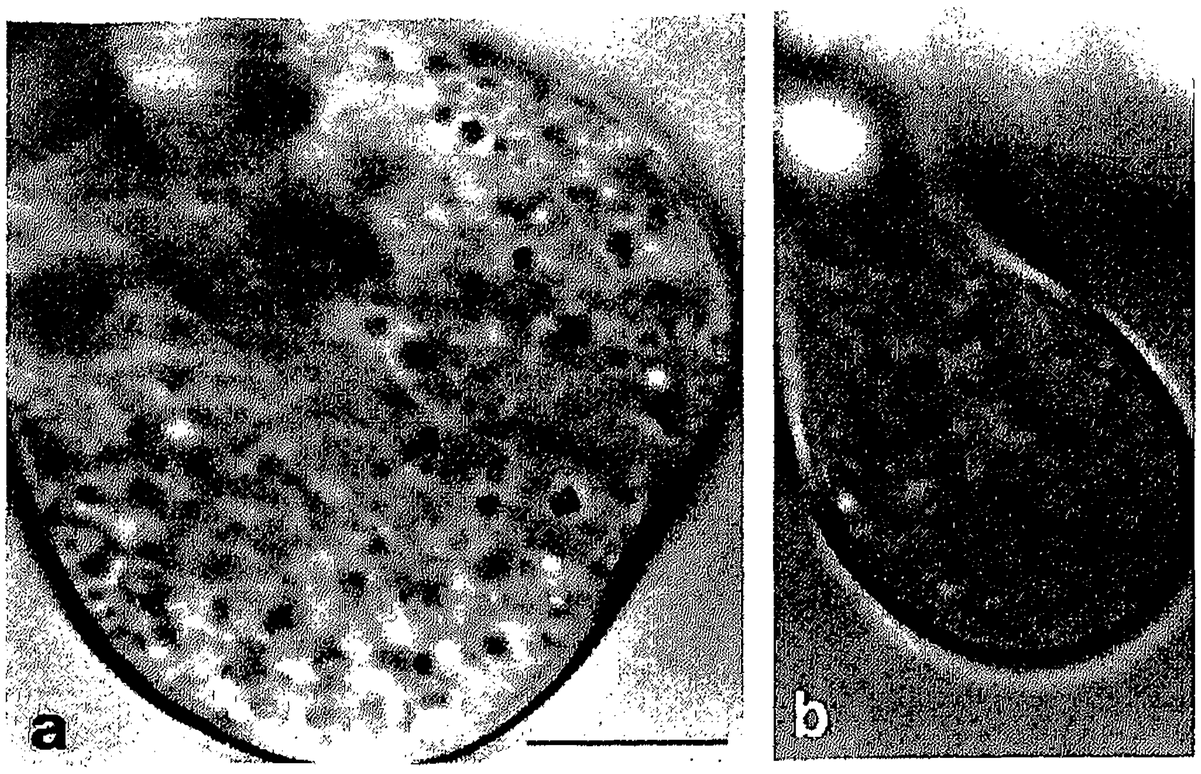


Fig. 2a,b Video microscopy of pollen tube tips in a four-day-old culture. **a** Detailed image of a tip in the process of bifurcation. **b** A small lateral branch containing numerous organelles, but no starch grains or vacuoles. *Bar* 25 µm

lateral branches could also form at various distances behind the tube tip (Fig. 2b). At day 6 and 7 a number of tubes showed swollen apices (Table 1). Growth was never seen in 7- to 10-day-old cells, although they still showed an intact structure and slow organelle movement. Growth could not be sustained or resumed by changes in the medium.

General cellular organization of pollen tubes

The cytoplasm of young unbranched tubes showed a hyaline cap of variable size at the tip (Fig. 1a; see also Fig. 5a) and contained numerous starch grains and other, unidentifiable, organelles. The hyaline cap gradually disappeared and was no longer observed in 4-day-old tubes (Fig. 1c,e), although some of the tubes were still growing.

During growth, aggregates of starch grains progressively migrated from the pollen grain into the tube and later into the branches (Fig. 1c,e). The distance between the tip(s) of the tube and these aggregates was variable. Tubes or branches with starch grains in the extreme tip had ceased growing. After 6 days of culture, the pollen grain and the basal part of the main tube were devoid of starch grains in a third of the cell population. The starch grains disaggregated in some tubes (Fig. 1g).

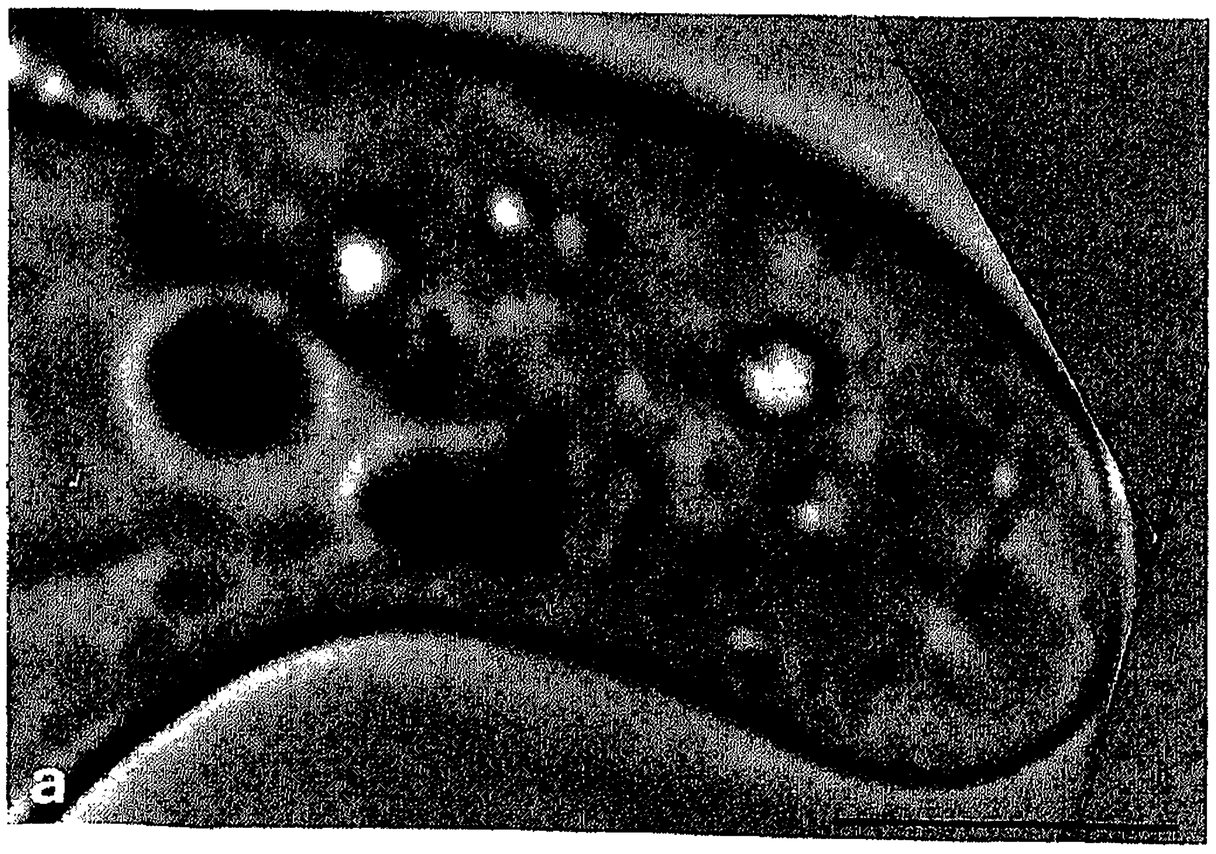
Small vacuoles, 2.7–7.0 µm in diameter, appeared in the cytoplasm of 2-day-old tubes (Fig. 1c). No correlation was seen between the presence of vacuoles and the occurrence of a hyaline cap. The vacuoles became larger as growth proceeded (Fig. 1e) and were also present in tubes during the process of branching. Except for the small branches (Fig. 2b) and the area containing the starch grains, 6- and 7-day-old tubes were highly vacuolized.

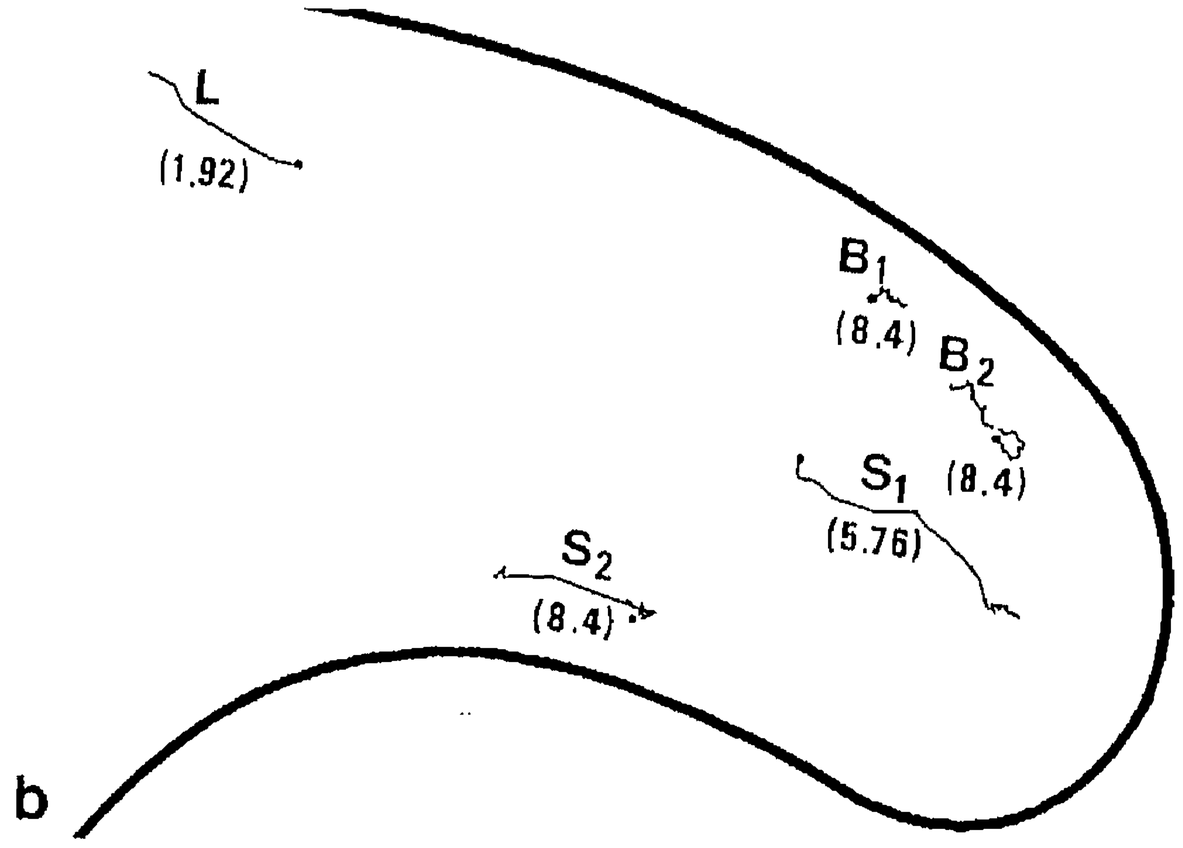
The tube nucleus (TN) could be stained with DAPI and sometimes appeared as a clear area (average diameter 9 µm) in the middle of an aggregate of starch grains (Fig. 1c–f). The TN was located at a variable position in the main tube or in one of the branches (Fig. 1d,f). The antheridial cell (AC) remained located in the grain (Figs. 1d,f, 5b).

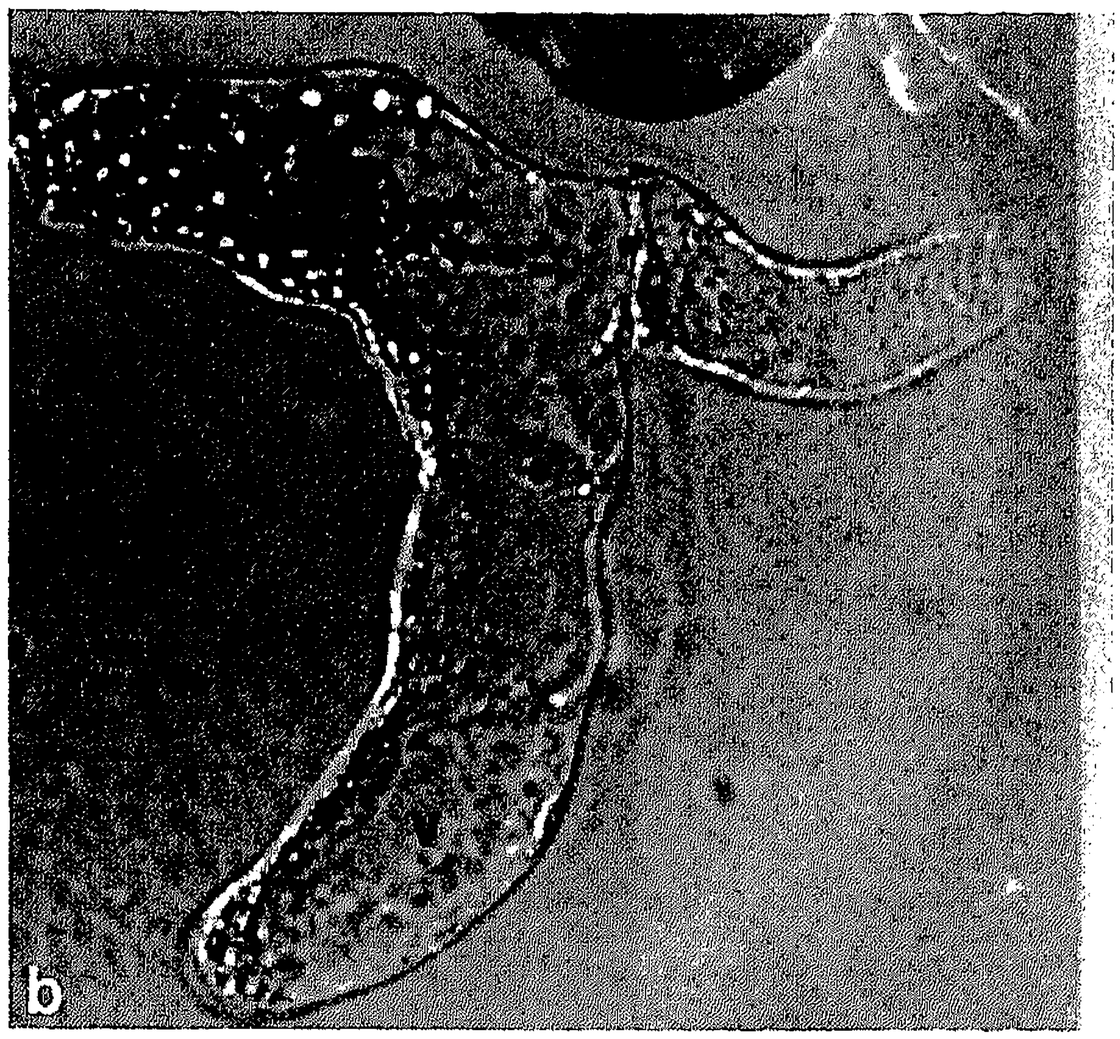
Organelle movement

Brownian-like movements of all organelles, except starch grains, were common in all pollen tubes. A subset of the organelles transiently showed linear movements (Fig. 3a,b), but this was not observed in all cells. From accelerated-play modes of video recordings, three patterns of organelle movement could be distinguished: in 1- and 2-day-old tubes, most frequently, a fountain-like pattern and occasionally, a reverse fountain-like pattern; in older and branched tubes, a circulatory pattern. Organ-

Fig. 3a, b Types of organelle movements in a branched pollen tube after 3 days of culture. **a** A video microscopic image showing the organelles in the apex of one branch and **b** illustration of the corresponding trajectory of selected organelles. The starting point of each trajectory is marked by a dot with the numbers in brackets indicating the duration of single tracings in seconds. Types of movement shown: *L* linear movement, *B1* brownian-like movement confined to one spot, *B2* brownian-like movement with a slow progression, *S1* saltatory movement with linear movement followed by brownian-like movement, *S2* saltatory movement with brownian-like movement followed by linear movement. *Bar* 10 µm







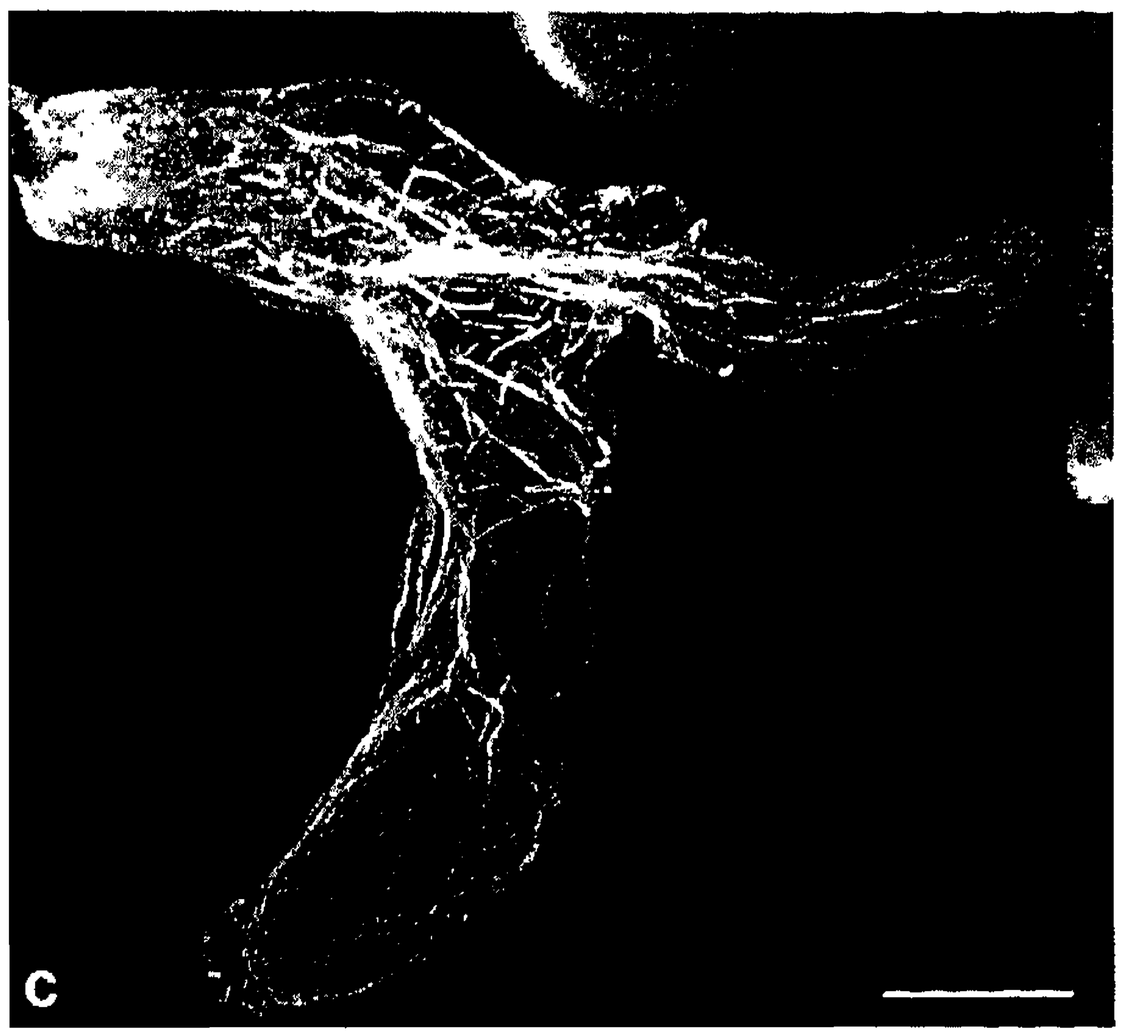


Fig. 4a–c Confocal laser scanning microscopy of AF stained with rhodamine-phalloidin. **a** One-day-old pollen tube. Maximum projection of five optical median sections taken at 1 µm *z*-intervals showing AF in the entire pollen tube including the extreme tip. **b,c** Three-day-old branched pollen tube. **b** Non-confocal transmission microscopy image (*v* vacuole). The pollen grain is located exactly left of the image. **c** Projection of 30 optical sections showing the extensive actin network in the whole cell. *Bar* 25 µm

elles located in the vicinity of the starch grains rarely showed linear movement. In highly vacuolated cells the organelle movement was mostly brownian-like, although extensive linear movements were also seen in some transvacuolar strands and cortical areas.

Changes in shape of small- and middle-sized vacuoles were rather frequent, and transvacuolar strands could completely relocate within 2 min. Conversely, motion of the TN and the AC could not be detected within 10 min.

Cytoskeleton

AF stained with rhodamine-phalloidin appeared as longitudinal arrays in both the peripheral and the central part of the tube (Fig. 4a,c). With the permeabilization method used, AF were also evident in the very tip of the tube or the branches (Fig. 4a,c). Bundles of microfilaments (MF) and bundles of MT with a more or less axial orientation were visible by electron microscopy (Fig. 5g). Few MT and MF were found in glancing and oblique sections in the cortex. MF could co-localize with MT (Fig. 5g). Associations, but no physical connections, were seen between cytoskeletal elements and organelles (not shown).

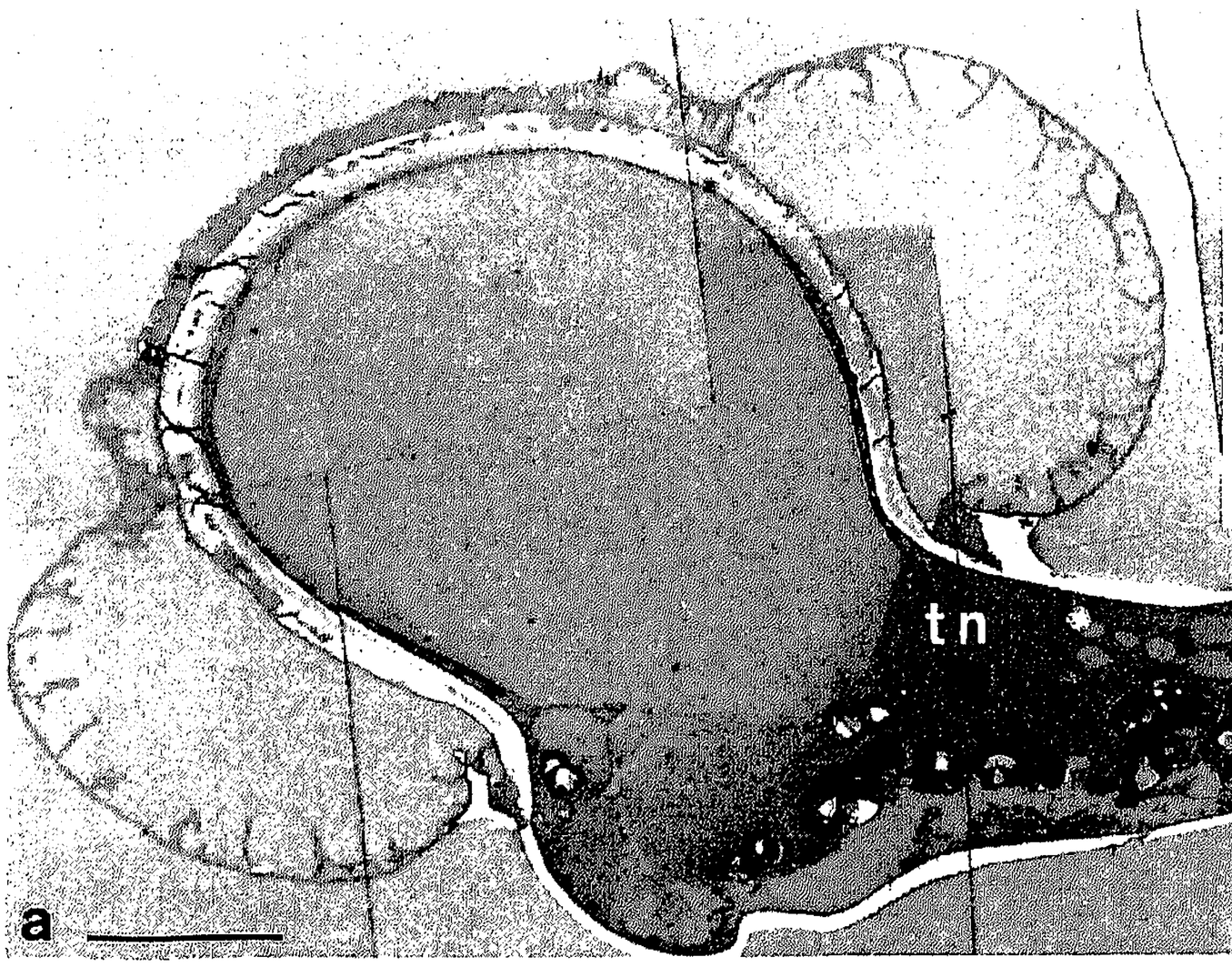
Ultrastructure and organelle distribution

FF-FS resulted in good preservation of the ultrastructure, especially cytoskeletal elements, coated pits (CP), coated vesicles (CV) and intercisternal filaments of the dictyosomes, which were not observed after chemical fixation. The plasma membranes were straight and smooth after FF-FS (Fig. 5f), while they were shrivelled and showed numerous invaginations and vesicles after glutaraldehyde fixation (Fig. 5i). Both cell wall and SV had low electron density after chemical fixation, indicating extraction. Shrinkage of *P. sylvestris* pollen tubes occurred during FF-FS, as was observed by gaps and cracks in the cytoplasm (Fig. 5a).

The growing tips of non-ramified (Fig. 5a) and ramified pollen tubes were mostly similar, although no quantitative evaluation could be established due to large variations in size and morphology. Organelles in the AC (Fig. 5b) were ultrastructurally equivalent to those in the tube, except for the absence of vesicles budding from the dictyosomes.

The cell wall thickness ranged from 0.4 µm in the tip to about 0.65 µm near the grain. In chemically fixed tubes, a clear layering of the wall was found (Fig. 5i), and in oblique FF-FS sections, layers with differently oriented fibres were also seen (not shown).

Large electron-translucent spaces were present between the organelles. The mitochondria were oval-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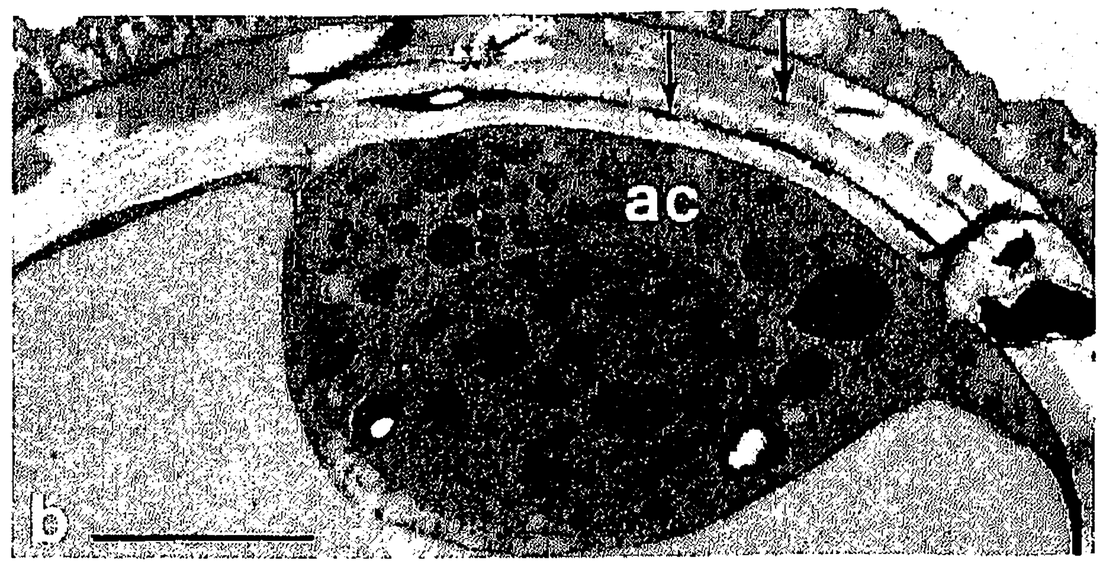
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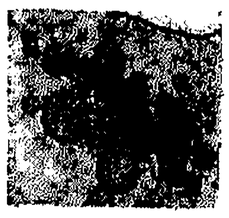

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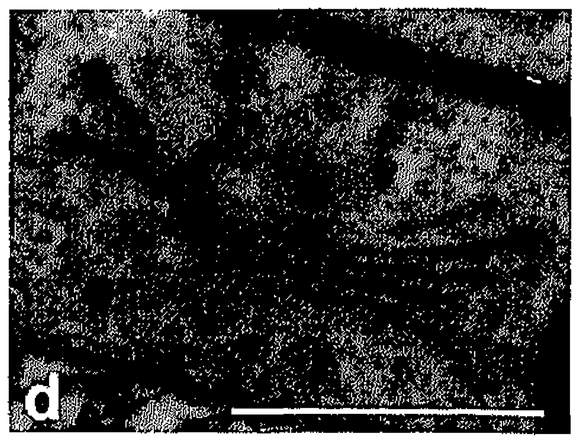
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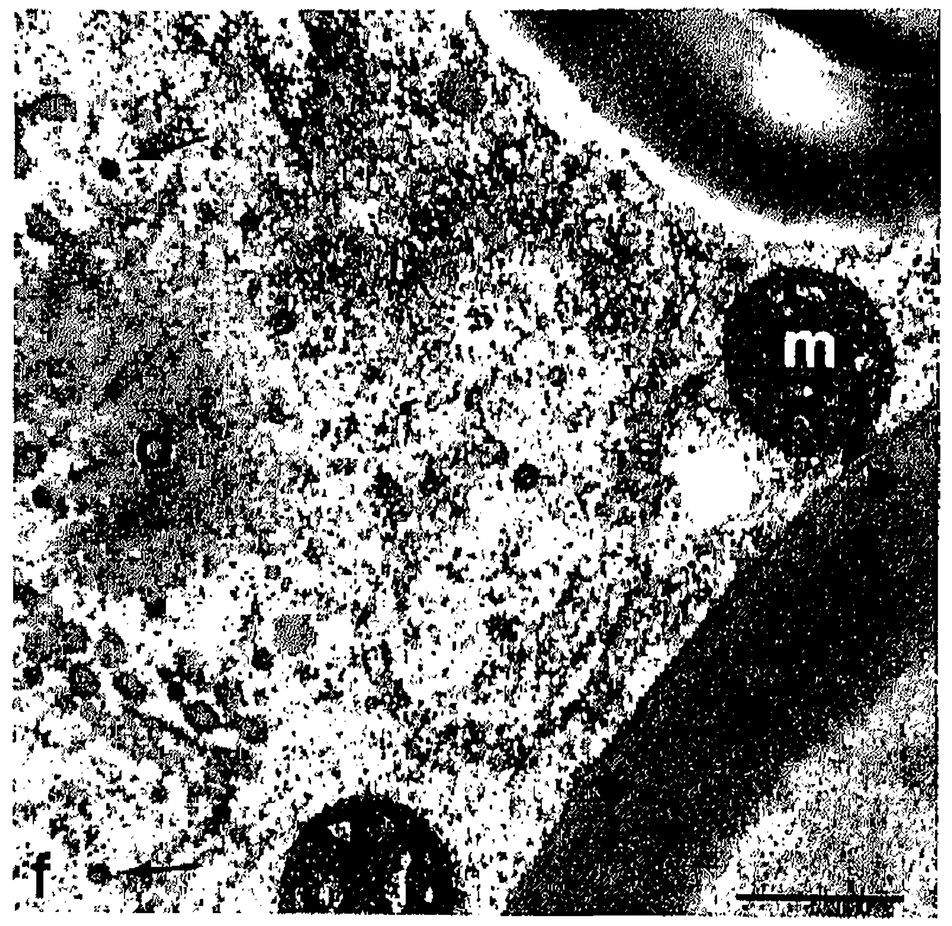
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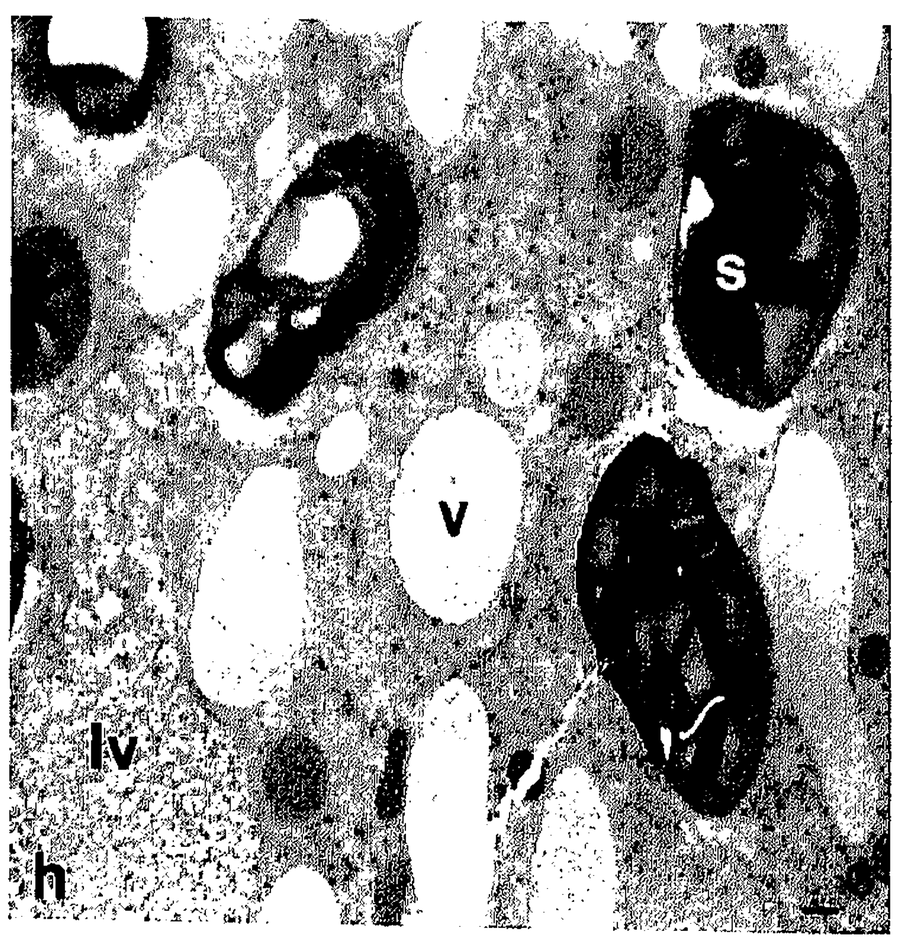
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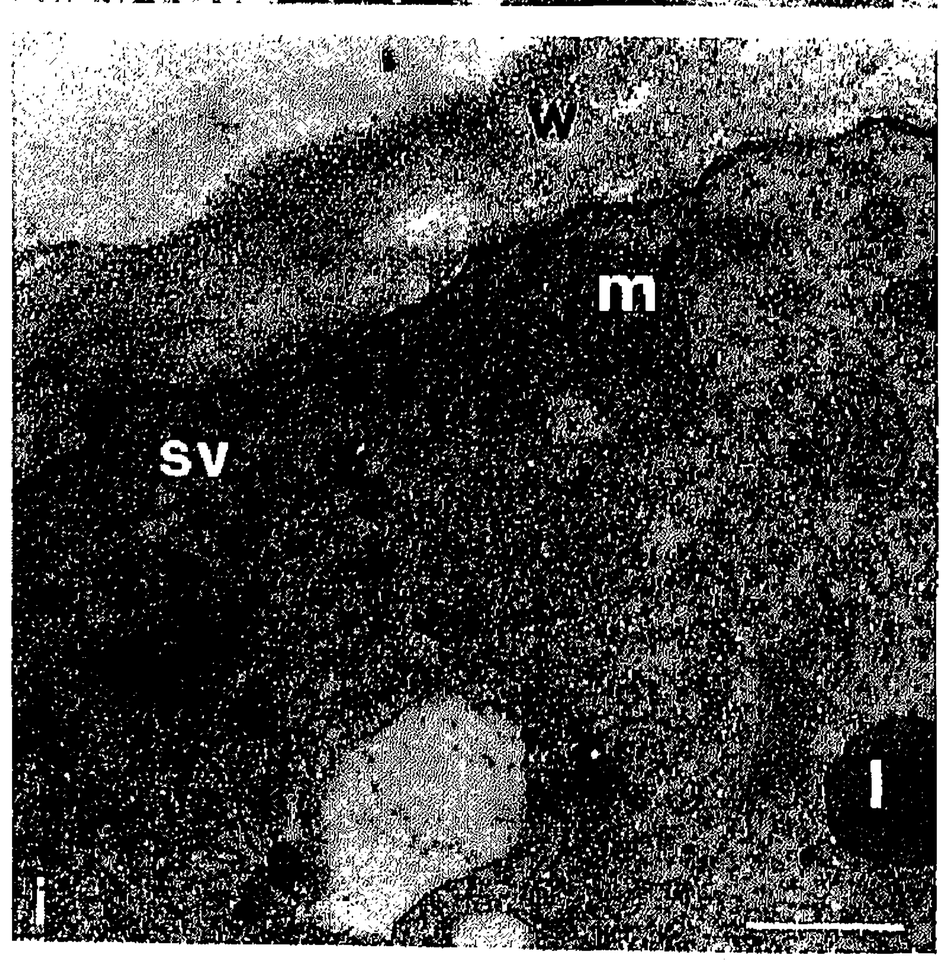
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shaped with a diameter of 0.45 μm and a maximal length of 1.2 μm (Fig. 5c,f). The cristae were relatively small and irregularly shaped, and the large matrix stained darkly. Outside the tip, mitochondria density increased towards the periphery, giving rise to rows of mitochondria along the tube wall (Fig. 5a,c). Fluorescent staining of the mitochondria with $DiOC_6(3)$ confirmed the high number of mitochondria near the cell wall, especially in cells with little linear movement.

Most dictyosomes had a 0.6–1.3 μm diameter and contained six to eight cisternae (Fig. 5c). Cis- and trans-cisternae and a typical trans-Golgi network (TGN) (Fig. 5c) could be distinguished. A number of dictyosomes (5–10%) were incomplete; some consisted of only a single cisterna still containing budding vesicles (Fig. 5c). Intercisternal filaments were observed in the central part of the cisternae (Fig. 5d). Some of the vesicles budding from the dictyosome were covered with a dark coating (Fig. 5c). Dictyosomes showed no preferential orientation or distribution. Only one type of Golgi vesicle (average diameter 0.1 μm) occurred, which had a secretory activity (Fig. 5e,i). Growing tips contained a considerable number of SV but not a conspicuous vesicle cone or vesicle-filled region.

The plasma membrane contained typical 80- to 140-nm-wide CP (Fig. 5f) with a density of 0.25 CP/μm membrane in an average section. A distinct distribution or accumulation was not seen, although CP seemed to be more abundant in the subapex of some young tubes. CV were occasionally observed in the vicinity of the dictyosomes (Fig. 5f). In addition, circular multivesicular bodies (MVB) (diameter about 0.32 μm) containing an average of eight vesicles were abundantly present throughout the cytoplasm (Fig. 5g).

The intermembrane distance of the rough endoplasmic reticulum (RER) varied between 0.01 μm and 0.08 μm (Fig. 5g). A preferential orientation or distribution of the RER could not be established. The starch grains (diameter 4–7 μm) were distorted during sectioning because of the presence of amylose/amylopectin (Fig. 5h). They were usually found in clusters with other organelles dispersed among them. Sections of pollen tubes contained large numbers of nearly circular (diameter about 0.9 μm) electron-dense granules, presumably lipid bodies (Fig. 5h). These granules were distributed throughout the cytoplasm except in the tips of young tubes. In some young tubes, a layer of lipid bodies was seen in the cortical region of the cytoplasm that was filled with starch grains. Numerous electron-translucent vacuoles with a circular to elongated form (long axis 0.5–4.7 μm) occurred throughout the cytoplasm (Fig. 5h). Also, some larger and more electron-dense vacuoles were present (Fig. 5h).

Fig. 5a–i Ultrastructure of *P. sylvestris* after FF-FS (**a–h**) and chemical fixation (**i**). **a** Overview of an unbranched pollen tube, showing the air bags, the tube nucleus (*tn*), starch grains (*s*), and small, electron-translucent vacuoles (*v*). Mitochondria formed rows along the plasma membrane (*arrows*). Shrinkage during preparation is visible from the cracks (*) in the tip. **b** The antheridial cell (*ac*) and remnants of the two prothallial cells (*arrows*) located in the grain of the tube in **a**. **c** Detail of **a**. Mitochondria (*m*) have irregularly shaped cristae and darkly stained matrices. The dictyosome (*d*) shows a complete cis-trans differentiation, whereas dictyosome (*i*) consists only of a trans-Golgi network-like cisterna. The inset shows a typical trans-Golgi network. The white lining is artificially induced by the irradiation in the electron microscope. **d** Dictyosome with intercisternal filaments. **e** Secretory vesicles (*arrowheads*) are budding from the dictyosome (*d*). Some vesicles are covered with a dark coating (*arrow*). **f** Coated pits (*arrowheads*) on the plasma membrane in the pit between two branches. Some coated vesicles (*arrows*) are located near a dictyosome (*d*). *M* Mitochondrion. **g** Part of the cytoplasm showing multivesicular bodies (*b*), rough endoplasmic reticulum (*er*) and a microtubule (*mt*), partly aligned with a microfilament (*arrow*). **h** Small, electron-translucent vacuoles (*v*) appear together with larger, more electron-dense vacuoles (*lv*). *l* Lipid body, *s* starch grain. **i** The cell wall (*w*) and secretory vesicles (*sv*) are little electron dense due to chemical fixation. The wall seems to consist of many layers. *m* Mitochondrion, *l* lipid body. *Bars:* **a** 10 μm, **b** 5 μm, **c–i** 0.5 μm

Discussion

Germination, growth and ramification

The slow germination and growth rate of *P. sylvestris* pollen tubes and their tendency to ramify is consistent with other in vitro studies on pine pollen of various species (Tanaka 1955; Willemse 1971a; Gong et al. 1993). In vitro pollen germination required about 1 day of culture, which is not surprising considering that in vivo germination may take several days (Willemse 1968; Singh 1978). The cessation of growth after 6 days of culture has not been reported earlier and may correspond to the long natural resting stage that the microgametophyte undergoes in vivo before the actual fertilization takes place (Willemse 1968; Willemse and Linskens 1969; Singh 1978).

We could not confirm the relationship between the position of the nucleus and growth or ramification found by Tanaka (1956). Ramification is a general but not exclusive feature of gymnosperm pollen tubes and is probably related to their role or origin as a haustorium (Johri 1992) and their long reproductive cycle (Singh 1978). Angiosperm pollen tubes may ramify as well at some stage of their development (Derksen et al. 1995b). The process of bi- or trifurcation in *P. sylvestris* is an example of renewed relaxation of the tube wall and focused vesicle fusion.

Organelle movement and cytoskeleton

Most organelle movement in *P. sylvestris* is brownian-like. A small percentage of the organelles have a saltatory movement showing a circulatory or fountain-like streaming pattern. These features also sometimes occur in angiosperm pollen tubes, in particular after culture for more than 1 day or when growth has been inhibited

(Derksen et al. 1995b). However, this type of streaming pattern clearly differs from the classical pattern of young angiosperm pollen tubes growing in vitro, in which all organelles participate in a vigorous reverse-fountain streaming pattern (Iwanami 1959; Pierson and Cresti 1992; Derksen et al. 1995b). This difference does not seem to be due to a structural difference in the AF or myosin present: as in angiosperms (AF: Pierson and Cresti 1992; Derksen et al. 1995b; myosins: Miller et al. 1995; Yokota et al. 1995), pollen tubes of *P. sylvestris* show an abundant axial network of AF (this study; Terasaka and Niitsu 1994) and myosins at the surface of organelles (Terasaka and Niitsu 1994).

The low incidence of MT in glancing sections of well-preserved FF-FS material observed in *P. sylvestris* is very unusual compared to other interphase cells (Lloyd 1989; Derksen et al. 1990). Slow growth and absence of tip zonation after the early stages of development in growing *P. sylvestris* pollen tubes may be correlated to a poor MT cytoskeleton, comparable to colchicine-treated *N. sylvestris* pollen tubes, as reported by Joos et al. (1994).

Ultrastructure:
P. sylvestris versus angiosperm pollen tubes

The preferential occurrence of mitochondria close to the cell wall may be due to the more aerobic conditions in the cortical cytoplasm, since diffusion of oxygen in the cell is limited and organelle movement is slow. The dark condensed matrix with irregularly folded cristae of the mitochondria, also observed by Willemse (1971a), can be compared to the low-energy-state condensed conformation of rat liver mitochondria (Hackenbrock 1968) and may indicate low respiratory activity. The activity of the mitochondria is probably not limited by the availability of substrate, as the tubes are filled with starch grains and lipid bodies. We did not observe a conversion of lipid bodies or starch grains into P-particles as seen in lily pollen tubes (Miki-Hirosige and Nakamura 1982). The pollen tubes in our sucrose-rich medium contained very large aggregates of starch grains, suggesting de novo formation. However, no indications for synthesis or digestion of starch grains have been observed, although these features have been reported for growth in vivo (Willemse and Linskens 1969) or as a result of the sugar content of the culture medium (Tanaka 1955).

The dictyosomes generally showed all elements known to occur in angiosperm pollen tubes (Derksen et al. 1995a and references therein). The short stacks or single TGN-like cisternae observed may result from a slow turnover of the dictyosomes, or may relate to functional differences. The dictyosomes did not show any specific zonation or accumulation in the tube, unlike those in angiosperms (Derksen et al. 1995a,b).

Formation of the primary wall solely by Golgi vesicles is supported by the fact that these SV had the same electron density as the cell wall (see also Willemse 1971a) and the same sensitivity for extraction during chemical fixation. The same phenomenon has been reported for tobacco pollen tubes (Derksen et al. 1995a). Relatively few CP were found at the plasma membrane and, in contrast to tobacco (Derksen et al. 1995a), no distinct accumulation of CP was observed, except for incidental records within the region close to the growing tip. Low incidence of CP is in accordance with expectations for a slowly growing cell, where there is proportionally less urgency for retrieval of membrane material secreted in excess (Picton 1981) or maintenance of a differentiated composition of the plasma membrane between the growing tip and the rest of the tube (Derksen et al. 1995a,b).

Lack of orientation in the RER is probably related to the absence of a vigorous cytoplasmic streaming. MVB occurred in much higher densities than in angiosperm pollen tubes. It has been proposed that MVB could be involved in signal transduction in the style (van Cutsem and Messiaen 1994), but this is unlikely for *P. sylvestris* pollen grown in vitro. Two types of vacuoles occurred, but the significance of this remains unclear. Extensive vacuolization and relatively little cytoplasm in the growing tip and around the nucleus has been reported for both in vivo and in vitro grown tubes (Tanaka 1955, 1956; Willemse and Linskens 1969; Willemse 1971a). The occurrence of vacuoles in the tip with concomitant growth was also observed in old angiosperm pollen tubes (Iwanami 1959) and in colchicine-treated *N. sylvestris* pollen tubes (Joos et al. 1994).

Conclusions

Pollen tubes of *P. sylvestris* differ from those of most angiosperms in that they show natural ramification and a slow, mostly brownian-like movement of the organelles. Furthermore, they almost completely lack a tip-to-base zonation of large organelles. These observations demonstrate that a distinct organelle zonation in the apical region and a reverse-fountain-like streaming pattern are not a requirement for tip growth. Instead, the organization of *P. sylvestris* pollen tubes may relate to its slow growth and low metabolic activity, while the organization in angiosperm pollen tubes may relate to fast growth meeting ecophysiological conditions.

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References

Chamberlain CJ (1966) The male gametophyte. In: Gymnosperms, structure and evolution. Dover, New York, pp 306–320

Cutsem P van, Messiaen J (1994) Biological effects of pectic fragments in plant cells. Acta Bot Neerl 43:231–245

Derksen J, Wilms FHA, Pierson ES (1990) The plant cytoskeleton: its significance in plant development. Acta Bot Neerl 39:1–18

Derksen J, Rutten T, Lichtscheidl IK, Win AHN de, Pierson ES, Rongen G (1995a) Quantitative analysis of the distribution of organelles in tobacco pollen tubes: implications for exocytosis and endocytosis. Protoplasma 188:267–276

Derksen J, Rutten T, Amstel A van, Win AHN de, Doris F, Steer MW (1995b) Regulation of pollen tube growth. Acta Bot Neerl 44:93–119

Driessen MNBM, Derksen JWM, Spieksma FThM, Roetman E (1988) Pollenatlas van de nederlandse atmosfeer. Onkenhout, Hilversum, The Netherlands

Gong M, Zhang SX, Li XS, Yang ZH, Cao ZX (1993) The catabolism of lipids and changes of fatty acid composition in the germinating pollen of *Pinus yunnanensis* and effects of calcium and calmodulin. Chin J Bot 5:159–167

Hackenbrock CR (1968) Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. Proc Natl Acad Sci 61:598–605

Heath IB (ed) (1990) Tip growth in plant and fungal cells. Academic Press, San Diego, Calif

Iwanami Y (1959) Physiological studies on pollen. Yokohama Municipal Univ (C-34, Biol 13) 116:1–137

Johri BM (1992) Haustorial role of pollen tubes. Ann Bot 70: 471–475

Joos U, Aken J van, Kristen U (1994) Microtubules are involved in maintaining the cellular polarity in pollen tubes of *Nicotiana sylvestris*. Protoplasma 179:5–15

Kroh M, Knuiman B (1982) Ultrastructure of cell wall and plugs of tobacco pollen tubes after chemical extraction of poly-saccharides. Planta 154:241–250

Linskens HF (1967) Pollen. Handbuch der Pflanzenphysiologie XVIII:368–406

Lloyd CW (1989) The plant cytoskeleton. Curr Opin Cell Biol 1:30–35

Miki-Hirosige H, Nakamura S (1982) Process of metabolism during pollen tube wall formation. J Electron Microsc 31:51–62

Miller DD, Scordilis SP, Hepler PK (1995) Identification and localization of three classes of myosins in pollen tubes of *Lilium longiflorum* and *Nicotiana alata*. J Cell Sci 108:2549–2563

Picton JM, Steer MW (1981) Determination of secretory vesicle production by dictyosomes in pollen tubes of *Tradescantia* using cytochalasin D. J Cell Sci 49:261–272

Pierson ES, Cresti M (1992) Cytoskeleton and cytoplasmic organization of pollen and pollen tubes. Int Rev Cytol 140:73–125

Singh H (1978) Embryology of gymnosperms. Borntraeger, Berlin

Tanaka K (1955) The pollen germination and pollen tube development in *Pinus densiflora* Sieb. et Zucc. I. The effect of storage, temperature and sugars. Sci Rep Tohoku Univ Ser 4 B 21:185–198

Tanaka K (1956) The pollen germination and pollen tube development in *Pinus densiflora* Sieb. et Zucc. II. The tube growth and tube nucleus. Sci Rep Tohoku Univ Ser 4 B 22:219–224

Terasaka O, Niitsu T (1994) Differential roles of microtubule and actin-myosin cytoskeleton in the growth of *Pinus* pollen tubes. Sex Plant Reprod 7:264–272

Traas JA, Doonan JH, Rawlins DJ, Shaw PJ, Watts J, Lloyd CW (1987) An actin network is present in the cytoplasm throughout the cell cycle of carrot cells and associates with the dividing nucleus. J Cell Biol 105:387–395

Willemse MThM (1968) Development of the micro- and macrogametophyte of *Pinus sylvestris* L., an electron microscopic investigation. Acta Bot Neerl 17:330–331

Willemse MThM (1971a) Gamétogenèse male de *Pinus sylvestris*: morphologie et évaluation approximative des organites. Ann Univ ARERS 9:127–132

Willemse MThM (1971b) Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. I. Morphological changes from zygotene until prometaphase I. Acta Bot Neerl 20:261–274

Willemse MThM (1971c) Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. II. Morphological changes from prometaphase I until the tetrad stage. Acta Bot Neerl 20:411–427

Willemse MThM (1971d) Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. III. Morphological changes during the tetrad stage and in the young microspore. A quantitative approach to the changes in the population of cell organelles. Acta Bot Neerl 20:498–523

Willemse MThM, Linskens HF (1969) Développement du microgamétophyte chez le *Pinus sylvestris* entre la méiose et la fécondation. Rev Cytol Biol Vég 32:121–128

Yokota E, McDonald AR, Liu B, Shimmen T, Palevitz BA (1995) Localization of a 170 kDa myosin heavy chain in plant cells. Protoplasma 185:178–187