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Cytological aspects of in vitro androgenesis in wheat (*Triticum aestivum* L.) using fluorescent microscopy

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Summary. Two winter wheat genotypes ('Diószegi 200' and 'Mv 15') were compared for their in vitro androgenic capacity. On average, the induction frequency of embryogenic structures was 71.7% in 'Diószegi 200' and only 4.3% in 'Mv 15'. The haploid induction ability of the two genotypes differed considerably, with 'Diószegi 200' being much higher. The difference in the in vitro inductability of the microspores may result from genetic differences which are manifested in the survival rate of the microspores during the culture period and their adaptability to in vitro conditions. Special DNA fluorochromes were suitable for studying the different pathways of in vitro androgenesis. Our data indicate that the repeated equal divisions of the microspore nucleus might lead to pollen embryo formation, and subsequent divisions of the vegetative portion of the pollen grain after the first asymmetric microspore mitosis can result in pollen callus formation.

Key words: *Triticum aestivum* – Cytology – In vitro androgenesis – Fluorescent microscopy.

Introduction

Although pollen grains are programmed genetically to complete fertilization by forming pollen tubes and male gametes, it has been demonstrated repeatedly that a small percentage of the microspores in cultured anthers can initiate an alternate developmental program of repeated cell divisions to form pollen embryos or calli with a haploid number of chromosomes. This phenomenon was first described by Guha and Maheshwari (1964, 1966) in *Datura innoxia* L. Since that time, in vitro androgenesis has been induced in a number of plant species (for reviews see Raghavan 1976; Sunderland and Dunwell 1977; Dunwell 1978; Vasil 1980; Heberle-Bors 1985; Raghavan 1986). In some species (*Datura innoxia* L. and *Hyoscyamus niger* L.), the sporophytic genetic program in the microspores is slightly repressed and can be easily activated by the trauma of excision and inoculation of the anthers. Pollen embryogenesis seems to be one of the most effective in vitro systems with which embryogenesis from single cells can be achieved practically in all higher plants including cereals (Heberle-Bors 1985).

In cereals, many aspects of in vitro microspore division and differentiation in culture have been studied (Ouyang et al. 1973; Sun 1978; Miao et al. 1978; Zheng and Ouyang 1980; Pan and Gao 1980; Sunderland and Evans 1980; Idzikowska et al. 1982; Henry et al. 1984; He and Ouyang 1985; Heberle-Bors 1985; Huang 1986). It has been discovered that, in the Gramineae, the same series of developmental pathways of pollen embryogenesis exist as in other plant species. The repeated division of the vegetative cell is one of the most common routes of pollen embryogenesis, but the generative cell or both the vegetative and generative cells can divide and contribute to the formation of the sporophytic outgrowth from the pollen grain. Zheng and Ouyang (1980) observed that the equal (symmetric) division of the microspore nucleus in wheat was the major pathway in the formation of pollen plants. He and Ouyang (1985) found a range of 0.2% to 18.4% in the frequency of equal divisions in wheat anthers inoculated at the mid- or late uninucleate microspore stage. Whether these different developmental pathways lead to different embryogenic structures (pollen calli or embryos) is uncertain. The limitations of

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the acetocarmine staining technique makes it difficult to distinguish the different nucleic types during the in vitro androgenic process. Recently, fluorescent methods (Coleman and Goff 1985; Hough et al. 1985) were introduced for staining nuclei in mature pollen, and these methods might be used for studying nuclear divisions in pollen cultures as well. The aim of the present work was to compare the dynamics of the embryogenic divisions occurring in anther culture of two winter wheat genotypes by means of fluorescent microscopy and to examine critically the relationship between the pathways of sporophytic development of the microspores and the quality of the resulting embryogenic structures (pollen embryos or calli).

Materials and methods

Two winter wheat genotypes, 'Diószegi 200' and 'Mv 15' were used. Spikes were collected from field-grown plants and sterilized in a 0.1% $HgCl_2$ solution. Anthers containing microspores in the mid-uninucleate stage were inoculated on P2 medium (Chuang et al. 1978) under aseptic conditions. About 1000 anthers from each genotype were cultured in Petri dishes at 29° C in the dark.

To observe the divisions occurring in vitro in the microspores, five anthers were taken randomly from the cultures of both genotypes at intervals of 1, 3, 8, 13, and 21 days after inoculation. The anthers were fixed in Carnoy solution (3:1 mixture of 96% ethanol and concentrated acetic acid) for 8 min and then stored at 4° C in 70% ethanol. At the time of examination, the microspores released from the anthers were stained with 1 μg/ml 4,6-diamidino-2-phenyl-indole (DAPI) according to Coleman and Goff (1985). Although DAPI staining is not a viability test, the degenerating microspores could be distinguished from the viable ones by their collapsed shape and lack of any fluorescence. The stained microspores were examined by means of fluorescent microscope OPTON, Ultraphot-III.

The remaining anthers were cultured for 30 days and then the embryogenic structures (pollen embryos or calli) formed (Fig. 1) were distinguished.

Results

In the 1-month-cultured anthers, a considerable difference in haploid induction capacity was found between the two genotypes. On average, the induction frequency of embryogenic structures was 71.7% (50.7% embryos and 21.0% calli/100 anthers) in 'Diószegi 200' and only 4.3% (3.3% embryos and 1.0% calli/100 anthers) in 'Mv 15'. These data indicate that in both genotypes, pollen embryo formation predominated. However, the ratio of embryo to callus production seemed to be different (approximately 2:1 and 3:1) in the two genotypes.

With respect to in vitro microspore development in the two genotypes, extensive degeneration was observed to occur in the microspore population during the first few days (Fig. 2). Almost 50% of the 'Mv 15' microspores became unviable within the first few days of culture; this increased to 90% by the third day. This tendency for degeneration was not as high in 'Diószegi 200' microspores during the same period. There was a second increase in mortality of the microspores of both genotypes after the 13th day, but approximately 5% of the microspores still remained viable on the 21st day. The degenerating microspores had a collapsed shape and did not show any fluorescence. After 8 days, the majority of the viable structures were continuously dividing in 'Diószegi 200', while 'Mv 15' microspores showed a higher tendency to divide between the 3rd and 8th day (Fig. 3). By the final sampling, all of the living microspores of 'Diószegi 200' had divided compared to only 64.2% of those from 'Mv 15'. During the 21-day culture period, 156 out of the 1518 (10.3%)

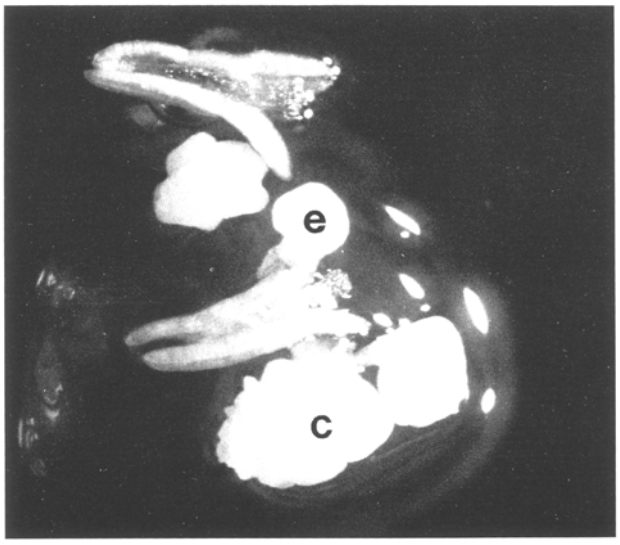

Fig. 1. Pollen embryo (*e*) and callus (*c*) on wheat anthers cultured for 1 month

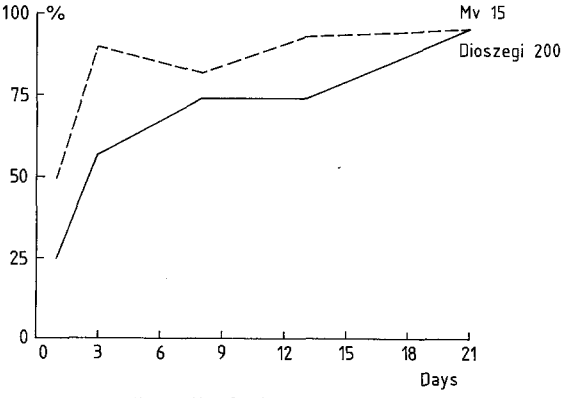

Fig. 2. Mortality (%) of microspores

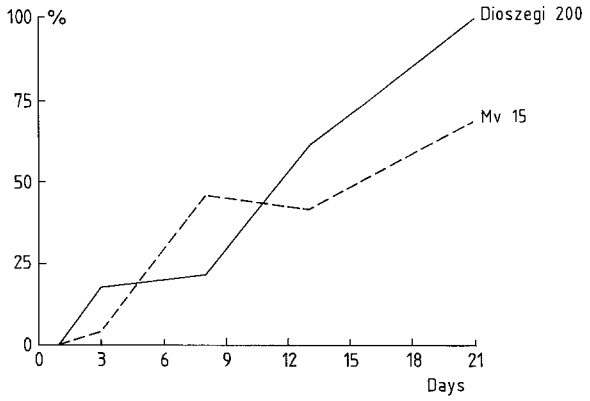

Fig. 3. Percentage (dividing microspores/living microspores × 100) of dividing microspores

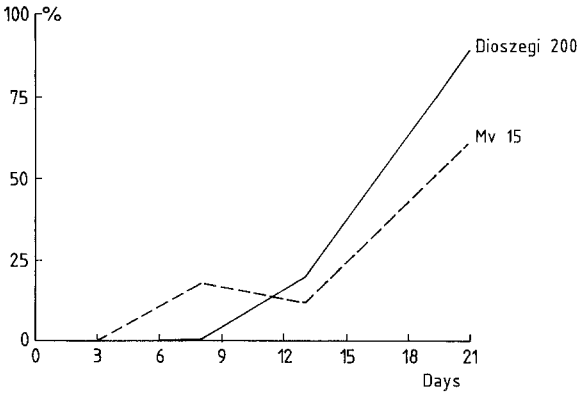

Fig. 4. Percentage of embryogenic pollen structures containing more than 3 nuclei

Table 1. Microspore development in anther culture. A, Diószegi 200; B, Mv 15; a, equally dividing microspores; b, unequally dividing microspores

Culturing period (days)	Genotype	No. of microspores observed	Living structures (%)	Living structures (%) containing							
				1	2		3		4		More nuclei	
					a	b	a	b	a	b	a	b
1	A	541	75.2	99.5	0.5	–	–	–	–	–	–	–
	B	1924	50.4	99.5	0.4	0.1	–	–	–	–	–	–
3	A	1399	43.7	82.4	10.2	7.0	0.4	–	–	–	–	–
	B	2481	10.2	95.6	4.4	–	–	–	–	–	–	–
8	A	871	26.1	78.4	11.9	6.2	2.6	0.4	–	0.4	–	–
	B	1237	18.3	54.0	23.0	–	3.5	1.8	2.7	0.9	10.6	3.5
13	A	1102	26.2	38.8	16.3	13.5	8.3	3.8	2.4	3.4	10.4	3.1
	B	605	7.1	58.1	20.9	–	9.3	–	2.3	–	9.3	–
21	A	1313	4.9	0.0	4.7	3.1	3.1	–	–	–	71.9	17.2
	B	580	4.5	30.8	–	–	7.7	–	–	–	61.5	–

'Mv 15' microspores had divided compared to 399 of the 1598 (25.0%) living microspores of 'Diószegi 200'. These data suggest that there was a significant difference between the two genotypes in the in vitro dividing capacity of the microspores remaining viable in culture; the dynamics of the divisions had also altered.

As the data in Table 1 indicate, the first microspore division could be observed as early as 1 day after the onset of culturing in both genotypes. The first mitosis produced either identical nuclei by equal division of the microspore nucleus or a vegetative-like and a generative-like nucleus arising from unequal division. DAPI-stained nuclei were easily recognizable, even visually, by the different intensities of their fluorescence. The further development of the cultured pollen grains was different in the two genotypes (Fig. 4). Pollen grains that had presumably developed sporophytically (containing at least 4 nuclei) appeared initially in greater numbers in the 8-day samples of 'Mv 15' very few of these pollen grains were present in 'Diószegi 200' samples at this point. However, from this time onwards, the number of embryogenic structures increased faster in 'Diószegi 200' than in 'Mv 15'. The proportion of multicellular pollen grains obtained during the whole culture period was considerably higher (2.18%) in 'Diószegi 200' than in 'Mv 15' (0.89%). This indicates that there are differences between the in vitro induction capacity of the two genotypes at the microspore level.

During the embryogenic development of the pollen grains, the equal and unequal divisions could be readily distinguished (Fig. 5). The uninuclear microspore completed the first mitosis either symmetrically or asymmetrically: symmetric division resulted in two equal daughter nuclei having the same DNA content; asymmetric division produced a vegetative-like and a generative-like nucle-

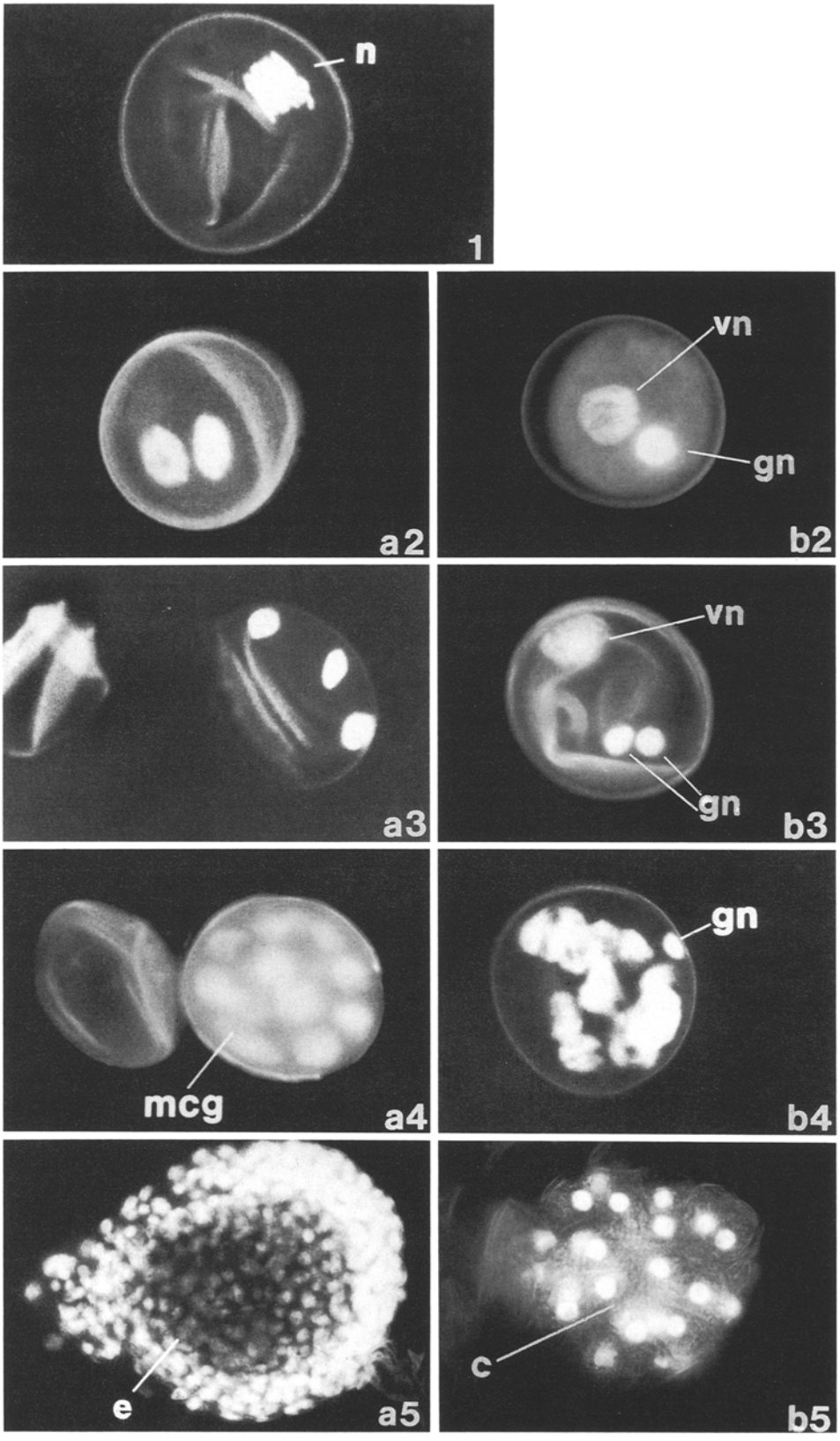

Fig. 5. Equal (*a2–a5*) and unequal (*b2–b5*) divisions occurring during wheat culture. *1* Microspore in late metaphase stage on the first day of culturing (×640). *a2* Equal division of microspore nucleus (×640); *b2* unequal division of microspore nucleus (×640); *a3* 3 nuclei originating from equal division (×640); *b3* dividing generative nuclei in 3-nucleate pollen (×640); *a4* multicellular pollen grain resulting from equal division (×640); *b4* multinuclear pollen grain with a single generative-like nucleus (×640); *a5* pollen embryoid (×200); *b5* callus developing from a pollen grain (×320). *n* Nucleus; *vn* vegetative nucleus, *gn* generative nucleus; *mcg* multicellular pollen grain, *e* embryoid, *c* callus

us having different DNA contents. During subsequent divisions of the asymmetric form, the vegetative nucleus (with a low DNA content) seemed to be the origin of multicellular structures. During the 21 days, 64.7% of the divisions in 'Diószegi 200' were equal and 35.5% unequal, while in 'Mv 15', 90.4% were equal and 9.6% unequal. After 30 days, this difference in division was manifested in different embryo/callus ratios. Thus a correlation may exist between the way microspores divide in culture and the formation of embryos or calli. Equal divisions may lead to pollen embryo development (Fig. 5a) and unequal divisions may result in pollen callus formation (Fig. 5b). The final resolution of this question will be obtained in cytological analysis of in vitro androgenesis in genotypes which primarily produce calli from anther culture by unequal divisions.

Discussion

In the present study, cytological evidence for different androgenetic capacities of microspores from two winter wheat genotypes was presented. Our data do not support one of the conclusions of Henry et al. (1984) that genotypic effects observed in wheat anther culture can only be related to the different abortion rates of the pollen embryos and not to the androgenic induction rate of the microspores. On the basis of our study, we suggest that the high embryo abortion rate which can occur in anther culture might not only be connected with genotypic differences as there is still a lack of information about the optimal culture requirements for the young pollen embryos, as also pointed out by Heberle-Bors (1985). Furthermore, we suggest that the induction capacity of the cultured microspores is not determined by pollen dimorphism or other factors, but depends on the flexibility of the microspore genome and its adaptability to the in vitro conditions. Since the male organs are sensitive to environmental changes, their ability to survive can be expressed by choosing the sporophytic way of development under stress conditions. In this case, the pretreatments and in vitro culture conditions function as stress factors for the microspores. Thus, the survival rate of the microspores at the beginning of the culture period and their stress tolerance can be important features.

The data so far available on the cytology of in vitro androgenesis in wheat (Zheng and Ouyang 1980; Sun et al. 1983; Henry et al. 1984; He and Ouyang 1985) does not indicate whether there is any correlation between the predominate way microspores divide in culture and embryo or callus formation.

The application of special fluorochrome techniques such as DAPI makes it possible to distinguish visually the vegetative and generative areas of the induced pollen grains. Such an exact differentiation was not always possible in the acetocarmine stained samples (Henry et al. 1984). The application of DNA-specific fluorescent dyes in different fields of pollen biology (Coleman and Goff 1985; Hough et al. 1985; Vergne et al. 1987) and biotechnology (Kyo and Harada 1986; Pace et al. 1987) is very advantageous.

Our present observations on the pollen embryo and pollen callus induction support the hypothesis of Zheng and Ouyang (1980) that equally dividing microspores are the main source of wheat haploids. In this study, equal divisions predominated in both genotypes, but in the case of 'Diószegi 200', which also produced calli in considerable numbers, the frequency of unequal divisions was relatively higher than in the other genotype. From this observation, we conclude that equal divisions of the wheat microspores can lead to pollen embryo formation while unequal divisions will result in pollen callus formation. Our recent ultrastructural studies on pollen embryogenesis in maize (Barnabás et al. 1987) and wheat (B. Barnabás, É. Szakács, K. Liszt, unpublished work) also support this hypothesis. We observed that a certain amount of multicellular pollen grains have morphological heterogenity in their cell construction. Some cells contain a dense cytoplasm with lipid accumulation. The cell walls are irregular, very thick, and without plasmodesmata. These cells stop functioning and degenerate. In the other type of cells of these multicellular grains, the cytoplasm has a low electron density and contains regular organelles and storage materials. Their cell walls are thinner, and they are connected by numerous plasmodesmata. These groups of cells seem to function and perhaps are able to proliferate with callus formation. We suggest that these multicellular structures originate from unequally divided microspores.

Perhaps further genetic and ultrastructural studies relating to the function of the microspore cytoplasm will provide satisfactory information in this critical research area.

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