Journal of Microscopy, Vol. 146, *Pt* 2, *May* 1987, *pp.* 157–162.
Received 6 *September* 1985; *revised* 6 *January* 1987; *accepted* 16 *January* 1987

A rapid methyl salicylate clearing technique for routine phase-contrast observations on female meiosis in *Solanum*

by E. JONGEDIJK, *Agricultural University Wageningen, Department of Plant Breeding (IvP), P.O. Box* 386, 6700 *AJ Wageningen, The Netherlands*

KEYWORDS. *Solanum*, clearing technique, methyl salicylate, phase-contrast microscopy, megasporogenesis, megagametogenesis.

SUMMARY

A rapid clearing technique, involving methyl salicylate clearing and phase-contrast microscopy, that enables large-scale observations on meiosis and embryo sac development in intact ovules of potato, was developed. With high resolution phase-contrast optics the details observed in cleared ovules were as well defined as in microtome sections and 'abnormalities' associated with desynapsis and 2n-megaspore formation were readily detected.

In potato this ovule clearing technique offers certain advantages over earlier clearing and staining–clearing techniques and permits bulk preparation of ethanol (70%) stored ovaries shortly (1·5–2 h) before observation.

INTRODUCTION

Conventional embedding–sectioning techniques have proven to be powerful tools in embryological research. Though they generally yield high-contrast preparations with excellent clarity and nicely maintain the topography of the tissue, they have some major constraints. Firstly, the production of microscopic slides is laborious. Secondly, the (quantitative) interpretation of the investigated phenomena is tedious because three-dimensional structures are often distributed over several sections and thus require reconstruction the full image.

Ovule clearing techniques have received renewed attention since a considerable improvement was achieved by Herr (1971). He developed a clearing-squash technique, involving a clearing fluid composed of lactic acid (85%), chloral hydrate, phenol, clove oil and xylene (2:2:2:2:1, w/w), suitable for phase-contrast observations in intact ovules of a wide range of plant species. In spite of the use of special optics, however, contrast and resolution have not been optimal for all species. In some, additional squashing of ovules (Herr, 1971; Smith, 1973; Rembert, 1977), changes in the clearing fluid formula and components (Herr, 1973a, b; George *et al.*, 1979; Franke, 1981), changes in the pretreatment of ovules (Herr, 1973a, 1985; Farence & Smith, 1975) or additional embedding–sectioning techniques (Lazarte & Palser, 1979) have been necessary to facilitate detailed observations, especially on megasporogenesis.

A second ovule clearing technique that could be used for observations on embryo sac development of Zephyranthaceae (Crane, 1978) and several grasses (Young *et al.*, 1979) involves methyl salicylate as the clearing agent and differential interference-contrast microscopy (Crane, 1978). Using methyl salicylate, staining–clearing techniques suited for normal bright field observations of female meiosis were recently put forth for *Medicago* (Pfeiffer & Bingham, 1983) and *Solanum* (Stelly *et al.*, 1984). These latter staining–clearing techniques do not require special optics and permit the production of high-contrast specimens. They still have, however, some serious drawbacks: it takes several days before preparations from ethanol-stored ovaries are available for quantitative analysis of meiotic stages, and the analysis itself is seriously hampered by frequently occurring overstaining of ovules as a consequence of size differences between ovaries and ovules within ovaries (Jongedijk, unpublished).

In potato these drawbacks could be overcome by the adoption of a simple methyl salicylate clearing technique, which includes the use of high resolution phase-contrast optics for observation.

MATERIALS AND METHODS

To evaluate the present clearing technique, observations on megasporogenesis and megagametogenesis in stained microtome sections and cleared ovules of diploid *Solanum tuberosum–S. phureja* hybrids (Jongedijk, 1985) were compared. To determine its potential for the detection of 'abnormalities' in megasporogenesis genotypes with 2n-megaspore formation and desynaptic mutants (Jongedijk, 1983; Ramanna, 1983) were included.

Both for clearing and sectioning, ovaries with different stages of ovule development were fixed in CRAF V (Berlyn & Miksche, 1976) for 24 h and next rinsed and stored in 70% ethanol.

For clearing, the ovaries were dehydrated through two 30 min rinses in absolute ethanol and then directly transferred into pure methyl salicylate. After 30–45 min the material was completely cleared. With the help of a dissecting microscope the ovary wall was removed, ovules were scraped off the placenta and mounted in a drop of methyl salicylate. If necessary, preparations could be made semi-permanent and kept for at least 2 years by sealing the coverslip edges with Canada Balsam (dissolved in xylene).

For microtome sectioning, the ovaries were dehydrated in a tertiary butanol–ethanol series (Gerlach, 1977) and embedded in Paraplast Plus (BDH). Sections were stained with safranin–fast green and mounted in Canada Balsam according to Gerlach (1977).

Microtome sections and cleared ovules were examined and photographed with bright field Köhler illumination using a Planapochromatic 40/1·0 oil immersion objective (Zeiss) and a Planapochromatic 63 PH3H/1·4 oil immersion objective (Zeiss) respectively. All photographs were taken with a Zeiss Photomicroscope II, equipped with an achromatic–aplanatic phase-contrast and interference-contrast condensor (N.A. 1·4) on Kodak Technical Pan Film 2415 using a blue or green filter.

RESULTS AND DISCUSSION

Depending on its developmental stage (the larger the ovules, the more easily they could be scraped off the placental tissue) cleared ovaries yielded 150–300 ovules suitable for analysis. As the risk of overstaining of ovules does not exist with the present technique, megasporogenesis and megagametogenesis could easily be analysed (Figs. 1A–I and 2A–E) in the majority of cases.

The successive stages of megasporogenesis and megagametogenesis (Figs. 1A, B, D, F, G, H and 2A, B, D respectively) could easily be recognized and deviations from the normal pattern associated with desynapsis (Figs. 1C, E, I) or 2n-megaspore formation (Fig. 2C) were readily detected. It is noteworthy to point out that even the origin (sexual or somatic) of additionally developing cells within the chalazal tissue of an ovule could be established (Fig. 2E). This is of particular importance for investigations on gametophytic apomixis, as

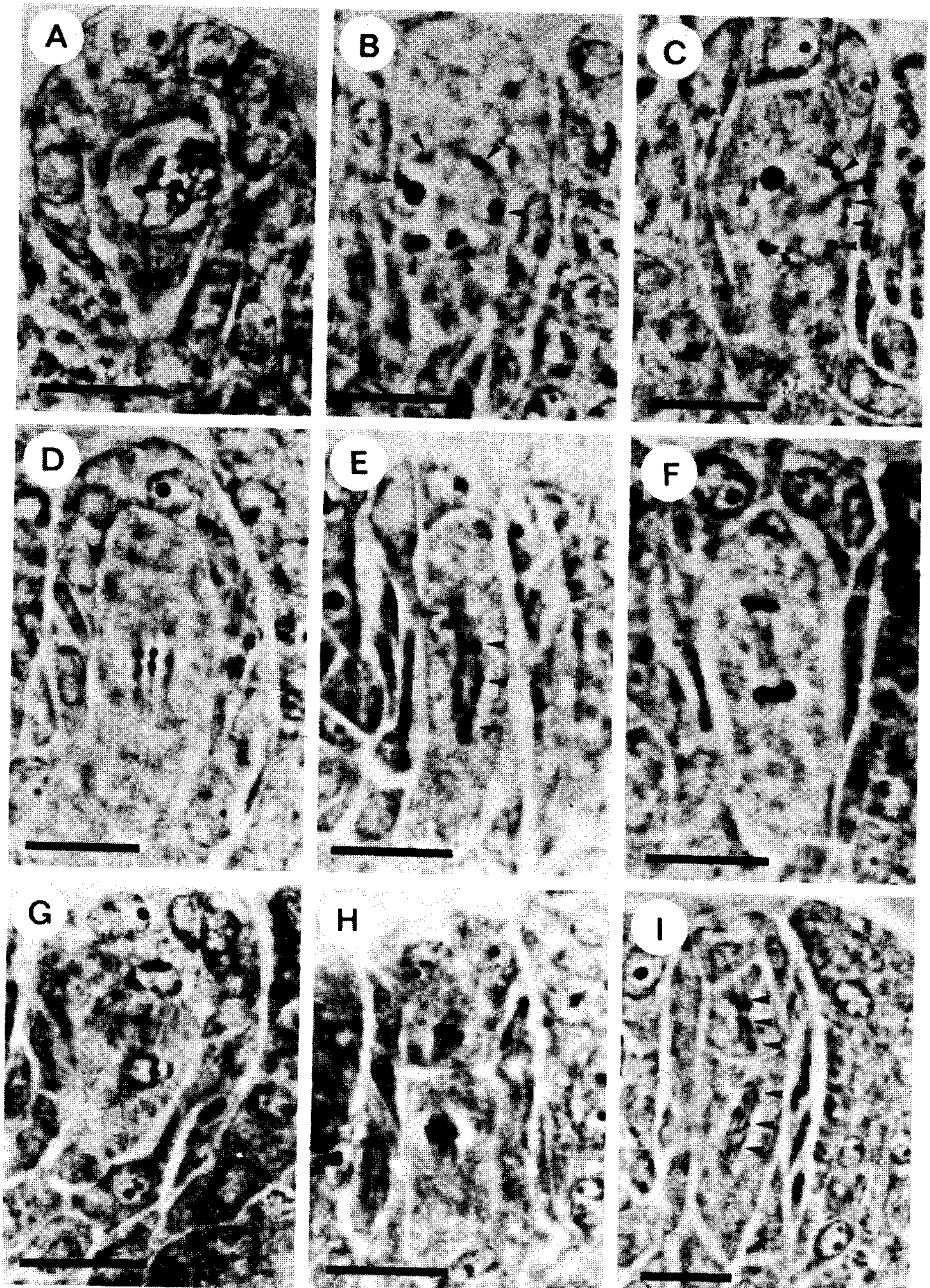

Fig. 1. Methyl salicylate cleared ovules showing different stages of megasporogenesis in diploid potato. Micropylar end at the top, bars represent 10 μm. (A) Pachytene. (B) Normal diakinesis; bivalents (arrows). (C) Desynaptic diakinesis; univalents (arrows). (D) Normal metaphase I; bivalents. (E) Desynaptic metaphase I; univalents (arrows) scattered. (F) Anaphase I. (G) Telophase I; cell plate formation. (H) Metaphase II. (I) Irregular metaphase II in a desynaptic plant.

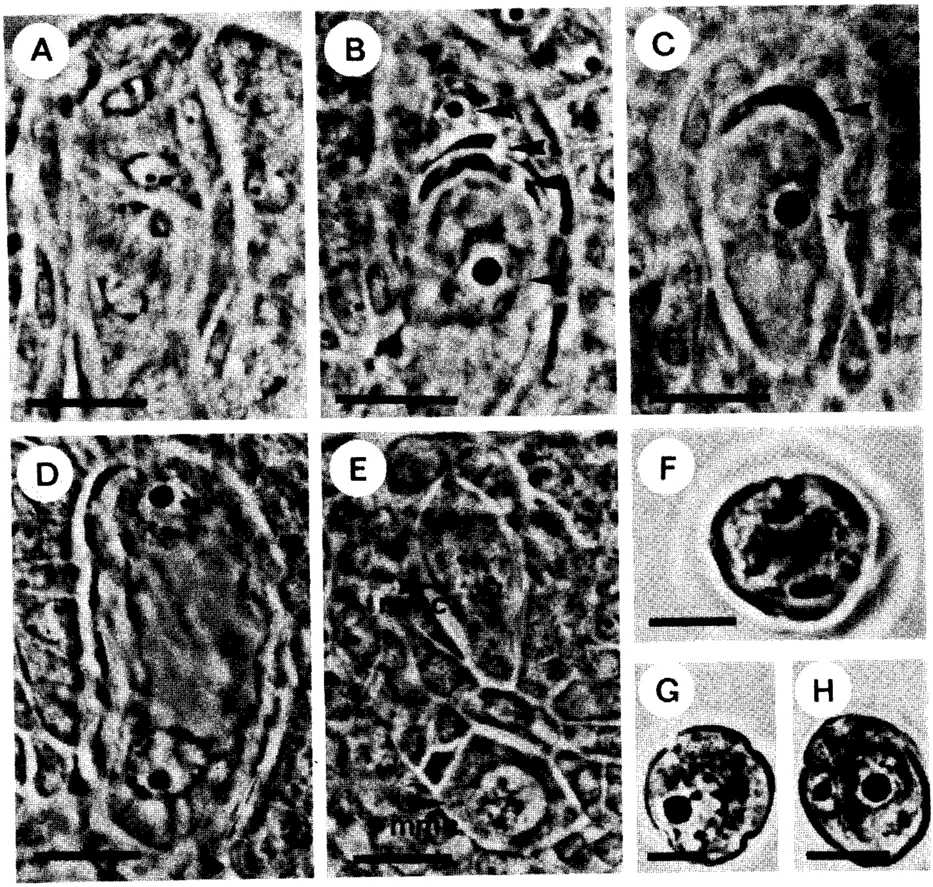

Fig. 2. Methyl salicylate cleared ovules (A–E) and pollen (F–H) showing different stages of megasporogenesis, embryo sac development and pollen development. Micropylar end at the top, bars represent 10 μm. (A) Telophase II; cell plate formation. (B) Tetrad of reduced (n) megaspores. (C) Dyad of unreduced (2n) megaspores. (D) 2-nucleate embryo sac. (E) Two non-adjacent megaspore mother cells (mmc); one mmc (sexual, pachytene) differentiated within the chalazal tissue. (F) First pollen mitosis; anaphase. (G) Uninucleate pollen; mitotic prophase. (H) Binucleate pollen.

it offers an opportunity to reliably trace the origin (sexual with diplospory and somatic with apospory) of additionally developing unreduced embryo sacs within the chalazal tissue.

Though shrinkage effects as a consequence of rapid dehydration and methyl salicylate infiltration as applied in this study may hamper cytological observations on megasporogenesis and megagametogenesis in many plant species, it did not do so in potato. In fact, even in embryo sac stages, which usually are most sensitive, shrinkage effects appeared to be minimal (Fig. 2D).

In Figs. 3A–F some stage of megasporogenesis and megagametogenesis as observed in microtome sections are shown. When these are compared to the phase-contrast images from cleared ovules it is obvious that the high contrast realized with the present technique competes well with that obtained in stained microtome sections. The same holds true for the maintainance of ovule topography. It proved to be the combination of excellent clearing properties of methyl salicylate, the maintenance of ovule topography and in particular the high contrast and resolution specifically obtained with the 63 PH3H/1·4 objective which

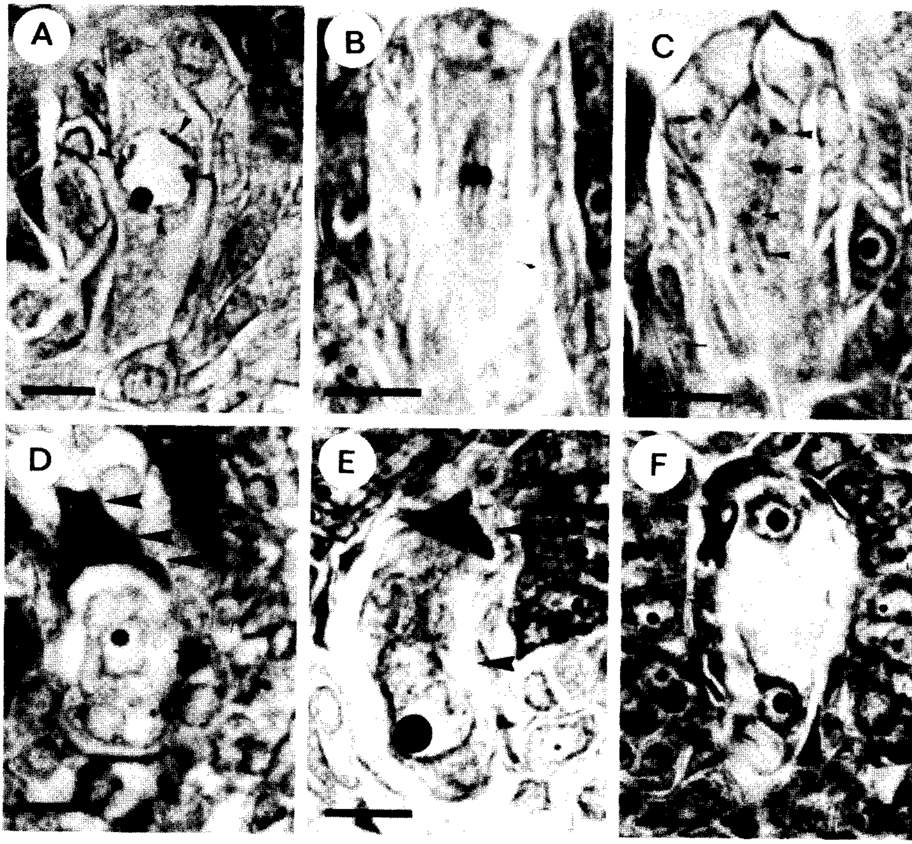

Fig. 3. Safranin–fast green stained sections of paraplast embedded ovules showing different stages of megasporogenesis and embryo sac development in diploid potato. Micropylar end at the top, bars represent 10 μm. (A) Diakinesis; bivalents (arrows). (B) Normal metaphase I; bivalents. (C) Desynaptic metaphase I; univalents (arrows) scattered. (D) Tetrad of reduced (n) megaspores. (E) Dyad of unreduced (2n) megaspores. (F) 2-nucleate embryo sac.

made the classification of megasporogenesis and megagametogenesis as easy in cleared potato ovules as in microtome sections. It should be mentioned that microsporogenesis and pollen development (Figs. 2F–H) could easily be analysed as well. The technique thus facilitates simultaneous study of micro- and megasporogenesis within a single flower bud.

The clearing effect of chemical agents may be caused by their high refractive index, their ability to dissolve the cell content or a combination of both (Gardner, 1975). The clearing effect of methyl salicylate is a consequence of its high refractive index (nD20=1·536–1·538), which apparently closely matches that of the cell walls (nD20 (cellulose)=1·55) in potato ovules. The relatively low refractive index of Herr's (1971) '4½-clearing fluid' might thus partly explain the poor results, which did not even approach those obtained by Herr (1971), when it was applied to potato ovules (Jongedijk, unpublished). Moreover, lactic acid, phenol and chloral hydrate are known to destroy the cytoplasm (Gardner, 1975) and thus negatively affect ovule topography and hamper cytological interpretation. Finally, the traces of water in the tissue (with Herr's technique the dehydration of ovules is far from complete) were found to reduce the clarity and contrast of preparations. With incomplete dehydration methyl salicylate cleared potato ovules were seriously blurred as well. Since the good contrast and resolution obtained with the 63 PH3H/1·4 objective was by far superior to that obtained with other phase-contrast objectives and differential interference-contrast microscopy, it seems justified to emphasize that the quality of the optical equipment is an essential part of clearing techniques and therefore deserves to receive serious consideration.

In potato the clearing technique adopted in this study overcomes the drawbacks imposed by earlier developed clearing and staining-clearing methods. The particular combination of methyl salicylate clearing and appropriate phase-contrast optics permits routine observations on megasporogenesis and megagametogenesis and bulk preparation of specimens from ethanol (70%) stored potato ovaries shortly (1·5–2 h) before observation.

ACKNOWLEDGMENT

I thank Professor J. G. Th. Hermsen, Professor J. Sybenga, Dr M. S. Ramanna and Dr J. H. de Jong for their critical comments on the manuscript.

REFERENCES

Berlyn, G.P. & Miksche, J.P. (1976) *Botanical Microtechnique and Cytochemistry*. The Iowa State University Press, Ames, Iowa.

Crane, C.F. (1978) Apomixis and crossing incompatibilities in some *Zephyranthaceae*. Ph.D. thesis, University of Texas, Austin. Cited in Young *et al.* (1979).

Farence, D.R. & Smith, B.B. (1975) Effects of chemical pretreatments on *Ludwigia alternifolia* L. ovules prior to immersion in Herr's clearing fluid. *Proc. Pa. Acad. Sci.* **49**, 89–91.

Franke, R. (1981) Eignung verschiedener Aufhellungsverfahren zur Beobachtung von Samenanlagen in intakten Fruchtknoten. *Arch. Züchtungsforsch.* **6**, 299–309.

Gardner, R.O. (1975) An overview of botanical clearing technique. *Stain Technol.* **50**, 99–105.

George, G.P., George, R.A. & Herr, J.M., Jr (1979) A comparative study of ovule and megagametophyte development in field-grown and greenhouse-grown plants of *Glycine max* and *Phaseolus aureus* (*Papilionaceae*). *Amer. J. Bot.* **66**, 1033–1043.

Gerlach, D. (1977) *Botanisch Mikrotechnik*, 2nd edn. George Thieme, Stuttgart.

Herr, J.M., Jr (1971) A new clearing-squash technique for the study of ovule development in angiosperms. *Amer. J. Bot.* **58**, 785–790.

Herr, J.M., Jr (1973a) The cytological effects of several fixatives on the ovules of *Cassia abbreviata* Oliver var. *granitica* Bak. *New Botanist*, **1**, 118–126.

Herr, J.M., Jr (1973b) An analysis of methods for permanently mounting ovules cleared in four-and-a-half type clearing fluids. *Stain Technol.* **57**, 161–169.

Herr, J.M., Jr (1985) The removal of phlobaphenes for improved clearing of sections and whole structures. (Abstract). *Amer. J. Bot.* **72**, 817.

Jongedijk, E. (1983) Selection for first division restitution 2n-egg formation in diploid potatoes. (Abstract). *Potato Res.* **26**, 399.

Jongedijk, E. (1985) The pattern of megasporogenesis and megagametogenesis in diploid *Solanum* species hybrids; its relevance to the origin of 2n-eggs and the induction of apomixis. *Euphytica*, **34**, 599–611.

Lazarte, J.E. & Palser, B.F. (1979) Morphology, vascular anatomy and embryology of pistillate and staminate flowers of *Asparagus officinalis*. *Amer. J. Bot.* **66**, 753–764.

Pfeiffer, T.W. & Bingham, E.T. (1983) Abnormal meiosis in alfalfa, *Medicago sativa:* cytology of 2n egg and 4n pollen formation. *Can. J. Genet. Cytol.* **25**, 107–112.

Ramanna, M.S. (1983) First division restitution gametes through fertile desynaptic mutants of potato. *Euphytica*, **32**, 337–350.

Rembert, D.H., Jr (1977) Ovule ontogeny, megasporogenesis and early gametogenesis in *Trifolium repens* (Papilionaceae). *Amer. J. Bot.* **64**, 483–488.

Smith, B.B. (1973) The use of a new clearing technique for the study of early ovule development, megasporogenesis and megagametogenesis in five species of *Cornus* L. *Amer. J. Bot.* **60**, 322–338.

Stelly, D.M., Peloquin, S.J., Palmer, R.G. & Crane, C.F. (1984) Mayer's hemalum–methyl salicylate: a stain-clearing technique for observations within whole ovules. *Stain Technol.* **59**, 155–161.

Young, B.A., Sherwood, R.T. & Bashaw, E.C. (1979) Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. *Can. J. Bot.* **57**, 1668–1672.