Improved Nuclear Staining of Asparagus Microspores for Cytological Analysis

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Abstract. Clear visualization of asparagus (*Asparagus officinalis* L.) microspore nuclei with common stains such as acetocarmine or DAPI is difficult, hindering cytological analyses. The addition of saturated aqueous ferric chloride solution to Carnoy's I fixative (30 $\mu\text{L}\cdot\text{mL}^{-1}$) resulted in clear visualization of nuclei. A distinct nucleus was observed in uninucleate cells and the vegetative and generative nuclei were clearly visible in binucleate microspores. This method can be used reliably for determination of asparagus microspore developmental stage. Chemical name used: 4',6-diamidino-2-phenylindole-2HCL (DAPI).

When producing doubled-haploid plants through anther or microspore culture, microspore developmental stage at the time of anther excision and culture is critical for embryo formation and plantlet regeneration (Pechan and Keller, 1988; Prakash and Giles, 1987). Acetocarmine squashes are used routinely with fresh anthers to determine microspore nuclear developmental stage; however, this procedure is not appropriate for asparagus due to the thick microspore exine and many starch grains in the cytoplasm. Fixation of asparagus anthers in a glutaraldehyde fixative (GF) allowed faint staining of the nucleus with carmine stains (Feng and Wolyn, 1991). GF generally is not recommended for squash methods, and the fixation procedure involves time-consuming dehydration of anthers with a series of ethanol concentrations. Also, GF is not suitable for fluorescent staining, an important tool in studying the nuclear development of the microspores. We here report that the addition of ferric chloride to the anthers in Carnoy's I fixative permits very clear staining of the nuclei in asparagus microspores with acetocarmine and the fluorescent stain DAPI.

Materials and Methods

Plants of asparagus genotype G203 were grown in a growth room at 22 °C day/16 °C night. Flower buds of varying sizes (1.8–3.0 mm) were collected and used for fixation. Two anthers from each bud were placed in each of three fixative treatments:

1) Carnoy's I fixative (Sharma and Sharma, 1980) containing 3 absolute ethanol : 1 glacial acetic acid (v/v), for 24 h.

2) Carnoy's II fixative (Sharma and Sharma, 1980) containing 6 absolute ethanol : 3 chloroform : 1 glacial acetic acid (v/v), for 24 h.

3) Carnoy's I fixative for 30 min, followed by addition of saturated aqueous solution (30 $\mu\text{L}\cdot\text{mL}^{-1}$) of ferric chloride (4 g·mL⁻¹) to fixative-containing anthers, and incubation for an additional 23.5 h.

After fixation, anthers were transferred to 70% ethanol and stored at 4 °C for all treatments.

For acetocarmine squashes, the anthers were placed on a slide in a drop of stain (Gurr,

1963), then cut open, and the microspores were teased gently from the anther wall tissue. A coverslip was placed over the microspores and then the slide was heated for 1–2 s over a flame, blotted on a bibulous paper, and viewed with a light microscope.

For fluorescent staining, the DNA-specific fluorochrome DAPI was used. The stock solution (1 mg·mL⁻¹ in distilled water) was stored in darkness at –20 °C. Stock solution (5 μL) was diluted in 1 mL of McIlvaine's citrate-phosphate buffer at pH 7.0 (Troyer, 1980) to prepare the working solution. Anthers were placed on the slide in a drop of stain and cut open to release the microspores. The slides were kept in darkness for 10 min. The anthers were removed and a coverslip was applied. The slides were observed with a fluorescence microscope (Nikon, Tokyo). For each of the fixation methods, 30–40 anthers were examined.

Results and Discussion

Binucleate microspores that were stained after fixation in the standard Carnoy's I and II fixatives did not show a clear distinction between the nucleus and the cytoplasm with the acetocarmine stain, although a faint generative nucleus occasionally was present (Fig. 1 A and B). In uninucleate microspores, the cytoplasm was dark and the nucleus was not visible (not shown). The addition of ferric chloride to Carnoy's I fixative markedly improved the nuclear staining of the asparagus microspores. Uninucleate microspores stained

Fig. 1. Staining of asparagus microspores with acetocarmine. (A) binucleate microspore fixed with Carnoy's I fixative; (B) binucleate microspores fixed with Carnoy's II fixative; (C–E) fixation with Carnoy's I fixative plus ferric chloride; (C) uninucleate microspores; (D) binucleate microspores with empty exine shells (arrows); (E) binucleate microspore extruding from the exine. Bar = 40 μm .

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Fig. 2. Staining of asparagus microspores with 4',6-diamidino-2-phenylindole-2HCl (DAPI). (A) binucleate microspores fixed with Carnoy's I fixative; (B) binucleate microspores fixed with Carnoy's I fixative plus ferric chloride. Bar = 20 μ m.

with acetocarmine showed a distinct nucleus (Fig. 1C), while the vegetative and the generative nuclei were both visible in the binucleate microspores (Fig. 1D). In many binucleate microspores, the exine was present as an empty shell (Fig. 1D), or the intact cellular contents were partially extruded from the exine (Fig. 1E). In both cases, cells were larger than those of microspores not fixed with ferric chloride. Despite these changes, distortion of the cellular contents was absent. In anthers fixed with the standard Carnoy's fixative, all the binucleate microspores were intact.

Study of the nuclear development in the cultured microspores is facilitated with fluorescent staining. Asparagus microspores have thick autofluorescent walls that usually prevent visualization of the nuclear material. When using the standard fixatives (Carnoy's I and II) and DAPI staining, nuclei were not visible in most of the microspores, but a faint generative

nucleus sometimes could be seen in a binucleate microspore (Fig. 2A). In anthers that were fixed with ferric chloride and stained with DAPI, autofluorescence was absent, the generative and the vegetative nuclei were visible, and the exine appeared to have ruptured (Fig. 2B).

Staining of uninucleate and binucleate microspores was enhanced with the addition of ferric chloride to the fixative. The exines of binucleate microspores ruptured, while those of uninucleate microspores remained intact with this treatment. Ferric chloride likely enhanced uptake of the stain by the intact uninucleate microspores. Since the improved staining of binucleate microspores with ferric chloride was always associated with exine rupture, one can not determine if exine rupture was critical for enhanced staining of binucleate microspores, or if the ferric chloride treatments enhanced staining of these microspores

regardless of exine integrity. Very few reports exist regarding the use of iron in fixatives. Marks (1952) and McGoldrick et al. (1954) included small amounts of iron acetate in fixative for anthers of species with small chromosomes and for flower buds of *Cucurbita* species. Belling (1926) used a rusted needle during acetocarmine staining and observed that the iron acted as a mordant for the stain and promoted differentiation of the nuclear material and the cytoplasm. It is difficult to quantify the amount of iron required from a needle, and unplated iron needles are not readily available today. In this research the use of ferric chloride in the fixative enabled routine, rapid, and precise observation of nuclear stages in asparagus microspores.

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