



CYTOPLASMIC RESERVES IN GENERATIVE AND VEGETATIVE CELLS OF *HERMODACTYLUS TUBEROSUS* MILL.: CYTOCHEMISTRY, ESI AND EELS ANALYSES

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This study uses cytochemical tests, electron spectroscopic imaging and electron energy loss spectroscopy techniques to identify and localize the reserves inside the generative cell of *Hermodactylus tuberosus* pollen. Cytochemical probes applied to sections observed by light and transmission electron microscopy indicated that the generative cell contains large osmiophilic bodies probably made of phytic acid rich in P and Ca. The significance of the rich granulations in generative cells of *Hermodactylus* pollen is discussed in relation to floral biology and environmental conditions. In comparison, the vegetative cytoplasm contains (a) lipid droplets formed by unsaturated lipids and related to vacuoles, (b) lipid bodies with larger dimensions, irregular in shape and very rich in Ca, (c) bodies stained in polysaccharide tests as well as lipid probes tentatively identified as glycolipid granulations, and (d) small granules very rich in P and Ca interpreted as phytin granules.

Key words: *Hermodactylus*, pollen, reserves, cytochemistry, ESI, EELS.

INTRODUCTION

Pollen cytoplasm reserves are made up mainly of lipids, polysaccharides, starch or monosaccharides, and probably also proteins (Pacini, 1996; Piffanelli et al., 1998). Other pollen inclusions recently have received attention, as in the case of phytin or phytin-like globoid reserves (Butowt et al., 1997) rich in P, Ca, Mg; they are frequently found in the seeds of many plants, usually concentrated within inclusions called globoids inside the protein bodies (Greenwood and Bewley, 1984). A relation has been found between phytic acid content and style length: species with a style length of 5 mm or more have a significant amount of pollen phytic acid, suggesting its role in providing precursors for the cell-wall synthesis needed for rapid pollen tube elongation down the style of pollinated plants (Jackson et al., 1982). All the above-reported pollen reserves have been localized mainly in the vegetative cell cytoplasm, the

generative cell cytoplasm being destined for formation of sperm cells for egg and central cell fertilization (Tanaka, 1993). General and detailed reviews on the male gametophyte of spermatophytes have never referred to generative cell reserves (Cresti et al., 1984; McCormick, 1993), but in *Hermodactylus tuberosus* pollen the generative cell appears very rich in large electron-dense bodies whose composition and significance are still unknown.

This study attempts to identify and localize the reserves in generative cell cytoplasm of mature pollen of *Hermodactylus tuberosus* by cytochemical tests applied to light and transmission electron microscopy. In addition, electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) are used to test for the presence of nitrogen, phosphorus and calcium (N, P, Ca) in different compartments of the same pollen.

TABLE 1. TEM localization of lipids, proteins, carbohydrates and phytin-like reserves in cytoplasm of generative and vegetative cells of mature pollen of *Hermodactylus tuberosus*

Probes	Generative cytoplasm	Specificity	Vegetative cytoplasm
OTO	++	Lipids	++ Lipid bodies and lipid droplets
TCH - SP	+ -	Unsaturated lipids	++ Lipid droplets
SP	+ -	Proteins	+ -
Soloff	--	Membranes	++ Negatively stained vesicles
PA-TCH-SP	--	Polysaccharide with vicinal groups	++ Amyloplasts
H ₂ O ₂ -TCH-SP	--	Control	--
Aqueous PTA	--	Polysaccharides and proteins	++ Small vesicles
Acid PTA	++	Glycoproteins positively charged, phytin	++ Small vesicles and starch
Acetic acid	++	Phytic acid	+ - Vesicles and starch disappeared

MATERIALS AND METHODS

PLANT MATERIAL AND LIGHT MICROSCOPY (LM)

Pollen was gathered from *Hermodactylus tuberosus* L. (Iridaceae) grown in olive groves at Villa Mondragone, Monteporzio Catone Rome (Italy) (Grilli Caiola and Brandizzi, 1997). Fresh pollen grains and thin sections of samples prepared as for transmission electron microscopy were observed unstained and stained as follows: toluidine blue O 0.25% in water for acid polyanions (Knox et al., 1989); toluidine blue O after 0.1 N acetic acid treatment; Coomassie blue for proteins (Knox et al., 1989); iodide in aqueous potassium iodide solution (Lugol) for starch (Johansen, 1940); Sudan black for unsaturated lipids (Knox et al., 1989); and Nile blue sulphate for neutral lipid (Dunnigan, 1968). Unstained and stained sections were observed with interference Zeiss and Aristoplan Leitz microscopes. Photographs were made with Ektachrome 100 ASA or PANF 50 ASA film.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

For transmission electron microscopy the pollen was fixed overnight in 3% glutaraldehyde in 0.2 M phosphate buffer (PB), pH 7.2, post-fixed in 1% osmium tetroxide in PB overnight, dehydrated and embedded in Epon resin (Grilli Caiola et al., 2000). The thin sections were treated as follows: uranyl-acetate and lead citrate to contrast lipoprotein membrane (Venable and Coggeshall, 1965); 0.1 N acetic acid for 10 min, then uranyl-acetate to reveal phytic globoids (Greenwood and Bewley, 1984); periodic acid to deosmicate and then thiosemicarbazide proteinate silver (PA-TSC-SP) for 2 and 24 h to reveal polysaccharides with 1,2 hydroxyl groups according to Thiry (1967); thiosemicarbazide and silver protein (TCH-SP) for unsaturated

lipids (Seligman et al., 1966); phosphotungstic acid (PTA) in water to reveal protein groups and polysaccharide (Ferragiana and Marinozzi, 1979); phosphotungstic acid in chloride (acid PTA) for glycoproteins positively charged (Roland et al., 1972); permanganate-uranyl-acetate-lead citrate to reveal negatively stained membranes and vesicles (Soloff, 1973); and osmium-thiosemicarbazide-osmium (OTO) to enhance contrast of lipids (Seligman et al., 1966).

ELECTRON SPECTROSCOPIC IMAGING (ESI) / ELECTRON ENERGY LOSS SPECTROSCOPY (EELS)

To gather more information on cytoplasm reserves, N, P and Ca were detected by ESI and EELS (Canini et al., 2001). Ultrathin sections (<40 nm) for ESI and EELS analyses were placed on 600 mesh copper grids and observed without staining. The electron spectroscopic images (ESI) for localization of calcium, nitrogen and phosphorus in the specimens were taken as follows: for calcium at $\Delta E = 365\text{eV}$ just beyond the ionization edge (IE) ($\text{Ca}_{L_{2,3}}$ edge at $\Delta E = 346\text{--}359\text{eV}$), and at the pre-ionization edge (PIE) at $\Delta E = 315\text{eV}$ as reference carrying information on the background; for nitrogen ESI images were obtained at $\Delta E = 410\text{eV}$ just beyond the IE (N_k edge at $\Delta E = 401\text{eV}$), and at the PIE at $\Delta E = 370\text{eV}$ as reference; for phosphorus at $\Delta E = 165\text{eV}$ (edge $\text{P}_{L_{2,3}}$ $\Delta E = 153,5\text{eV}$) and at the pre-ionization edge (PIE) at $\Delta E = 115\text{eV}$. Inelastically scattered electrons of defined energy losses were imaged using a 20 eV energy window to obtain high-contrast darkfield images at energy losses between 150 and 250 eV. At such values most of the important biological elements, except carbon, contributed to the signal. Electron energy loss spectroscopy (EELS) was performed with an integrated photomultiplier with a

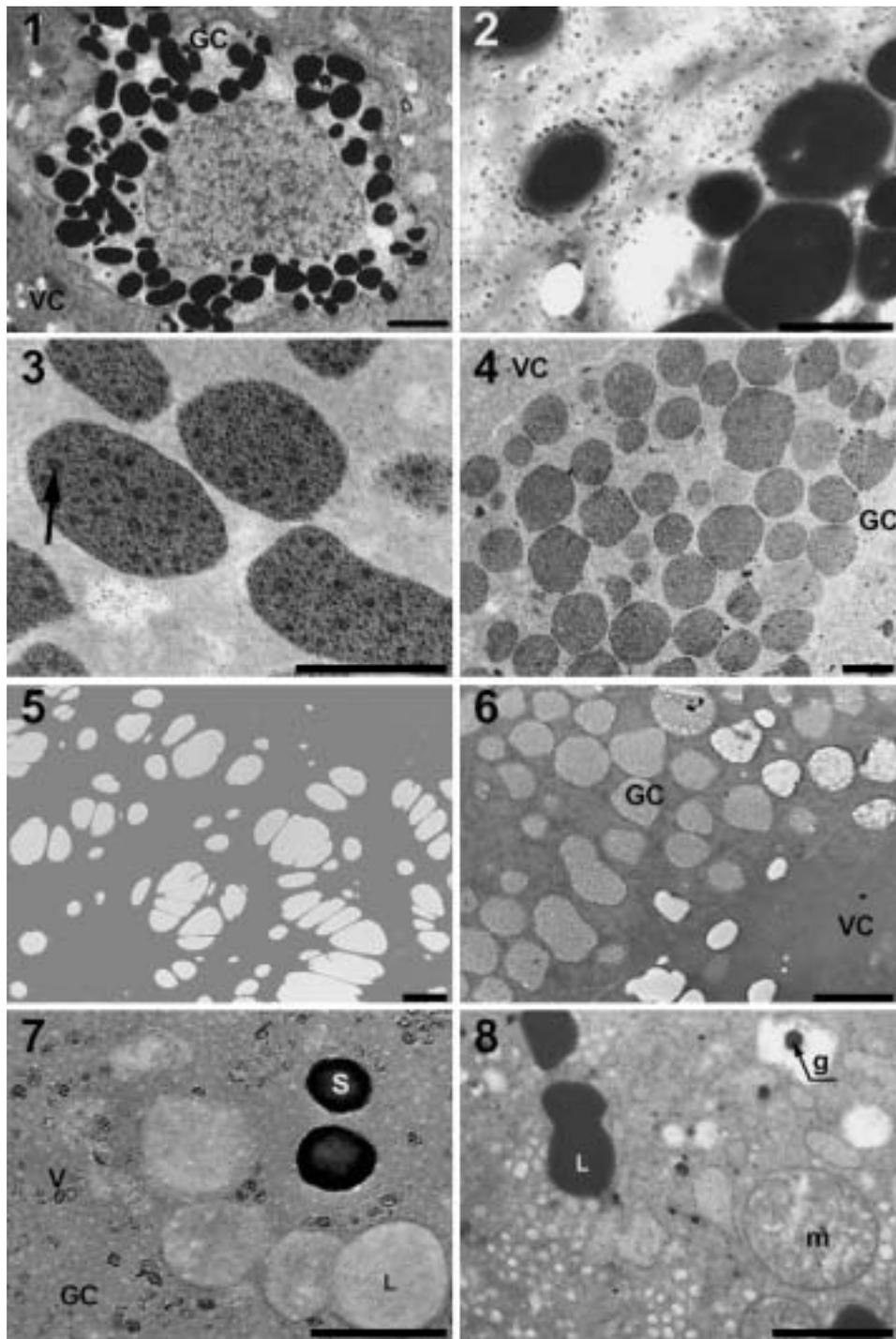


Fig. 1. Generative cell (GC) with central nucleus and large electron-dense granules in cytoplasm. VC – vegetative cell. Bar = 2 μm. **Fig. 2.** Electron-dense inclusions in GC after OTO staining. Bar = 1 μm. **Fig. 3.** After TCH-SP, GC granulations show numerous small bodies (arrow) more electron-dense inside homogeneous matrix. Bar = 1 μm. **Fig. 4.** After Soloff treatment, no membranes appear around the granules. GC – generative cell; VC – vegetative cell. Bar = 1 μm. **Fig. 5.** After acetic acid, granules are electron-transparent and dissolved in many cases. Bar = 2 μm. **Fig. 6.** PA-TCH-SP does not reveal the presence of polysaccharides in GC. Bar = 2 μm. **Fig. 7.** Starch (S) granules and numerous vesicles (V) PTA-positive in vegetative cell cytoplasm. Large lipid bodies (L) are unstained. Bar = 1 μm. **Fig. 8.** Lipid bodies (L), mitochondrion (m) and electron-dense globoids (g) in vegetative cytoplasm. Bar = 1 μm.

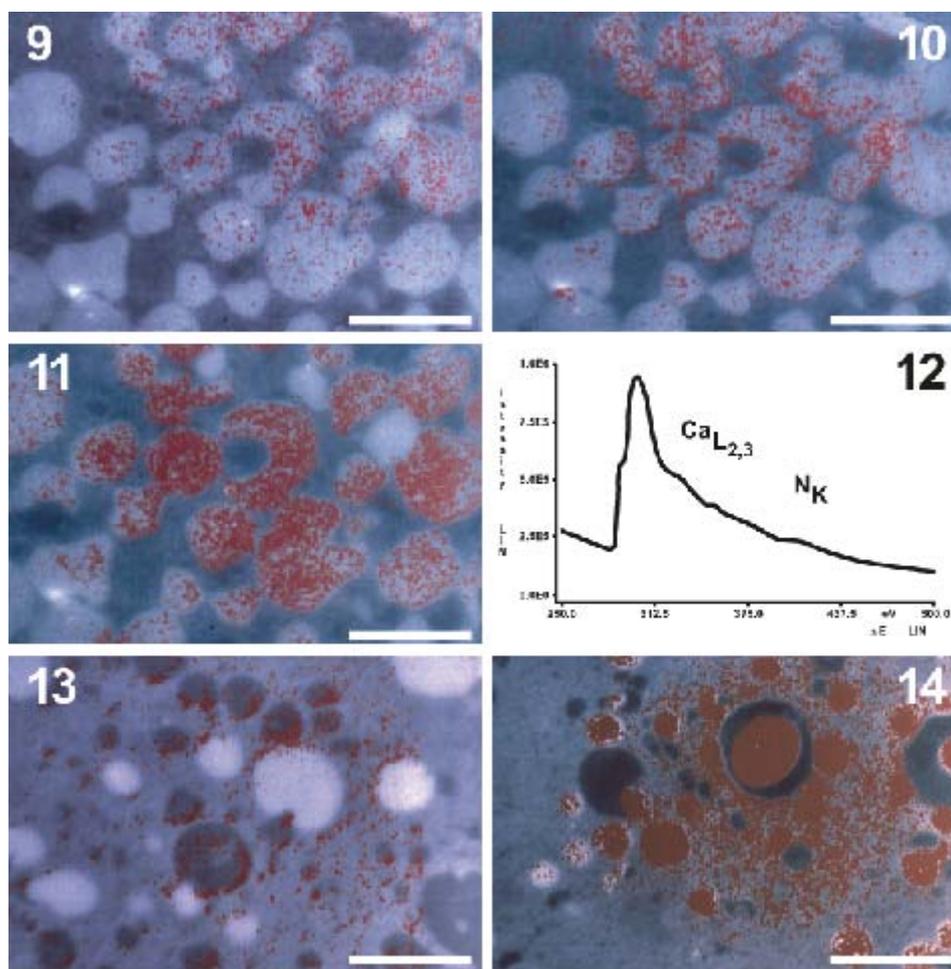


Fig. 9. Electron spectroscopic imaging showing localization of N on granules of generative cell (GC). **Fig. 10.** ESI showing Ca distribution on GC. **Fig. 11.** ESI showing very intense signals of P on GC. **Fig. 12.** EELS spectrum showing edges of Ca (at 365 eV) and N (at 410 eV) on granule of GC cytoplasm. **Fig. 13.** P signals after ESI on granules and cytosol of vegetative cell. **Fig. 14.** Very intense signals due to Ca localization by ESI on granules and cytosol of vegetative cell cytoplasm. Bar = 1 μ m in all figures.

90 μ m objective lens diaphragm and 100 μ m spectrometer aperture at a scan rate of 1 eV/channel and 30,000 \times magnification on 200 or 300 nm diameter areas of the ultrathin sections where Ca_{L2,3}, N_k and P_{L2,3} were localized. All ultramicroscopic observations were performed on sets of at least three subsamples.

RESULTS

CYTOCHEMISTRY OF CYTOPLASM RESERVES

The mature pollen grain of *Hermodactylus tuberosus* shows a vegetative nucleus and a large spindle spiral generative cell (Grilli Caiola et al., 2000). The latter, when stained with toluidine blue O, shows

two long, sharp arms filled with intensely stained granulations, whereas other inclusions are spread in the cytoplasm of the vegetative cell. Cytochemical probes used in light microscopy to identify the composition of generative cytoplasm reserves gave negative reactions to Sudan black, Nile blue, Lugol and Coomassie.

SUBMICROSCOPIC CYTOCHEMISTRY

The generative cell evidenced large electron-dense bodies different in size (from 0.10 to 2.8 μ m) and composition from the reserves of vegetative cytoplasm (Fig. 1). By OTO staining the generative cell inclusions remained intensely electron-dense (Fig. 2). TCH-SP treatment revealed the presence of

small roundish structures which were more electron-dense inside the matrix (Fig. 3). Soloff staining did not reveal membranes around the granules (Fig. 4). Acetic acid or acid PTA decolorized the generative inclusions (Fig. 5). After PA-TCH-SP, no positive reaction was obtained in granules of generative cells (Fig. 6). In both vegetative and generative cells, sections osmicated by SP treatment showed reserve granules formed by more and less electron-dense parts, often with a dissolved central area. PA-TCH-SP- and PTA-positive inclusions (Fig. 7) and small granules inside protein bodies (Fig. 8) were detected in vegetative cytoplasm (Tab. 1).

ELECTRON SPECTROSCOPIC IMAGING (ESI)/
ELECTRON ENERGY LOSS SPECTROSCOPY (EELS)

Very few nitrogen signals were obtained on granules (Fig. 9) in generative cells, whereas Ca (Fig. 10) and mainly P (Fig. 11) were very abundant. A spectrum showing edges of Ca and N (Fig. 12) confirmed the ESI results. P (Fig. 13) and Ca (Fig. 14) were also present on large and small roundish granules spread in the vegetative cytoplasm.

DISCUSSION

In mature pollen of *H. tuberosus* the generative cell accumulates numerous and large granules of different sizes but with the same composition. They start accumulating from the first stage of generative cell differentiation and appear very rich in P and Ca and less in N. On the basis of cytochemical tests and ESI and EELS signals we consider them an accumulation of phytin-like granules (Butowt et al., 1997) inside a lipoprotein matrix. The role of so many reserves in the generative cytoplasm could be to sequester high Ca content from vegetative cytoplasm. *H. tuberosus* pollen does not germinate easily in vitro on germination medium (Grilli Caiola and Brandizzi, 1997). According to Dafni and Werker (1982), Mediterranean geophytes allocate the necessary food storage for flowering and seed set during the previous photosynthetic period. This strategy enables them to invest relatively large resources in both flowers and seed set. *Hermodyctylus tuberosus* flowering occurs in winter or spring when humidity is 75–90% and diurnal temperature between -3°C and 16°C. However, sometimes the temperature may reach -12°C and this may affect both pollen maturation and release, as well as reduce pollination. Generative cell reserves could be signi-

ficant in assuring survival of the generative cell against environmental stresses. Cytochemical probes did not find evidence of reserves formed only or mainly of proteins, and ESI and EELS also revealed only a small amount of nitrogen in the reserves. The most abundant reserves of the vegetative cell are lipids, which accumulate in droplets or bodies. Many are small roundish vesicles carrying glycoproteins, localized mainly around lipid bodies or toward the periphery of the cytoplasm. They could be related to pollen wall development as well as to glycolipid accumulation or degradation. We interpret the PA-TCH-SP-positive bodies as partially lysed amyloplasts. The last type of reserve consists of small granules spread in the cytoplasm which are very rich in P and Ca. In view of their dimensions and composition they are considered phytin globoids. The presence of phytic acid has been reported in the pollen of many plants (Jackson et al., 1982; Butowt et al., 1997).

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REFERENCES

- BUTOWT R, RODRIGUEZ-GARCIA MI, ALCHE JD, and GORSKA-BRYLASS A. 1997. Calcium in electron-dense globoids during pollen grain maturation in *Chlorophytum elatum* R. Br. *Planta* 203: 413–421.
- CANINI A, LEONARDI D, RUGGERI S, CARNOVALI E, and GRILLI CAIOLA M. 2001. Intracellular localization of calcium, phosphorus and nitrogen in common bean seeds (*Phaseolus vulgaris* L. cv Borlotto) by SEM, ESI and EELS techniques. *Plant Biosystems* 135: 123–132.
- CRESTI M, CIAMPOLINI F, and KAPIL RN. 1984. Generative cells of some angiosperms with particular emphasis on their microtubules. *Journal of Submicroscopic Cytology* 16: 317–326.
- DAFNI A, and WERKER E. 1982. Pollination ecology of *Sterubergia clusiana* a (ker-gawler) spreng (Amaryllidaceae). *New Phytologist* 91: 571–577.
- DUNNIGAN MG. 1968. The use of Nile blue sulphate in the histochemical identification of phospholipids. *Stain Technology* 43: 249–256.
- FARRAGIANA T, and MARINOZZI V. 1979. Phosphotungstic acid staining of polysaccharides containing structures on epoxy embedded tissues. *Journal of Submicroscopic Cytology* 12: 263–265.
- GREENWOOD JS, and BEWLEY D. 1984. Subcellular distribution of phytin in the endosperm of developing castor bean: a possi-

- bility for its synthesis in the cytoplasm prior to deposition within protein bodies. *Planta* 160: 113–120.
- GRILLI CAIOLA M, and BRANDIZZI F. 1997. Pollen-pistil interactions in *Hermodactylus tuberosus* Mill. (Iridaceae). *Plant Biosystems* 131: 197–205.
- GRILLI CAIOLA M, BRANDIZZI F, and CANINI A. 2000. *Hermodactylus tuberosus* L. (Iridaceae) pollen organization before and after anther dehiscence. *Plant Biosystems* 134: 353–364.
- JACKSON JF, JONES G, and LINSKENS HF. 1982. Phytic acid in pollen. *Phytochemistry* 21: 1255–1258.
- JOHANSEN DA. 1940. *Plant microtechnique*. Mc Graw Hill, New York.
- KNOX RB, KENRICK J, JOBSON S, and DUMAS C. 1989. Reproductive function in the mimosoid legume *Acacia retinodes*: ultrastructural and cytochemical characteristics of stigma receptivity. *Australian Journal of Botany* 37: 103–124.
- MCCORMICK S. 1993. Male gametophyte development. *Plant Cell* 5: 1265–1275.
- PACINI E. 1996. Types and meaning of pollen carbohydrate reserves. *Sexual Plant Reproduction* 9: 362–366.
- PIFFANELLI P, ROSS JHE, and MURPHY DJ. 1998. Biogenesis and function of the lipidic structures of pollen grains. *Sexual Plant Reproduction* 14: 65–80.
- SELIGMAN A, WASSERKRUNG HL, and HANKER JS. 1966. A new staining method (OTO) for enhancing contrast of lipid-containing membranes and droplets in osmium tetroxide-fixed tissues with osmiophilic thiocarbonylhydrazide (TCH). *Journal of Cell Biology* 30: 424–432.
- SOLOFF BL. 1973. Buffered potassium permanganate-uranyl acetate-lead citrate staining sequence for ultrathin sections. *Stain Technology* 48: 159–165.
- TANAKA I. 1993. Development of male gametes in flowering plants. *Journal of Plant Research* 105: 55–63.
- THIÉRY J.P. 1967. Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *Journal of Microscopie* 6: 987–1018.
- VENABLE JH, and CÓGGESHALL R. 1965. A simplified lead citrate stain for use in electron microscopy. *Journal of Cell Biology* 25: 407–408.