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Rapid Assesment of Microspore Development Stage in Pepper Using DAPI and Ferric chloride

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Abstract

Clear visualization of pepper (*Capsicum annuum* L.) microspore nuclei with common stains such as acetocarmine or propionocarmine is difficult, hindering cytological analysis. The DAPI stain after the addition of ferric chloride solution to fixative resulted in clear visualization of nuclei. For clear visualization of nuclei and slight fluorescence of microspore wall, addition of 40-60 μL of ferric chloride solution to the 1 mL fixative was identified as most effective. At all stages of gametophytic developemt, the nuclei can be distinctly visualized. Starch granules does not interfere with the fluorochrome, and so the vegetative and generative nuclei were cleary visible in binucleate pollens. With its rapidity and reliability, this technique represents an efficient tool for routine staging or investigation of the nuclear status of the microspore during culture.

Introduction

When producing 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 (Gupta and Borthakur, 1987; Pechan and keller, 1988; Willcox et al., 1991). In order to understand the process of embryogenesis and callus formation in anther and microspore cultures, cytological studies must be conducted. Acetocarmine squashes are used routinely with anthers to determine microspore nuclear developmental stage and to study the cytological changes during microspore embryogenesis. However, this procedure is not appropriate for most plants due to the thick exine and many starch grains in the cytoplasm. And also acetocarmine-based methods are somewhat inconsistant and nonspecific, especially when they are applied to the late stages of pollen development.

Gupta and Borthakur (1987) suggested a staining technique using haematoxylin and iron-alum. The microspore nucleus of rice when stained in iron-alum-haematoxylin, assumed a deep grey color against the colourless cytoplasm. Uninucleate microspore and binucleate pollen stages were distinctly visible. Nevertheless, researchers have not found even this method very satisfactory because successive trials have to be performed before the desired contrast to be obtained. Using the Feulgen stain (Kott et al., 1988), the nucleus can be seen distinctly, but this technique is time-consuming and tedious, and hence may have only a limited application, especially when a large number of anthers are to be plated every day.

The use of DNA-specific fluorescent dye presents an alternative way to visualize pollen nuclei (Coleman and Goff 1985; Hough et al., 1985; Heslop-Harrison, 1992). Mature and living germinating pollen can be stained with mithramycin or 4'-6-diamidino-2-phenylindol (DAPI). The same probe has been used to characterize microspore and pollen nuclear division in *Brassica* (Singh et al., 1985; Fan et al., 1988) and asparagus (Peng et al., 1997) after fixation and also to study microspore development in cultured maize anthers (Pace et al., 1987; Pescitelli

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and Petolino, 1988). In addition, Otto and Tsou (1985) have recommeded DAPI for routine DNA measurements after a comparison of several fluorochromes. Nevertheless, there is no evidence that DAPI can readily pass through the thick membranes of intact microspores or pollen grains. Membrane permeabilization by Triton X-100 incorporated in the staining solution improved nuclear staining of corn and wheat microspore (Vergne et al., 1987). Recently, Ziauddin et al. (1997) reported that the addition of saturated aqueous ferric chloride solution to fixative resulted in clear visualization of nuclei in asparagus microspores.

For pepper pollen, no satisfactory technique is available in anther staging and cytological studies, because thick exines and dense cytoplasm filled with starch grains prevent visualization of nuclei with conventional stain such as acetocarmin. Accordingly, some problems are encountered when looking for a rapid and reliable assessment of the pollen stage.

In the present paper, we report that the addition of ferric chloride to the anthers in Canoy's fixative permits very clear staining of nuclei in pepper microspores with fluorescent stain DAPI. With this staining method, the pattern of pollen development from the microspore mother cell to the mature pollen grain was elucidated.

Materials and Methods

Plant materials

Plants of *Capsicum annuum* L. (var. Milyang-jare) were raised from seeds sown in soil in 15cm diameter plastic pots and kept in a growth chamber at 25/23℃ (day/night) under an 18h photoperiod provided by metal halide and cool white fluorescent lamps. Two weeks after germination, seedlings were transplanted in 21cm diameter pots and maintained with regular nutrients feeding under the same light and temperature conditions.

Staining of microspore

Anthers from flower buds of varying size were collected and fixed for 30 min in Carnoy's fixative containing 3 absolute ethanol : 1 glacial acetic acid (v/v) and transferred to 70% ethanol.

For propionocarmine squashes, the anthers were placed on a slide in a drop of stain, then cut open, and the microspores were teased gently from the anther wall tissue. A coverslip was placed over the microspores and then slide was heated for 1-2 s over a flame, blotted on a filter paper, and observed with a light microscope. If necessary, gentle pressure was applied on the coverslip to bring chromosomes into the same plane so that focusing helped.

For fluorescent staining, the DNA-specific fluorochrome 4'-6-diamidino-2-phenylindol-2HCl (DAPI, Sigma D-8417) was used. The DAPI solution was made according to the procedure of Coleman and Goff (1985). The DAPI was stored at 4℃ as a 1 mg/mL stock solution in distilled water. One microliter of stock solution was diluted in 1 mL distilled water to prepare working solution. Anthers were quickly dissected in this solution to release the microspores, and anther debris was removed. Microspres were incubated for 10-30 min in a humid chamber in darkness and a coverslip applied. Observations were made with a Olympus fluorescence microscope equipped with a 100 W high pressure mercury burner and U filter (main wave length 365 nm). Selected microspores were photographed using T-Max 400 film and developed with HC-110.

Triton X-100 was selected as the permeabilizing agent for microspore and pollen grains. The detergents was incorporated in the staining solution described by Vergne et al. (1987). One microliter of DAPI stock solution was diluted in 1 mL of 1% Triton X-100. Microspores were observed after being incubated for 15-30 min in a humid chamber in darkness.

For ferric chloride treatment, Ziauddin method was (1997) modified. A stock solution of 40% ferric chloride in distilled water was prepared and 300 μL of stock solution was diluted in 10 mL Carnoy's fixative to prepare working solution. Anthers were fixed in 1 mL Carnoy's solution for 30 min, followed by addition of 10-400 μL of ferric chloride solution to fixative containing anthers and incubated for I days. After fixation, anthers were transferred to 70% ethanol and stored at 4℃ for DAPI stain.

Results and Discussion

Effects of Ferric Chloride on DAPI Staining

Microspores stained with propionocarmine did not show a distinct nucleus. In many vacuolate microspores, the nucleus did not visible and seemed like empty. Binucleate pollens did not show a clear distinction between the nucleus and cytoplasm, although a faint generative nucleus was occasionary present (Figure 1A). When using the DNA-specific fluorochrome DAPI, nuclear stain was much better than the propionocarmine. However, pepper microspores have thick autofluorescent walls that usually prevent visualization of the nuclear material (Figure 1B).

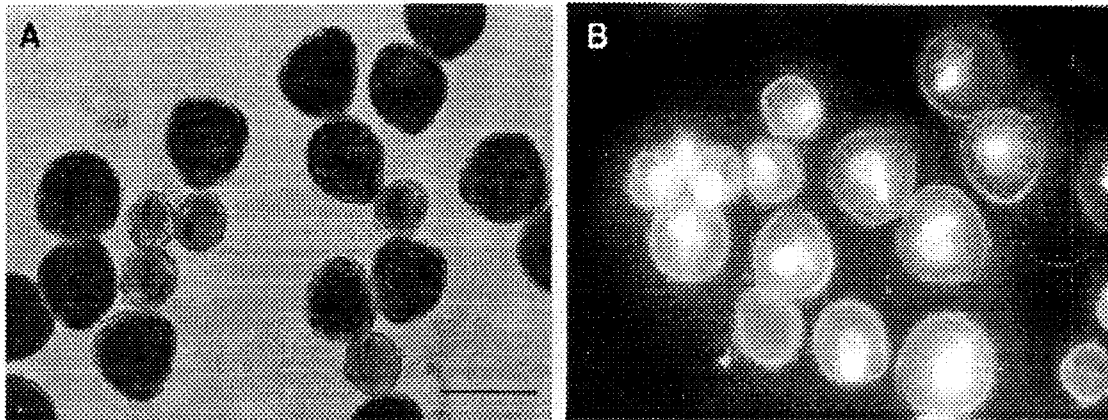

Figure 1. Staining of pepper pollens with propionocarmine and DAPI. (A) Propionocarmine staining of pollens. Binuclaeate pollens did not show a distinction between the nucleus and cytoplasm. (B) DAPI staining of pollens. Intensive wall autofluorescence appears. Scale bar (30 μm) in Figure A also applies to Figure B.

Table 1. Relative DAPI staining in microspore after the addition of different amount of ferric chloride solution to 1 mL fixative.

Ferric chloride (μL)	Nuclear staining	Wall auto-fluorescence
0	+	++
10	+	++
20	+	++
40	++	+
80	++	+
200	+	-
300	+	-
400	±	-

Symbols for ++, +, ± and - represents intense, detectable, obscure and none, respectively.

For membrane permeabilization, 1% Tritone X-100 was incorporated in the DAPI solution. However, Triton-X 100 in staining solution did not show any difference in both membrane permeabilization and nuclear visualization (data not shown).

To investigate the effects of ferric chloride on DAPI staining of microspore, 10-400 μL of ferric chloride solution was added to fixative containing anthers. The addition of 40-80 μL ferric chloride mrkedly improved microspore staining, but 10-20 μL and 200-400 μL addition did not improve microspore staining (Table 1). In anthers fixed with 10-20 μL ferric chloride and stained with DAPI, wall autofluorescence and nuclear staining were similar with untreated microspores (Figure 2A), while in anthers fixed with 200-400 μL ferric chloride, wall autofluorecsence was absent, and so, the outlines of the

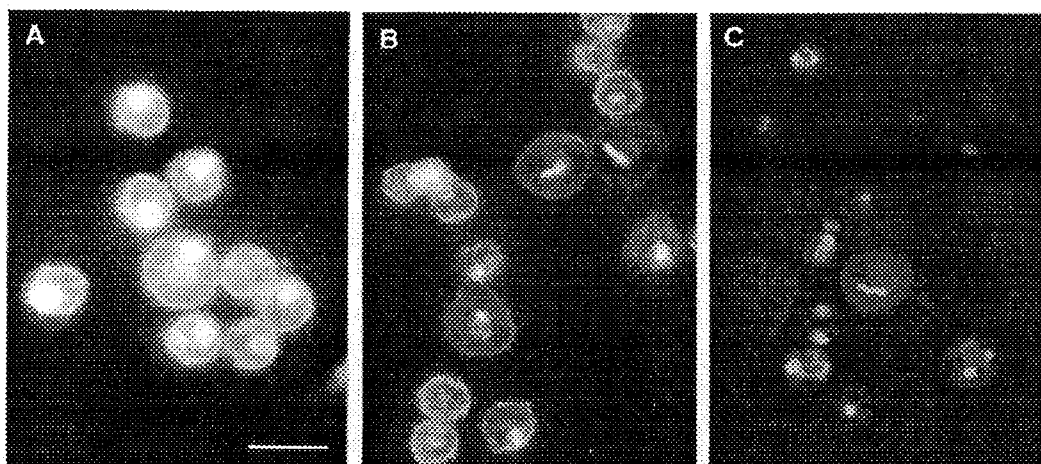

Figure 2. DAPI staining of pollen with ferric chloride treatment. (A) Pollens fixed with carnoy's fixative plus 20 μL ferric chloride; wall autofluorescence is almost same as control. (B) Pollens fixed with carnoy's fixative plus 40 μL ferric chloride; nuclei are very distinctive and the outline of pollen wall is clearly defined. (C) Pollens fixed with carnoy's fixative plus 300 μL ferric chloride; No wall autofluorescence presents and only faint nuclei appears. Scale bar (30 μm) in Figure A applies to all the figures.

wall were not defined (Figure 2C). In anthers that were fixed with 400 μL ferric chloride, only faint nuclei could be seen. When 40-80 μL of ferric chloride was added to the fixative, microspore exhibited brilliant nuclei, and the outlines of the walls are satisfactory defined by their slight autofluorescence (Figure 2B). Consistant nuclear visualization was achieved after 10-20 min of incubation. In the vacuolated microspore, the low-nuclear-cytoplasmic ratio prevents an immediate stain and a minimum 30 min incubation step is required before the observation of nuclear features. DAPI with ferric chloride treatment stains microspore nuclei very quickly, and the nucleus become very distinctive. Moreover, the bulk of microspores and pollen grains in one slide take up the stain uniformity as shown in Figure 1B, so that it is possible to investigate stage distribution within a anther.

The stain generally used in microspore staging is acetocarmine which in our experience dose not stain the nucleus and cytoplasm differentially (Kim and Kim, 1984). The visibility of the microspore nuclei in *Brassica napus* become slightly better when propionocarmine staining was employed (Kim, 1992). However, the microspore nucleus of pepper could not be clearly observed by simple propionocarmine staining. Accordingly, for clear visualization of microsppore nuclei, it was necessary to use the DNA-specific dye.

The fluorescence of the DAPI/DNA complex faded little when exposed to the UV light. This allows the observation of an microspore for several minutes and subsequent photography of the same field. The recorded data correspond, therefore, with the actual observations. In that sense, DAPI is superior to other DNA probes such as Hoechst 33258 or mithramycin, whose use requires antifading procedures (Franklin and Filion, 1985). However, difficulties encountered when the DNA-specific DAPI staining was employed because autofluorescence of microspore wall occurred very intensively, and nucleus stained only faintly.

For the enhanced microspore staining, Triton-X 100 was incorporated in staining solution. However, the effect of Triton-X 100 was no significant. We assume that fixed microspores are permeable to DAPI, and it is not required to use Triton X-100 for permeabilization. This finding is in agreement with Vergne et al. (1987) who reported that for fixed material, the incorporation of Triton X-100 in the staining solution was not required.

DAPI Staining of microspore was enhanced with the addition of ferric chloride to the fixative. This results is in agreement with Ziauddin et al. (1997) who reported the effects of ferric chloride. However, previous attempts to visualize nuclei in pepper microspores, as described by Ziauddin et al. have led to poor results apparently because of the high concentration of ferric chloride. In that concentration, microspore nuclei merely showed a slight fluorescence, and cytoplasm did not visible. By contrast, after the addition of diluted aqueous ferric chloride solution, microspore exhibited brilliant nuclei and slight wall autoflurescence.

Very few reports exist regarding the use of iron for the staining. 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 *Curcurbita* 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 needles are not readily available today. For microspore staging, Gupta and Borthakur (1987) used acetic acid-iron alum-haematoxylin and observed that the nucleus of rice microspore can be seen distinctly. Recently Ziauddin et al. (1997) reported nuclear staining of microspores and pollens was enhanced with the addition of ferric chloride to a fixative. The clarity of nuclear staining using the ferric chloride may be due to the act as a mordant. However, since the exines of some pollen ruptured with ferric chloride treatment, one can not determine if exine rupture was critical for enhanced staning of binucleate microspores, or if the ferric chloride treatments enhanced staining of these microspores regardless of exine integrity.

Development of Male Gametophyte

This technique permits sharp visualization of the nuclei, their disposition at the various stages of microspore and pollen development, and also offers the opportunity to monitor pollen development with accuracy during the entire male program in pepper.

Microspore mother cell, by a meiotic division, gives rise to microspore tetrads. The arrangement of microspore in a tetrad is tetrahedral (Figures 3A-C). Microspores liberated from the callose wall of the tetrad have an irregular contour, but they soon become almost spherical. At this stage, each microspore contains a centrally placed nucleus (Figure 3D). Soon after, however, the microspore expands in volume and acquires a distinct wall with rudiments of a pore. The initial expansion of the microspoe occurs without an apparent vacuolation, but later vacuoles appear and the nucleus is displaced from the centre toward one side of the cell (Figure 3E). The

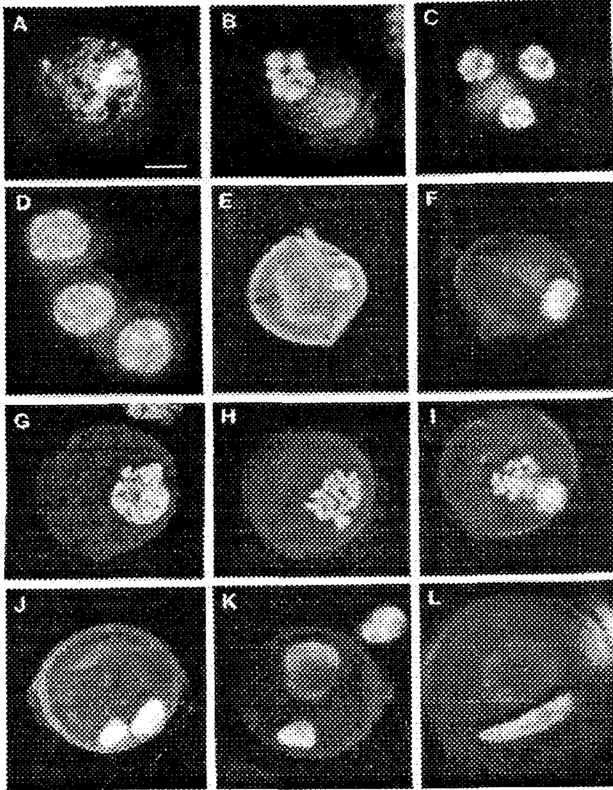

Figure 3. DAPI cytology of pollen development in pepper. (A) Microspore mother cell in meiotic prophase. (B) Meiosis. (C) tetrad. (D) Nonvacuolated microspore soon after release from the mother cell wall. (E) Vacuolated microspore before DNA synthesis. (F) Late microspore after DNA synthesis. (G-I) Microspore mitosis. (J) Pollen grain just after microspore mitosis. (K) Pollen grain with condensed generative nucleus and diffuse vegetative nucleus. (L) Mature 2-celled pollen, generative cell highly differentiated and appears as vermiform. Scale bar (10 μm) in Figure A applies to all the figures from B to L.

microspore nucleus remains in G1 phase of cell cycle. Without ferric chloride treatment, nucleus did not visible in most vacuolate microspore. By contrast, most vacuolated microspore after ferric chloride treatment exhibited distinct nuclei. After displacing the cytoplasm and nucleus to the periphery of the cell, most microspores had a large-diffused nucleus in G2 phase situated laterally close to the thick wall (Figure 3F). Immediately after this stage, some microspores were found at various stages of the mitosis (Figures 3G-I). The different phases of microspore mitosis are precisely identified because condensed chromosomes fluoresce intensely. After anaphase, initially, the nucleus of the vegetative cell is seen adjacent to the generative cell but it soon migrates from this position to the opposite end of the pollen grain (Figure 3J). After the migration of the vegetative nucleus, the more centrally located vegetative nucleus increased in size and its chromatin decondensed, while the lateral generative nucleus remained smaller with highly condensed chromatin (Figure 3K). The difference of vegetative and generative nuclei are precisely identified because condensed generative nucleus fluoresce intensely. This is the earliest sign that indicates the differentiation of vegetative and generative nuclei. Generative cell undergoes a lot of change in shape during pollen development. The cell elongates and in mature pollen it appears vermiform (Figure 3L).

Cytological study of pollen development of pepper *in vivo* and *in vitro* was very few (George and Narayanaswamy, 1973; Kim and Kim, 1984; Regner, 1996). Difficulties encountered were that conventional stains, such as acetocarmine and propionocarmine, were not suitable for developmental studies because thick exins and dense cytoplasm prevented visualization of nuclei. In our results, the visualization of all cytological stages is satisfactory, considering the difficulty in staining the nuclei with other methods. As starch does not interfere with the fluorochrome, this technique is specially useful for visualizing nuclei when starch deposition has begun within the pollen. The DAPI cytology of pollen development of pepper is in full agreement with earlier studies of stage assesment using acetocarmine (Kim and Kim, 1984). The terminal cytological events were comparable with those of other Solanaceous species (Bhojwani and Bhatnagar, 1999).

In this research, DAPI in conjunction with ferric chloride treatment provided in a simple easy method consistent and specific staining and satisfactory contrast at all stages from tetrad to mature pollen grain. Such a cytological tool will permit the definition and choosing of precise stages. Study of the nuclear development in the cultured microspores will be also facilitated with this fluorescent staining method.

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