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CYTOLOGICAL ASSESSMENT OF CARROT PLANTS OBTAINED IN ANTHER CULTURE

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Anthers of Feria F_1 and Narbonne F_1 carrot cultivars were cultured in vitro to induce androgenic embryos. To confirm the microspore origin of developed embryos, chromosome counts of root tip meristematic cells were made for each carrot plant obtained in anther culture. Using phase contrast technique and fluorescence microscopy, cytological changes of microspores during culture leading to proembryo formation were documented in the first days after anther placement on the induction medium. More than 90% of the carrot plants obtained in anther cultures had no haploid chromosomes.

Key words: Anther cultures, embryo, microspore, chromosome number, ploidy.

INTRODUCTION

Carrot is among the vegetables most cultivated and consumed in Poland; it is used in food processing and increasingly in the pharmaceutical industry. New cultivars need to be bred for high yield and uniformity (Górecka et al., 2005a). These requirements are met by F_1 hybrids, conventional breeding of which can take as long as 10 years. Anther culture and isolated microspore culture can shorten that process considerably. An additional advantage is that fully homozygous plants can be obtained (Maraschin et al., 2005).

Immature pollen grains (microspores) are programmed to differentiate into gametes, but in culture in vitro some of these cells may be induced to divide and form embryoids under the influence of various stress factors (Pauls et al., 2006) which induce various mechanisms and substances to work. These include oligosaccharides, peptides and arabinogalactan proteins, which during zygotic embryo development transfer signals between the embryo and the endosperm, which functions not merely as the host for the developing embryo (Matthys-Rochon, 2005). Homozygous plants can be regenerated from androgenetic embryos (Wang et al., 2000).

The Institute of Vegetable Crops in Skierniewice has for many years conducted experiments to obtain androgenetic plants of head cabbage, Brussels sprouts and carrot (Górecka, 1998; Krzyżanowska and Górecka, 2004; Górecka et al., 2005b). To confirm whether carrot plants obtained in this way had been formed from microspores, cytological methods were used to follow the process of embryo formation in carrot anther culture, and to determine the ploidy of the plants regenerated from those embryos.

MATERIALS AND METHODS

Carrot plants of the cultivars Feria F_1 and Narbonne F_1, generated from vernalized roots, were grown under controlled conditions in a phytotron chamber under a 16 h photoperiod at 20°C (day)/16°C (night). For anther culture, flower buds were taken from developed but not yet flowering umbels. Microspore development was observed in anthers isolated from those buds, at the following stages: tetrad, early, mid, late uninucleate and early binucleate (Fig. 1).

For anatomical studies of microspore development, the starting material for culture consisted of anthers having the majority of their microspores at the optimal stage for induction of androgenesis, that

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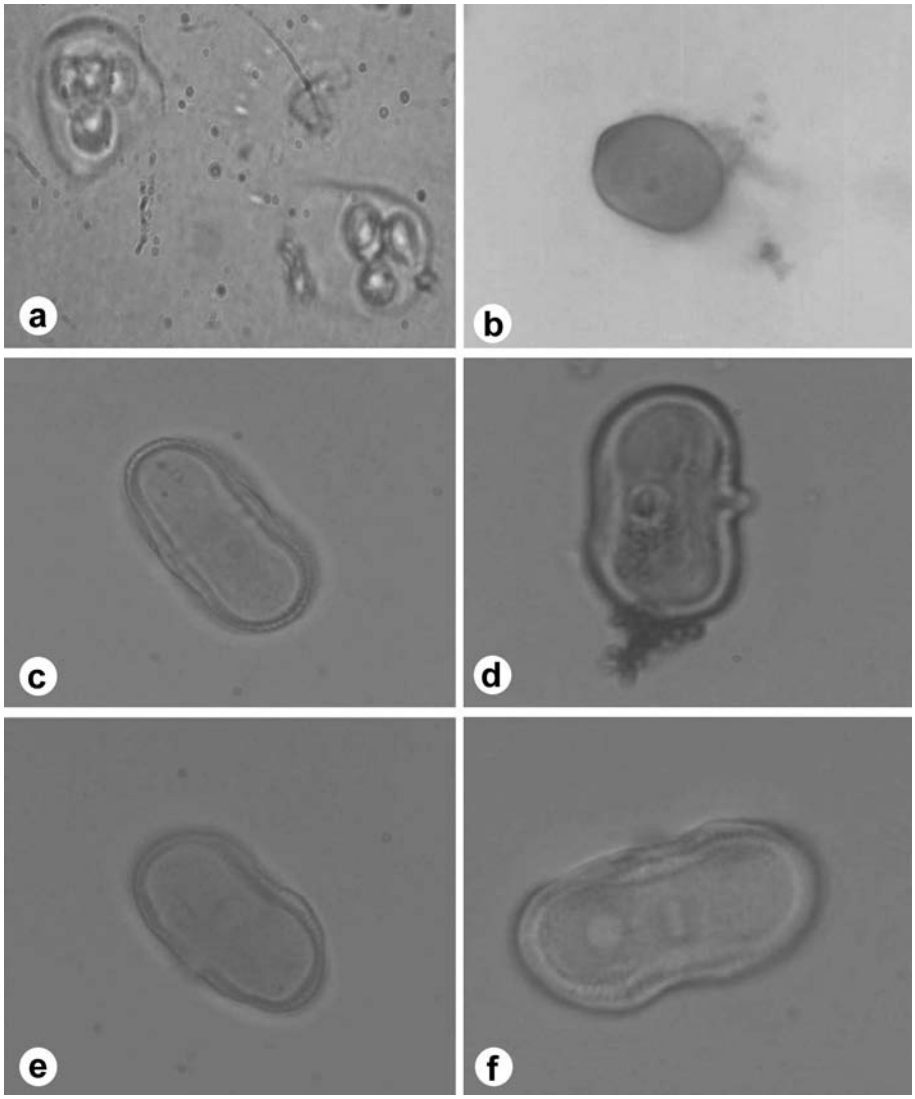

Fig. 1. Developmental stages of microspores in carrot anthers cultured in vitro. (**a**) Tetrad, (**b**) Early uninucleate, (**c**) Mid uninucleate, (**d**) Late uninucleate, (**e**) Early binucleate, (**f**) Late binucleate. ×1600.

is, uninucleate; this was judged by anther length (Kiszczak et al., 2005). Cultures were set up on B_5 medium (Gamborg et al., 1968) as modified by Keller et al. (1975), used for carrot anther culture by Andersen et al. (1990). The anthers were placed in 100 ml Erlenmeyer flasks, 40 anthers per flask. Cultures were kept in the dark at +27°C until embryos appeared. Every day for 28 days of Narbonne F_1 anther culture, and for 60 days of Feria F_1 culture, anthers were taken for cytological studies. They were fixed in Carnoy's mixture (absolute ethanol and glacial acetic acid, 3:1 v/v) for 24 h, then rinsed 3 times in 96% ethanol and 3 times in 80% ethanol for 15 min. Thus prepared anthers were stored in 70% ethanol.

To establish whether the embryos were formed from microspores or anther structural tissues, microspores in fixed anthers were examined after 1, 2, 3, 4, 5, 9, 13, 17 and 21 days of culture under phase contrast and fluorescence microscopes. Squashed, unstained specimens were examined under a Nikon Eclipse 200 microscope with phase contrast. Feulgen-stained specimens desiccated on dry ice and sealed with Canada balsam were examined with a Nikon Optiphot-2 fluorescent microscope (green light, G-2A excitation filter, λ=565 nm). All images were recorded with a SONY DXC 950 CCD camera.

To determine the ploidy of the regenerated carrot plants, the chromosomes in meristematic cells of young root tips were counted in metaphase. Root growth tips were sampled while the plants were being removed from glassware for planting. To get good slides with clearly visible chromosomes, the root tips

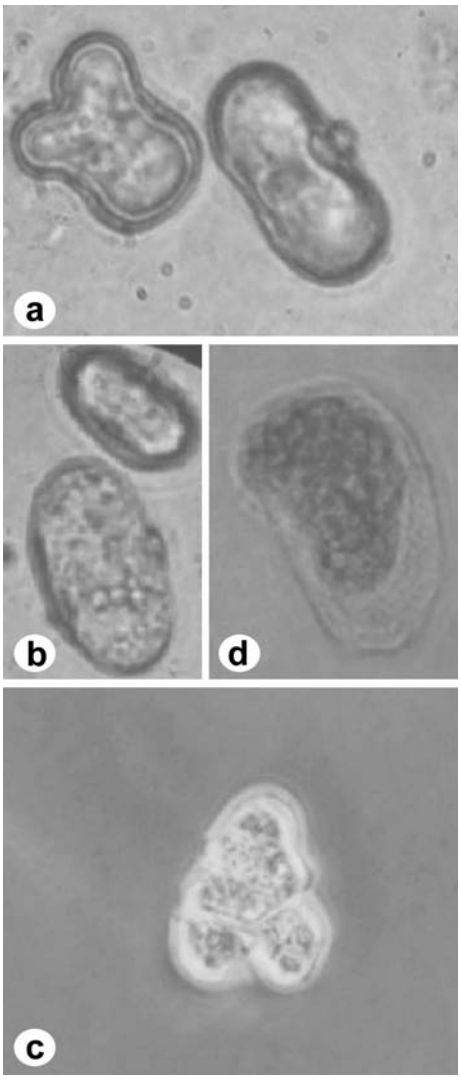

Fig. 2. Embryo formation in carrot anther culture (phase contrast microscopy). (**a**) Altered microspore shape, (**b**) Enlarged microspore observed after 1 day of culture, (**c**) Few-celled structures, (**d**) Proembryo. ×1600.

were pretreated with 0.25% aqueous solution of colchicine for 6 h, fixed in Carnoy's mixture for 1 h, and after rinsing stored in 70% ethanol. The fixed root tips of androgenetic carrot plants were stained with acid fuchsin and used to prepare squashed slides desiccated on dry ice and sealed with Canada balsam. These permanent preparations were used to count chromosomes in cells arrested at metaphase.

RESULTS AND DISCUSSION

After 1 day of culture a few enlarged microspores could already be seen by phase contrast microscopy in both carrot cultivars. Some of the Narbonne F_1 microspores changed in shape. From the second day of culture these microspores divided, and few-celled structures were formed. Further divisions began to produce multicellular structures from the third day of culture (Fig. 2). Proembryos could be seen on day four or five of culture. After that time all the stages of embryo formation could be seen in a single anther (from first divisions of microspores to proembryos). These developmental stages were observed in anthers of both cultivars. In anatomical studies of microspores during anther culture of *Datura innoxia*, Nitsch and Norreel (1973) observed the formation of proembryos on the fifth day of culture.

In their work on androgenesis in barley, Maraschin et al. (2005) divided the formation of embryos from microspores into three stages: (1) acquisition of embryogenic competence (increase in the volume of the microspore), (2) initiation of division and first divisions, and (3) embryo formation. Tyukavin et al. (1999) made similar observations in carrot anthers during culture, and they found that embryos could also be formed from callus of anther structural cells. This occurs in anthers with tetrads predominant and with tissues not yet fully differentiated. The anthers we used in the present work also contained microspores in the tetrad stage but with the uninuclear stage predominant; we found no evidence of embryos forming from anther tissues.

After the second day of culture, new fractions of small cells with small, intensely fluorescing nuclei were seen around the microspores, away from the anther structural tissues. The number of these cells increased with the duration of culture. On day four of culture their walls thickened, the nuclei enlarged, and fluorescence decreased. Proembryos were soon formed, and then embryos (Fig. 3). The same changes were noted in anthers of both cultivars. During culture the number of cells forming proembryos increased very rapidly. By day 21 of culture there were so many of them in the anthers that it was impossible to distinguish individual proembryos in specimens prepared as described above, so it was decided not to examine anthers cultured for longer than 21 days.

We identified 98% of the androgenetic plants regenerated from embryos obtained in carrot anther culture as diploids. Cells with chromosomes numbers lower or higher than 2n=18 were observed sporadically. Tyukavin et al. (1999) found spontaneous doubling of chromosome number in early stages of androgenesis (proembryo stage), particularly when secondary embryogenesis was taking place; the plants regenerated from carrot androgenetic embryos were comprised mainly of diploid cells, with 10% of the cells haploid. They also noted numerous mitotic mutations at the stage

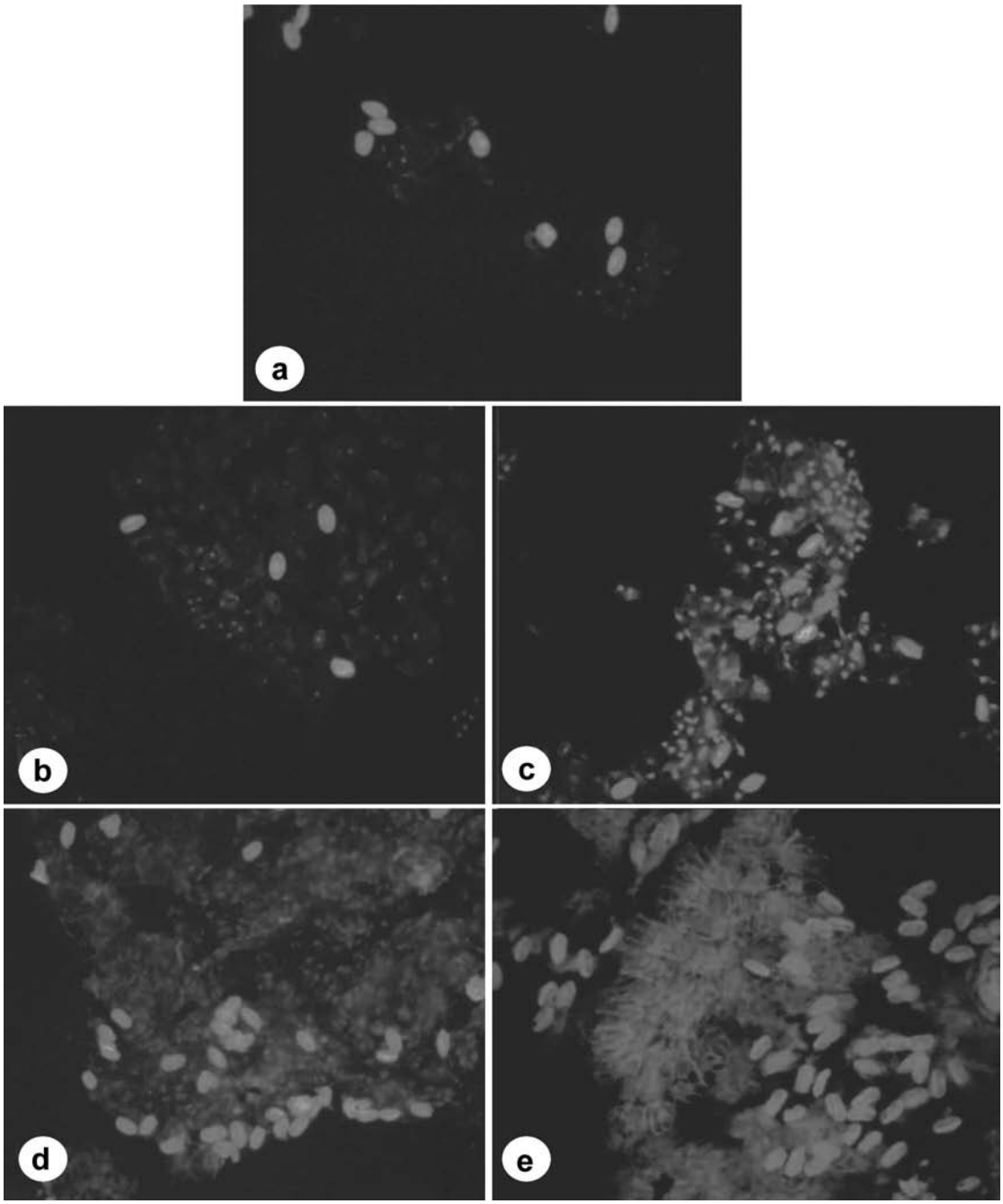

Fig. 3. Embryo formation in carrot anther culture (fluorescence microscopy). (**a**) New fractions of small cells with small, intensely fluorescing nuclei around microspores, (**b**) More small cells after longer culture, (**c**) Walls of small cells thickening, nuclei enlarging, (**d**) Proembryos, (**e**) Embryos. ×1600.

of secondary embryoid formation. Diploidization stabilized the genetic system and increased its adaptive potential.

During their three-year studies on carrot anther culture, Andersen et al. (1990) obtained different proportions of haploid plants each year. In the first year 33.3% of the plants were haploids, in the second year all the plants were either tetraploids or diploids, and in the third year 4% were haploids.

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