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A QUICK ENZYME SQUASH TECHNIQUE FOR DETAILED STUDIES ON FEMALE MEIOSIS IN *SOLANUM*

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ABSTRACT. A simple enzyme squash technique that enables detailed studies of meiosis in potato ovules has been developed. Fixation of ovules in iron-propionic-ethanol followed by enzymatic maceration and squashing in acetocarmine yielded numerous well preserved megasporocytes with nicely spread chromosomes. Resolution was sufficient, allowing detailed analysis of chromosome pairing and chiasma formation and readily permitting distinction between normal and desynaptic mutant plants. Whereas the use of previously developed ovule squash techniques has been restricted to cytogenetic analyses of plant species with relatively large megasporocytes and large chromosomes, the present technique is potentially more useful for analyses of species with small megasporocytes and small chromosomes.

Information on meiotic chromosome pairing and chiasma formation in angiosperms is largely based on analyses of microsporocytes. The relative paucity of cytogenetic information on megasporocyte meiosis may be attributed to the following: (i) microsporocytes are produced in much larger quantities than megasporocytes and (ii) the technical difficulties of preparing and analyzing megasporocytes. Preparation and analysis of megasporocyte meiotic specimens by conventional embedding-sectioning techniques is laborious, and cytogenetic analyses are complicated because three-dimensional structures are often distributed over several sections, thus requiring reconstruction of the full image. Recently developed staining-clearing (Stelly *et al.* 1984) and clearing (Herr 1971, Jongedijk 1987) techniques facilitate preparation of intact ovules but fail to yield specimens amenable to detailed analyses of karyotype, chromosome pairing, or chiasma formation.

Most ovule squash techniques developed to date basically consist of hydrochloric acid maceration and staining with Feulgen (Hillary 1940), acetocarmine (Bradley 1948, D'Cruz and Reddy 1967), acetic-lacmoid (Haque 1954) or acetic-orcein stains (Darlington and La Cour 1966). Though they have successfully been applied to study early megagametophyte development in a variety of plant species, their use for detailed observations on chromosome pairing and chiasma formation in megasporogenesis has been restricted to a limited number of plant species with large megasporocytes and large chromosomes (Darlington and La Cour 1966, Sharma and Sharma 1972). Improved squash techniques applicable to species with small chromosomes would thus be desirable. A favorable cytological effect of enzymatic maceration was first noted by Emsweller and Stuart (1944), who used 1% clarase to improve the spreading of chromosomes in microsporocyte squashes of tetraploidized *Lilium longiflorum*. Enzymatic maceration has since been used to enable

detailed chromosome studies in microsporocytes (Narayan 1976, Stack 1982, Loidl 1984), root tips (McKay and Clarke 1946, Chayen and Miles 1954, Setterfield *et al.* 1954, Schwarzacher *et al.* 1980, Pijnacker and Ferwerda 1984) and megagametophytes (Forbes 1960) when appropriate spreading could not be achieved by "standard" techniques. Its use to enable cytogenetic analyses of megasporocyte meiosis in species with small chromosomes, however, has not been reported.

In this article a simple technique for preparing squashes of enzyme-macerated ovules that enables detailed cytogenetic analysis of megasporocyte meiosis in a genus with small chromosomes (*Solanum*) is reported.

MATERIALS AND METHODS

To evaluate the merits of the present enzyme squash technique, megasporogenesis was studied in diploid ($2n = 2x = 24$) *Solanum tuberosum*-*S. phureja* hybrids (Jongedijk 1985). To determine its potential for the detection of "abnormalities" in chromosome pairing and chiasma formation both genotypes with normal chromosome synapsis (*Ds.*) and desynaptic mutants (*dsds*) (Jongedijk 1983, Ramanna 1983) were included.

Fixation. Flower buds were fixed in a freshly prepared solution of either propionic acid (saturated with ferric acetate) and ethanol (1:3, v/v) or glacial acetic acid and ethanol (1:3, v/v-Carnoy) for at least 48 hr (4–5 C) up to several months (–20 C). Removal of the calyx, corolla and anthers improved the penetration of the fixative.

Maceration. Under the dissecting microscope intact placentas were removed from the fixed buds and isolated in a drop of fixative. The placentas were rinsed twice for 20 min in a 0.1 M citric acid-sodium citrate buffer (pH 4.4–4.8) and macerated in a solution 10% with respect to pectinase (Sigma P-5146) and 1.5% with respect to cellulase (Onozuka R-10) in citrate buffer (pH 4.4–4.8) at 37 C for 4–5 min. The enzyme solution was subsequently removed from the tissue by two 20 min rinses with the citrate buffer.

Preparation of slides. With a Pasteur pipette $\frac{1}{3}$ – $\frac{1}{5}$ of a single placenta was transferred to a clean slide, excess buffer removed and a drop of 2% acetocarmine added. The tissue was next carefully divided into small pieces, slightly warmed over a gas flame and left for 1–2 min. A coverslip was then added and the tissue was gently squashed until the cells were well separated and evenly distributed over the slide. After this the slides were heated without boiling over a gas flame, left for 1–2 min on a plate at 30–40 C and further squashed by pressing the coverslip under filter paper without slipping until satisfactory spreading of megasporocytes was achieved. When necessary, preparations were made permanent by keeping them overnight in a mixture of *n*-butyl alcohol and glacial acetic acid (3:1, v/v), followed by a 30–60 sec immersion of the detached coverslip and the slide in absolute *n*-butyl alcohol and remounting in Euparal.

Besides the pressure applied to the coverslip, the degree of spreading of megasporocytes depends on the amount of stain used and the amount of

material on the slide. Removing as much debris as possible while isolating placentas, avoiding excess stain and especially squashing only small pieces of a placenta at a time significantly improved spreading. The staining of chromosomes has been consistently better after iron-propionic-ethanol fixation.

All stages of megasporogenesis were examined and photographed with bright-field Köhler illumination using a Zeiss Planapochromatic 63 PH3H/1.4 oil immersion objective. Photographs were taken with a Zeiss Photomicroscope II equipped with an achromatic-aplanatic phase-contrast and interference-contrast condenser (N.A. 1.4) on Kodak Technical Pan Film 2415 using a blue or green filter.

RESULTS AND DISCUSSION

Ovule squash techniques developed to date have not permitted detailed studies of chromosome pairing and chiasma formation in megasporocytes of species with small chromosomes. Small ovules are difficult to handle and megasporocytes generally fragment or severely distort upon squashing before cells and chromosomes are sufficiently spread. Although many of the megasporocytes were inevitably lost for analysis with the present enzyme squash technique as well, a considerable number of well preserved and sufficiently spread megasporocytes per slide were obtained. Up to 40% of all megasporocytes could be analyzed in the best preparations.

Meiotic cells, especially those with first division stages, were easily distinguished from somatic cells by their relatively large size and characteristic appearance (Fig. 1A). They frequently were found associated with respective groups of nucellar cells, which were held together by the undigested cuticle.

The different stages of megasporogenesis were readily recognized, and the extent of chromosome pairing and chiasma formation easily determined. Comparison of corresponding stages from a normal synaptic plant and a desynaptic mutant (Figs. 1B–D and 2A–C, respectively) demonstrated that in megasporogenesis, desynapsis is characterized by normal chromosome pairing through pachytene and a falling apart of bivalents by the time of diakinesis. A similar pattern of expression was previously noted in microsporogenesis (Ramanna 1983).

When compared to first division stages, second division stages are more transient and not as easily detected because of the smaller cell size. In addition, the interpretation of second division stages is seriously hampered as the two daughter cells formed after completion of the first meiotic division only rarely remain side by side. This presented a problem in deciding whether or not such cells are derived from the same megasporocyte. In plant species with tetrasporic embryo sac formation (*Lilium*, *Fritillaria* and others) such problems will not arise, however, as no cell wall is formed after completion of the first meiotic division.

As far as quantitative analysis of chromosome pairing and chiasma formation is concerned, it should be emphasized that in plant species with multiovular ovaries (such as potato) female meiosis generally is highly asynchronized. To

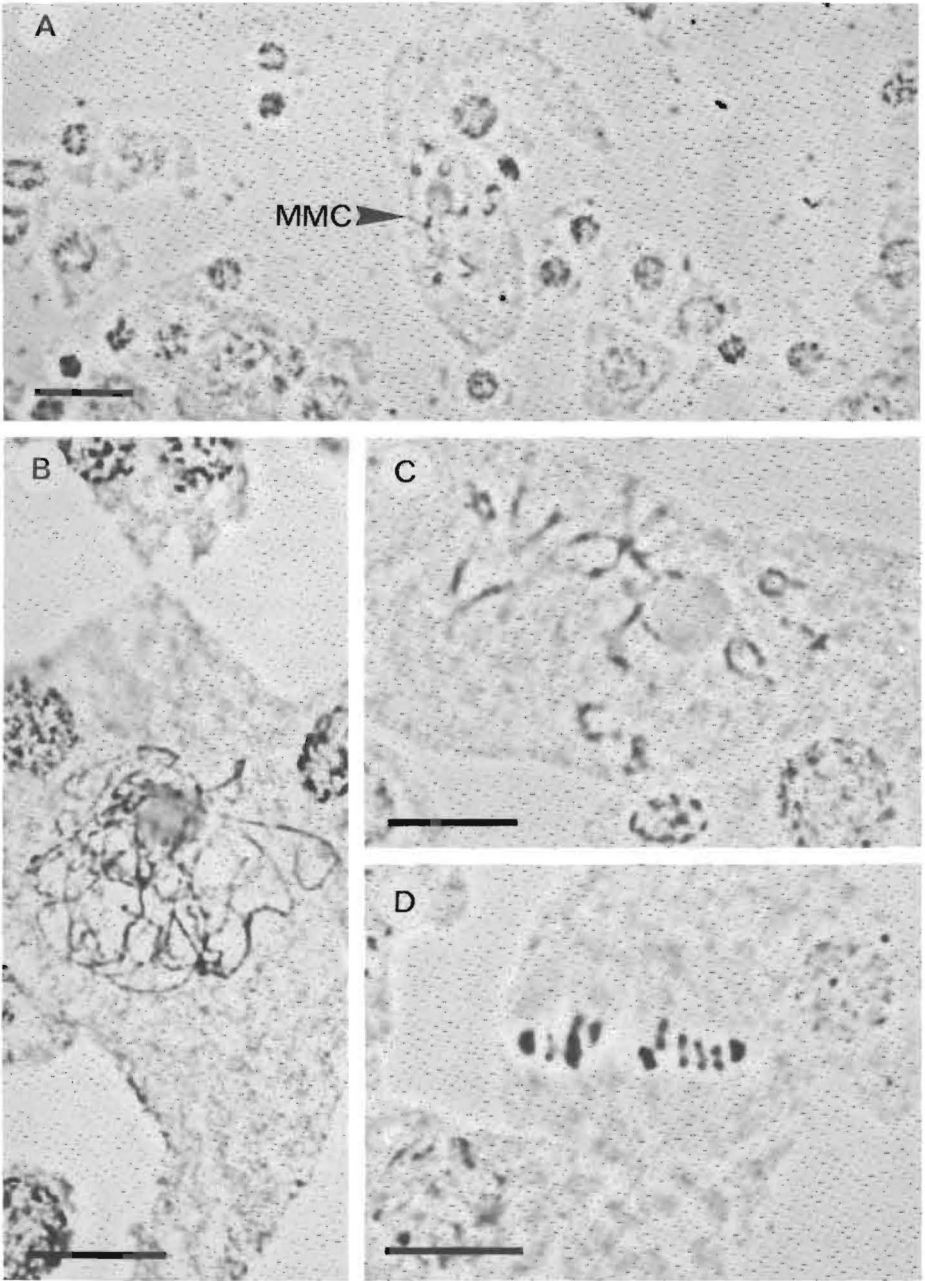


FIG. 1A-D. Acetocarmine squashes of enzyme digested ovules showing different stages of megasporogenesis in a diploid potato clone with normal synapsis. A) Typical megaspore mother cell (MMC) at diakinesis. B) Pachytene; normal chromosome pairing. C) Diakinesis; ring and rod bivalents. D) Metaphase I; ring and rod bivalents congregated at equatorial plate. Bars represent 10 μm .

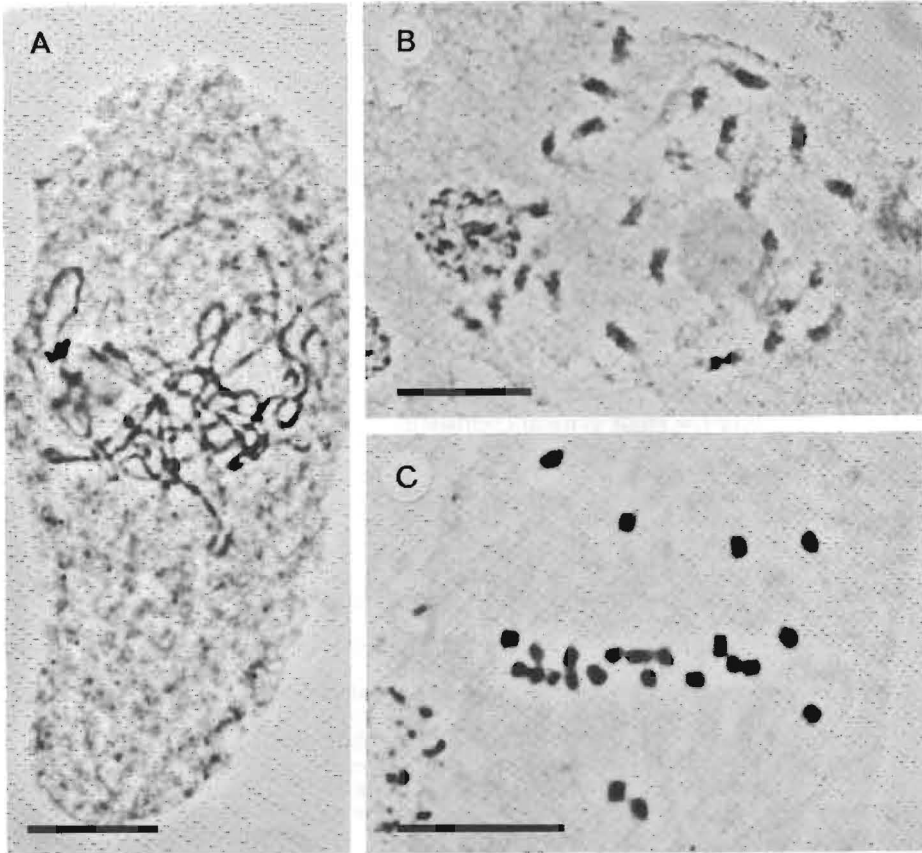


FIG. 2A-C. Acetocarmine squashes of enzyme digested ovules showing different stages of megalporogenesis in a desynaptic, diploid potato clone. A) Pachytene; normal chromosome pairing. B) Diakinesis; predominantly univalents. C) Metaphase I; predominantly univalents, note the predominant congregation of univalents at equatorial plate. Bars represent 10 μ m.

obtain a sufficient number of meiotic cells at a particular stage it may therefore be necessary to prepare several placentas, especially if the meiotic stage to be analyzed tends to be transient.

The success of the present technique for potato probably results from the enzymatic maceration procedure. In most earlier ovule squash techniques hydrochloric acid was used for maceration. Hydrochloric acid, in dissolving the pectic salts of the middle lamella, ruptures the connection between cells, but cell walls, though softened, and the elasticity of the cytoplasm are largely maintained. With the pectinase-cellulase solution used for maceration in this study both middle lamellas and cell walls are digested and the elasticity of the cytoplasm is largely destroyed. Cell walls and cytoplasmic elasticity are known to thwart the flattening of cells and spreading of chromosomes upon squashing (Emsweller and Stuart 1944, Narayan 1976). While hydrochloric acid maceration methods may suffice for plant species with large chromosomes, the

enzyme squash technique reported here seems much more appropriate for species with small chromosomes, since flatter preparations are needed for equivalent cytogenetic observations.

Finally, it should be mentioned that the present technique is expected to be of limited usefulness in the analysis of megagametophyte development. Megagametophytic stages generally are so large and the cytoplasm is so highly vacuolated that even gentle squashing causes their collapse. In that case, however, the use of recent "protoplast" techniques for the isolation of intact mature megagametophytes (Zhou and Yang 1982, Zhou 1985, Hu *et al.* 1985) might be considered.

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