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Germination and Early Tube Development in vitro of *Lycopersicum peruvianum* Pollen: Ultrastructural Features*

M. Cresti, E. Pacini, F. Ciampolini, and G. Sarfatti
Università di Siena, Istituto di Botanica, Via P.A. Mattioli, 4 I-53100 Siena, Italy

Abstract. Morphologic changes occurring during pollen grain activation and ultrastructural features of *Lycopersicum peruvianum* Mill. pollen tube during the first stages of growth in vitro have been studied. The more evident morphologic changes during activation, in comparison to those already described for mature inactive pollen, concern dictyosomes, rough endoplasmic reticulum (RER), and ribosomes. The dictyosomes are very abundant and produce "large" and "small" vesicles. Near the germinative pores both types of vesicles are present, while all along the remaining cell wall only the large type is observed. These latter react weakly to Thiéry's test and probably contain a callose precursor necessary for the deposition of a callosic layer lining at first only the inner side of the functioning pore and occasionally the other two pores, and subsequently the entire inner surface of the cell wall. The small vesicles, highly positive to Thiéry's test, are present only near the pores and could be involved in the formation of the pectocellulosic layer of the tube wall. The setting free of RER cisterns, which in the mature inactive pollen were aggregated in stacks, coinciding with polysome formation and resumption of protein synthesis, is in accord with the hypothesized role of RER cistern stacks as a reserve of synthesizing machinery. The pollen tube reaches a definitive spatial arrangement soon after the generative cell and vegetative nucleus have moved into it. At this stage four different zones that reflect a functional specialization are present. In the apical and subapical zone two types of dictysosome-originated vesicles, similar to those found in the activated pollen grain, are present. Their role in the formation of the callosic and pectocellulosic wall layers seems to be the same as in the activated pollen grain.

Key words: Early tube development – In vitro germination – *Lycopersicum* – Pollen.

Introduction

In studies performed in our laboratory on the genetic and ultrastructural aspects of self-incompatibility in *Lycopersicum peruvianum* (De Nettancourt et al., 1973a, b, 1974) we noticed what could be interpreted as very early morphologic differentiation between compatible and incompatible pollen tubes, while still in the stigmatic tissue. To obtain more definitive information about this phenomenon, we studied in detail the morphologic changes occurring during pollen grain activation and during the first stages of tube growth in vitro. In particular, we considered the following topics:

1. Pollen grain ultrastructural changes during activation.
2. Opening of germinative pore and pollen tube emission.
3. Zones present in the pollen tubes.
4. Callosic and pectocellulosic wall formation.

Materials and Methods

Pollen grains were gathered from mature anthers of *Lycopersicum peruvianum* plants, supplied by the Laboratorio Valorizzazione Colture Industriali of the Italian National Committee for Nuclear Energy (CNEN), and grown in the Botanic Garden of Siena University. Fresh pollen grains were sown on the medium recommended by Brewbaker and Kwack (1964) with 15% saccharose.

Preparation of Samples for Transmission Electron Microscopy

Fixation timings of pollen grains and tubes after seeding were: every 5 min from 10 to 45 min and every half hour from 1 to

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Abbreviations: ER = endoplasmic reticulum; RER = rough endoplasmic reticulum

3 h 30 min. The materials were centrifuged for a few seconds and the medium decanted and prefixed in 3% glutaraldehyde in cacodylate buffer 0.066 M, pH 7.2, at 4° C for 30 min. The samples were abundantly rinsed in the same buffer and postfixed in 1% osmium tetroxide in cacodylate buffer 0.066 M, pH 7.2, for 1 h, dehydrated through increasing concentrations of ethanol, and embedded in a low-viscosity resin (Spurr, 1969). Sections were cut on a LKB ultramicrotome Ultrotome III, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM 100B electron microscope.

To localize reducing polysaccharides, some of the material was fixed with glutaraldehyde only, and embedded as described. Sections, collected on gold grids, were treated with periodic acid-thiocarbohydrazide (TCH) silver proteinate (Thiéry, 1967).

Preparation of Samples for Scanning Electron Microscopy

Fresh pollen tubes were placed in Freon 113 and transferred to a Bomar apparatus. The Freon was substituted with liquid CO_2 which was then brought to the critical point. The material was then mounted on aluminum stubs with electron conductive glue, coated with gold-platinum, and examined with a JEOL JEM 200 scanning electron microscope.

Preparation of Samples for Optical Microscopy

Germinated pollen was fixed and dehydrated as for electron microscopy followed by embedding in glycol methacrylate (Feder and O'Brien, 1968). Sections 2–3 μm thick were cut and stained with periodic acid-Schiff (PAS) for localizing insoluble polysaccharides (Jensen, 1962). For fluorescence microscopy unfixed germinated pollen was stained with Wasserblau (Linskens and Esser, 1957) and examined to control pollen tube growth and to detect callosic walls and callose plugs. For localizing nucleic acids pollen tubes were fixed with Carnoy fixative and stained with acridine orange (Bertalanffy, 1962). The optical microscope used was a Zeiss RA equipped with super pressure mercury lamp HBO 50 AC.

Results

Activation and Germination

Lycopersicum peruvianum pollen grains hydrate as soon as they are put into the medium, becoming roundish and extruding three semispherical germinative pores about 2 μm in diameter, which are not visible in dry pollen. Pollen tubes appear generally about 45 min after seeding.

In comparison to the situation in the inactive pollen grains (see under Discussion), several cellular organelles appear to change during the activation period, and a layer of callose begins to be deposited below the pores. In pollen grains collected 20–30 min after seeding, most of the ribosomes are gathered in often long polysomes; the RER cisternae are free in the cytoplasm; the clearly evident dictyosomes (about which more will be said later) seem to be in a process of rapid division and are preferentially situated in the peripheral zone of the pollen grain where they form a considerable quantity of vesicles. The roundish plastids have a few lamellae; small vacuoles, which seem to originate from RER cisternae dilations, are present (Fig. 1).

Coming back to dictyosomes, it must be noted that they are particularly numerous and active near the germinative pores (Fig. 2) where they produce two types of vesicles. The most abundant vesicles are relatively larger (0.15–0.3 μm), their contents reacting weakly to Thiéry's test, and they migrate toward the plasmalemma with which they unite. Where this occurs, beyond the plasmalemma a thin, irregular layer is present that reacts positively when tested for callose but not when tested with Thiéry's reagent. This callosic layer, which continues for a short distance also around the pores, has been mainly observed below the pore from which the pollen tube emerges (Fig. 3); however it can occasionally be observed also below the other two pores. The vesicles of the other type are smaller (0.02–0.04 μm), not very abundant, and strongly positive to Thiéry's test; they seem to migrate toward the pore but it is difficult to follow them beyond the plasmalemma.

Also along the walls between the germinative pores (Fig. 4) dictyosomes are present; they are less numerous than in the proximity of the pores and seem to produce only vesicles of the larger type, weakly positive to Thiéry's test, and merging into the plasmalemma. As a result all around the grain a well-defined callosic layer is formed, but with a slight delay in comparison with what occurs below the functioning pore.

At the moment of germination (Figs. 5 and 6) the pore opens like a porthole door; in fact the thicker portion of the pore, protruding through the corresponding opening of the exine, breaks off along a large part of its edge forming a kind of door that opens toward the outside. The portion of the edge that remains attached to the intine functions as a hinge.

The pollen tube has a diameter a little larger than the pore (about 4–6 μm) and its apex is swollen (Fig. 7). The pollen tube wall is formed by two layers: a pectocellulosic external one, continuous with the intine, and an internal callosic one. At this stage the callosic wall is present also in the pollen tube apex (we have not been able to observe at what moment the tube apex looses this apical callosic layer, which as we shall see later, is not present in the older tubes).

During the first phases of germination the pollen grain cytoplasm slowly flows into the tube; later on a central vacuole is formed confining the cytoplasm to a peripheral zone. The pollen grain cytoplasm re-

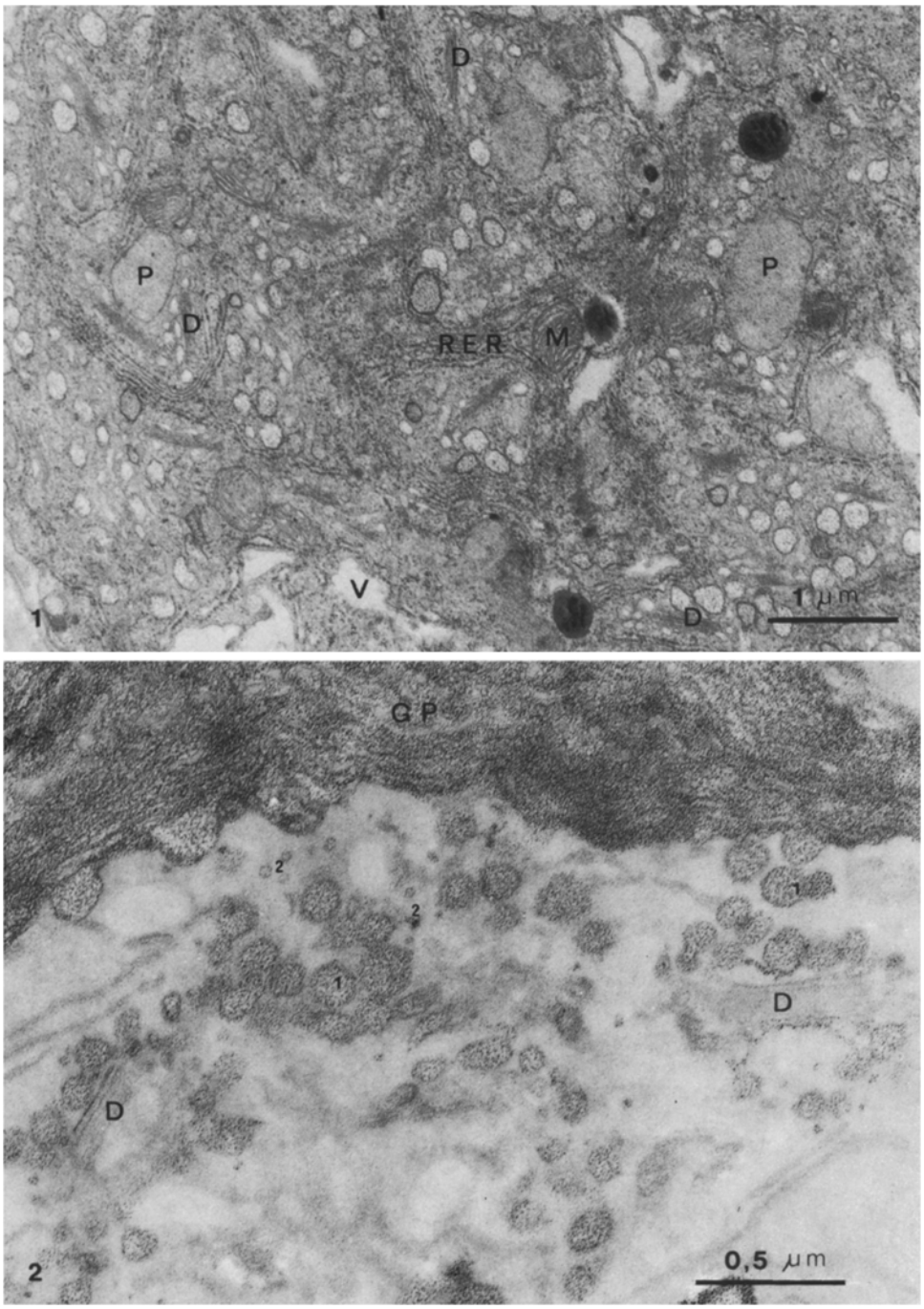

Fig. 1. *Lycopersicum peruvianum* pollen grain during activation (20 min after seeding). GA-OsO_4 fixation. General aspect of cytoplasm; notice the numerous vesicles produced by the dictyosomes (*D*), the mostly free RER cisterns, the nondifferentiated plastids (*P*). *M* mitochondria; *V* vacuoles; *L* lipidic bodies

Fig. 2. Pollen grain during activation (30 min after seeding). GA fixation; Thiéry's test. Dictyosomes near germinative pores (*GP*) produce large (*1*) and small (*2*) vesicles. The large vesicles migrate toward the plasmalemma, their limiting membrane fusing with it

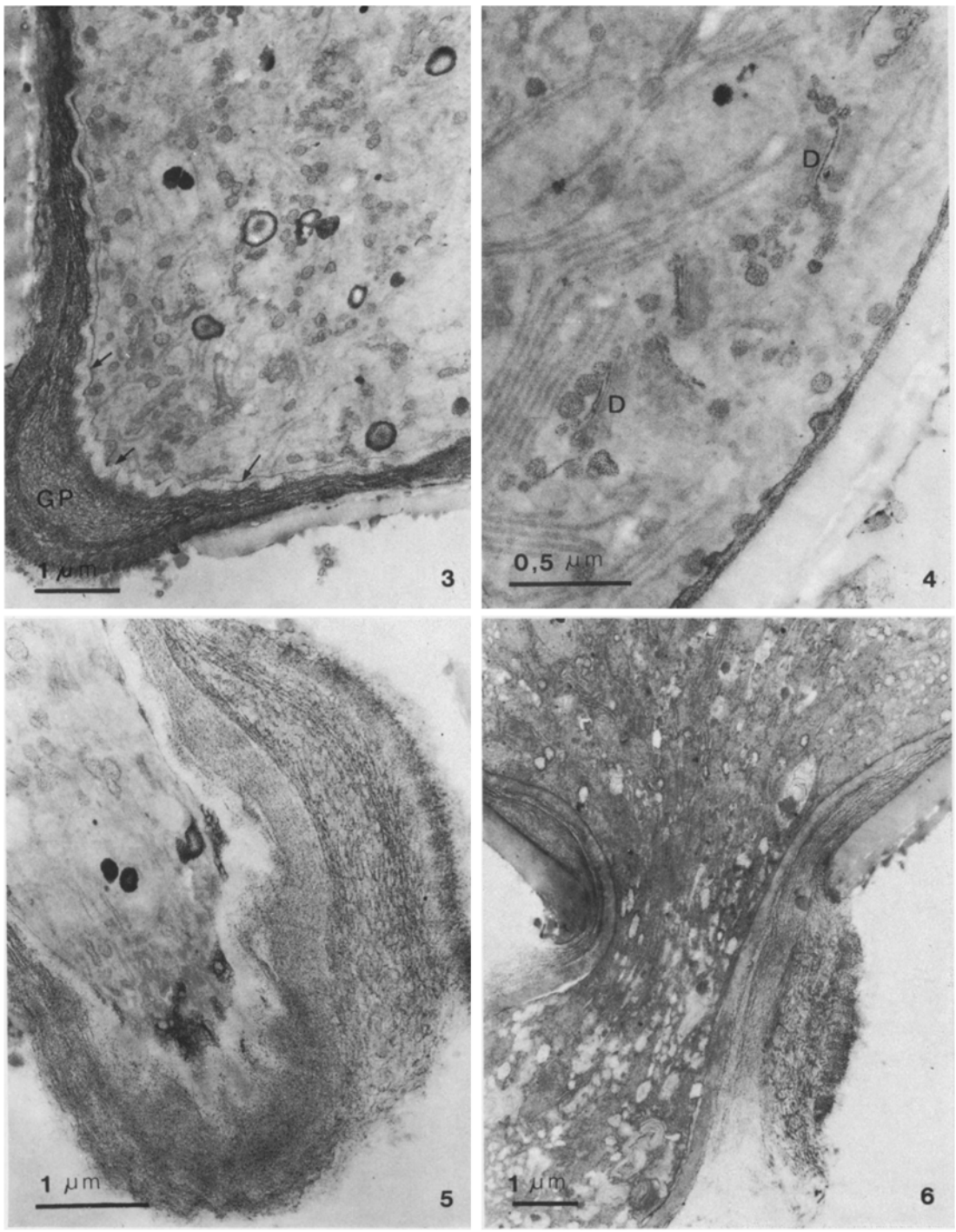

Fig. 3. Pollen grain shortly before the germination (45 min after seeding). GA fixation; Thiéry's test. A callosic, Thiéry test-negative layer (arrows) originating from the large dictyosomic vesicles is observed below the pore from which the pollen tube emerges

Fig. 4. Pollen grain shortly before germination (45 min after seeding). GA fixation; Thiéry's test. All along the wall between the germinative pores the dictyosomes form only large vesicles. In this zone a callosic layer will be evident only after germination

Figs. 5 and 6. Pollen grains at two germination stages (45 and 60 min after seedling). **Fig. 5** GA fixation; Thiéry's test. **Fig. 6** GA-OsO_4 fixation. The germinative pore opens like a porthole door. At these early stages a callosic layer is always present at the pollen tube apex

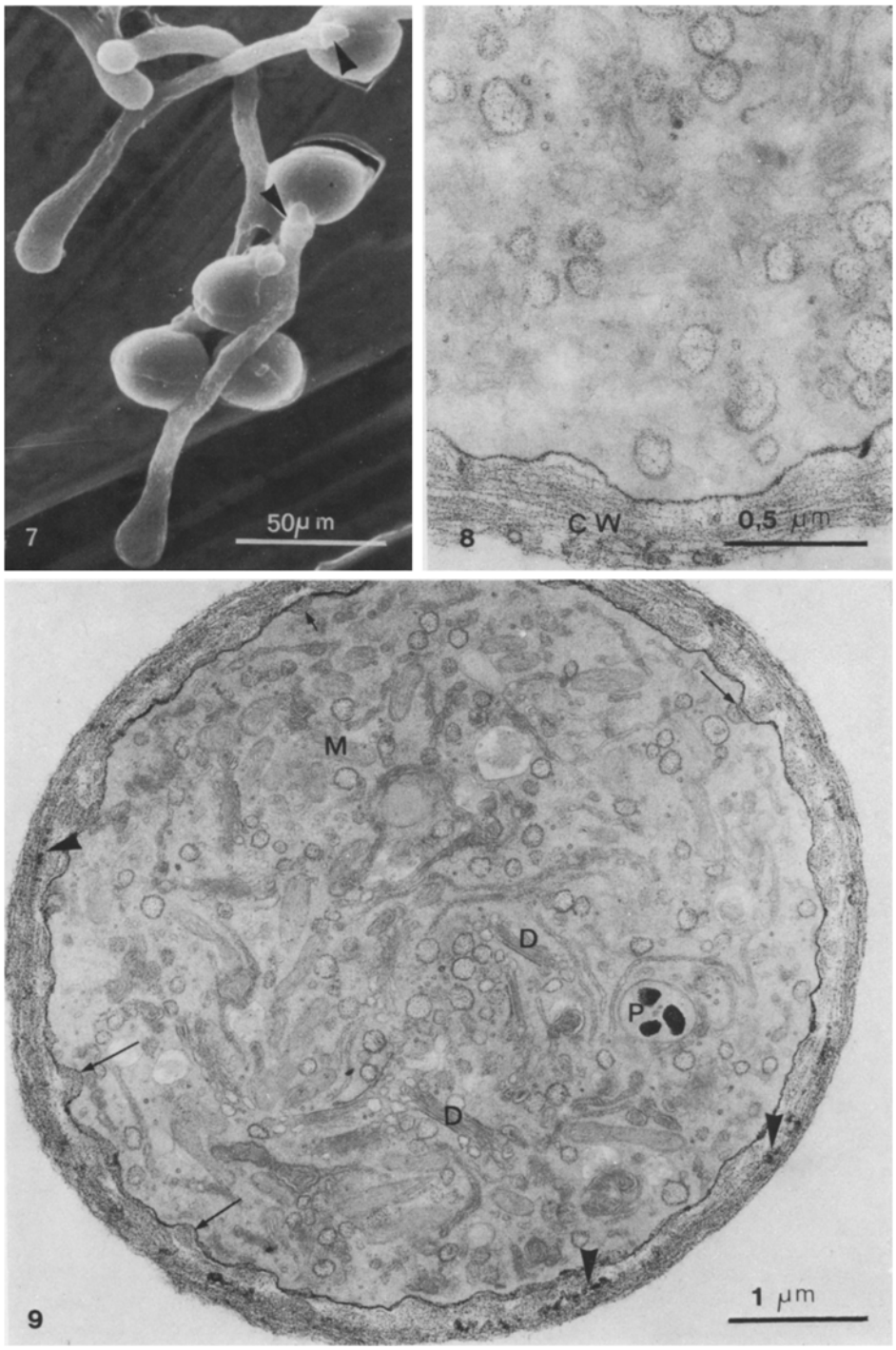

Fig. 7. Pollen and pollen tubes observed with SEM. The tube apex is swollen; the outwardly turned germinative pores (arrow heads) can be seen at the base of pollen tubes

Fig. 8. Cross section of the pollen tube apex (1 h 30 min after seeding). GA-OsO_4 fixation. Only a thin pectocellulosic wall (*CW*) is present. The section has been cut fairly near to the subapical zone; this explains why a few organelles besides vesicles are present

Fig. 9. Cross section of the pollen tube subapical zone (3 h after seeding). GA fixation; Thiéry's test. Besides several organelles numerous vesicles are present. The large vesicles are seen uniting with the plasmalemma (arrows), their content forming the not yet evident callosic layer; the content of small vesicles can be observed inside the pectocellulosic wall layer (arrow heads)

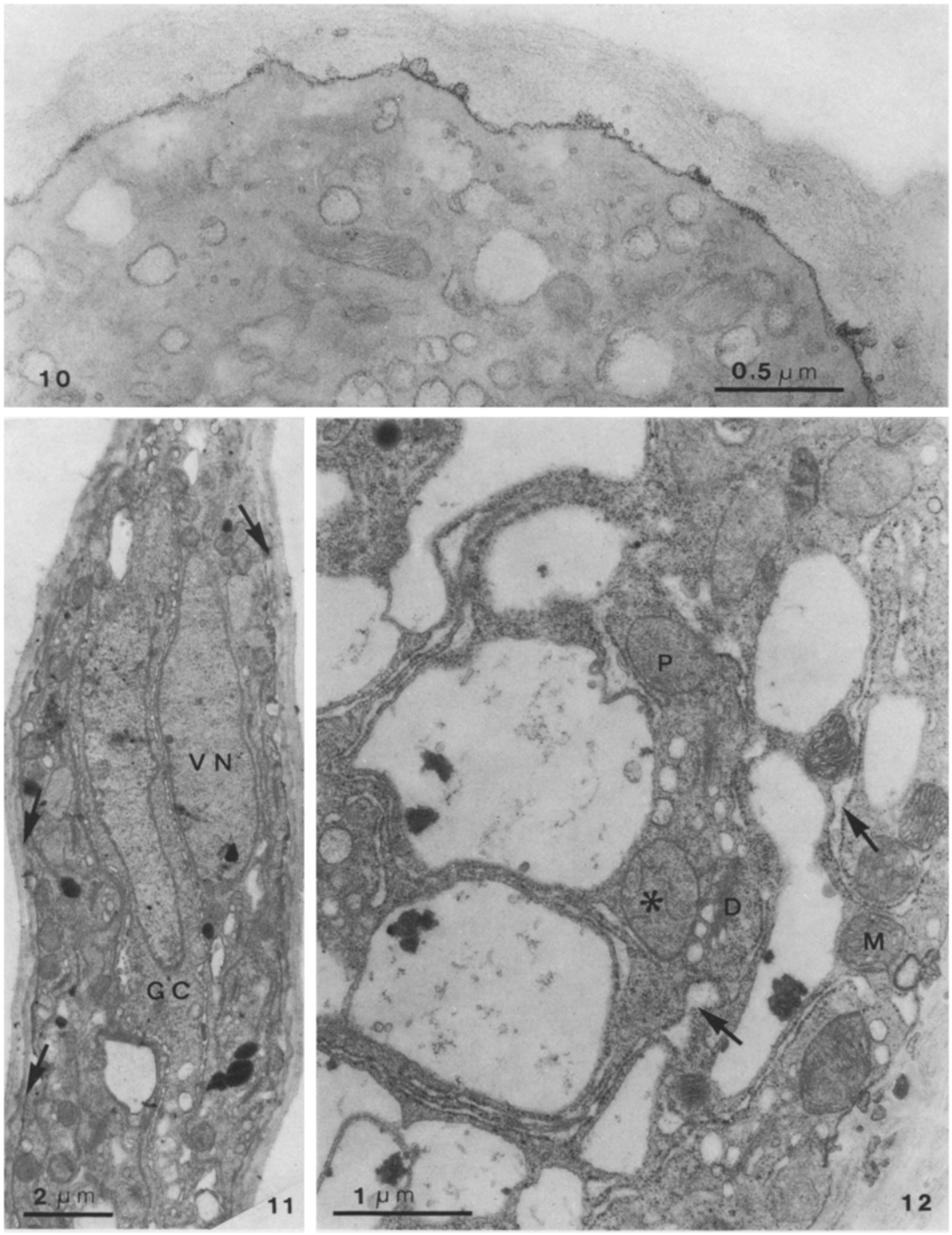

Fig. 10. Pollen tube zone-timing and type of fixation as in Figure 9. Higher magnification to show the migration of dictyosome vesicles of the small type to the pectocellulosic wall

Fig. 11. Longitudinal section of the nuclear zone (3 h after seeding). GA-OsO_4 fixation. The generative cell (*GC*) and vegetative nucleus (*VN*) can be seen. The callosic layer is now evident (arrows)

Fig. 12. Vacuolization zone. GA-OsO_4 fixation (3 h 30 min after seeding). Large vacuoles are present derived from ER cistern dilations (arrows). Small membranous structures (asterisk) are present inside the plastids

mains active, and its dictyosomes continue to produce the large types of vesicles that merge into the plasmalemma and form an uninterrupted callosic wall.

The Pollen Tube

The final organization of the pollen tube occurs as soon as the latter reaches a length of about 150 μm, shortly after the generative cell and the vegetative nucleus have moved into it. At this stage four zones can be clearly observed:

1. Apical or growth zone.
2. Subapical zone.
3. Nuclear zone.
4. Vacuolization and callosic plug formation zone.

Apical or Growth Zone. The apical zone is also the growing zone; this fact was already known but we checked it again by the charcoal powder technique used for *Lilium* (Rosen et al., 1964). This zone is 2 to 4 μm long; it is clearly distinguishable because it is slightly swollen (Fig. 7) and abundantly stained with PAS. The wall is single and formed by a thin layer of fibrils; the fluorescence technique does not indicate the presence of a callosic layer (Fig. 8).

Both types of vesicles, already observed in the activated pollen grain, are present; the small vesicles (Fig. 8; in this section cut fairly near to the subapical zone, some organelles besides the dictyosome vesicles are present) still have a granular content slightly labeled after treatment with periodic acid TCH and silver proteinate. Both types of vesicles have been observed in contact with the wall, but now the smaller ones are visible also inside the wall material itself. In this zone of the tube no other organelles are present.

Subapical Zone. The tube presents an external wall, staining with the PAS reaction, and a callosic inner wall that toward the apical zone forms only a very thin layer. The cytoplasm is rich in organelles: elongated mitochondria with clearly evident cristae, plastids differentiated into amyloplasts containing one or two starch grains, lipidic bodies, and very long profiles of endoplasmic reticulum. Very active dictyosomes, formed by 5–7 cisternae, are particularly abundant. They produce here, as in the activated pollen grain, numerous vesicles of both types. While the content of the smaller vesicles seems to pass through the plasmalemma and the callosic layer, apparently dissolving into the pectocellulosic layer, the larger ones have been ovserved in contact with the plasmalemma with which they often are seen to fuse (Figs. 9 and 10).

In longitudinal sections most of the cytoplasmic components appear parallel to the long axis of the tube except near the tip where they are more randomly distributed. The dictyosomes, as well as the RER profiles, are ranged near the wall.

Nuclear Zone. This zone shows not only the presence of the vegetative and generative nuclei but also of the (now clearly evident) callosic wall (Fig. 11).

The generative cell is very elongated, spindle-shaped, and curved at the extremities. The "wall" of the generative cell is formed by two membranes, the portion between them being sometimes swollen and presenting fibrillar material. The nucleus is ranged in the median portion where it takes up the greater part of the cell. The chromatin is rather uniform except for the more electron-dense masses near the nuclear membrane. In the cytoplasm, mitochondria, dictyosomes, short RER profiles, and little vacuoles are present. The ribosomes, mainly free, are randomly distributed. In the median part of the generative cell bundles of microtubules are present.

The vegetative nucleus, situated near the generative cell, has many lobes and shows an uniform electron density. Dictyosomes, longitudinally ranged RER profiles, amyloplasts, mitochondria, and lipidic bodies are present in considerable quantity.

Many little vacuoles have been observed in this zone.

After staining with acridine orange the generative nucleus, examined with the fluorescence microscope, is more deeply stained than the vegetative one.

Vacuolization and Callosic Plug Formation Zone. Behind the nuclear zone, the vacuoles are much more abundant and larger. They originate from RER enlargements and inside them we can observe small electron-dense masses (Fig. 12). The callosic wall is very thick, while the pectocellulosic wall thickness is unchanged. Roundish mitochondria with clearly evident cristae, plastids with some lamellae, long RER cisterns, and dictyosomes producing only large vesicles are present in the cytoplasm. This zone ends with the callosic plug, the formation of which occurs in the same way as that already described for *Petunia hybrida* pollen tube grown in vivo (Cresti and Van Went, 1976).

Discussion

Activation and Germination. Activation of *Lycopersicum peruvianum* pollen grain in vitro takes about 45 min, only a few seconds being necessary for its hydration. On the other hand in vivo inhibition needs

15 min and germination occurs 3 h 30 min after pollination (Pacini and Sarfatti, 1976). Processes leading to in vitro germinations are quicker than in vivo, probably because in the culture medium, H_2O and the sugar and mineral salts dissolved in it are immediately available without the intervention of cell walls and stigmatic exudate. Even if in vitro and in vivo activation needs different times, in both cases it seems to start immediately upon hydration, as has also been shown, at least for enzymatic activation, in *Petunia hybrida* pollen (Stanley and Linskens, 1964).

From a morphologic point of view our data show that during activation many cellular organelles undergo considerable changes in comparison with what has been observed in inactive ripe pollen (Cresti et al., 1975). The more evident changes consist of the setting free of the cisternae of the RER stacks present in mature pollen; in the considerable production of vesicles by dictyosomes; in the formation of a thin callosic layer, strictly related to dictyosomic activity and developing at first below the pore from which the tube originates; in the aggregation or ribosomes in polysomes, as already observed in *Petunia* by Linskens et al. (1970); and in the formation of lamellae inside the plastids. On the contrary, mitochondria, lipidic bodies, generative cell, and vegetative nucleus do not seem to undergo evident modifications.

We explain the disassembly of the stacks of RER cisternae, one of the more dramatic events occurring during activation, as the morphologic aspect of the activation of the machinery needed for protein synthesis, which had been stored in an inactive form. It is well known that synthesis of the various RNAs ceases before morphologic ripening of the pollen grain is completed (Steffensen, 1966; Mascarenhas and Bell, 1970; Linskens et al., 1971; Süss and Tupy, 1976), and, what is more important, that pollen can germinate and the tube can grow up to a certain length when incubated in the presence of a transcription inhibitor such as actinomycin D (for reviews see Rosen, 1968 and Mascarenhas, 1975) and therefore in the probably complete absence of newly synthesized RNA. This means that the necessary RNAs had been stored during pollen formation, probably in order to insure a rapid resumption of synthetic activity. In our previous paper on *Lycopersicum peruvianum* mature pollen (Cresti et al., 1975) we described the presence of stacks of RER cisternae, which are formed during the very last stage of maturation (unpublished data) and hypothesized that such stacks could represent a reserve of inactivated synthesizing machinery. The rapid disassembly of the stacks on resumption of growth seems to confirm such a hypothesis.

In the activated pollen grain two types of dictyosomic vesicles having different content and size are present. The dictyosomes, particularly abundant near the germinative pore, as well as later on in the apical and subapical zones of the pollen tube, produce both types of vesicles, while all along the remaining pollen grain wall only the large vesicles are present. These latter vesicles, reacting weakly to Thiéry's test, whose limiting membrane unites with the plasmalemma, could contain a callosic precursor that polymerizes as soon as it accumulates between plasmalemma and intine of germinating pollen or between plasmalemma and the preexistent wall of the tube. It must be noted that in the activated pollen dictyosomes are more abundant and a thin callosic layer develops first below the pore from which pollen tube will issue; the callosic layer will be completed later all around the remaining wall where dictyosomes are less frequent. It must be pointed out that although the mechanism of callosic wall formation is not yet well understood, the participation of dictyosome vesicles seems probable (Morre' et al., 1971; Helsper et al., 1977), particularly as far as microspore mother cells are concerned (Echlin and Godwin, 1968). The other type of vesicle present in the activated pollen, that is, the small one, reacting strongly to Thiéry's test, is located only near the germinative pores. While we have no direct evidence of the fusion of the vesicular content with the pore, we have been able to observe the content of the same type of vesicle inside the pectocellulosic wall of the growing pollen tube, and we suggest therefore that in both cases the role of the small vesicles is to carry polysaccharidic material necessary for wall formation.

Finally we wish to remark that two types of vesicles have been observed also in *Lilium longiflorum* pollen tube (Rosen and Gawlik, 1966) both being respectively larger than those we observed in *Lycopersicum peruvianum*. However, in *Lilium*, the function of these vesicles seems to be different; according to Rosen, in fact, while the large vesicles are related to the formation of new wall and plasmalemma, the small ones, of unknown origin, contain RNA.

Spatial Arrangement of the Pollen Tube. The final organization of the pollen tube takes place when it reaches a length of about 150 μm, soon after the generative and vegetative nuclei have moved into it, and probably soon after the callosic layer has disappeared from the tube apex, an event that we have not been able to observe. The different zones present in the pollen tube reflect a functional spezialization. In fact the apical zone is the growth region, while in the subapical zone, rich in organelles, the production of vesicles and metabolites occurs. These materials migrate toward the apical zone by means of cytoplasmic streaming (Franke et al., 1972); in the subapical zone

the callose layer is morphologically evident, also if its formation must start in the apical zone. The nuclear zone is the intermediate part of the pollen tube; it is most important because it contains the vegetative nucleus and, especially, the generative nucleus and later on the gametes. The vacuolization zone represents the transition between the active and the inactive part of the pollen tube. This inactive portion starts beyond the callosic plug. In general our data confirm and extend those already reported for *Lilium* (Rosen et al., 1964) and *Petunia* pollen tube grown both in vitro (Sassen, 1964) and in vivo (Cresti and Van Went, 1976), and correspond to our as yet unpublished data for *Lycopersicum peruvianum* pollen tubes growing in vivo.

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