Chapter 20
Carrot Doubled Haploids

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Abstract Anther culture of over 20 carrot cultivars was studied. The uni-nucleate stage proved optimal for embryogenesis for which bud length was used as a surrogate. Large genotypic variation in culture response was found. Anther culture at 27°C in the dark without sub-culturing had a significant beneficial impact on embryogenesis. Embryos were obtained regardless of donor plant culture; however, plants grown in glasshouse conditions produced more embryos than those from the field. Secondary embryogenesis was induced in some cultures and in one case, 102 plants were obtained from one embryo (on B_5 medium without hormones) over a 12 week period. Regenerated plants were planted into peat and acclimatized to glasshouse and growth room conditions. Cytological and cytometric studies revealed that over 90% of regenerated plants possessed a doubled chromosome complement and isozyme analysis showed that 96–100% were homozygous. Anatomical studies confirmed that embryos had formed from microspores.

Keywords Anther culture, secondary embryogenesis, cytometric studies, isozyme analysis

Introduction

The carrot is a plant species of increasing economic importance in Poland and other parts of the world. It has great value in that it is available raw all year round. In addition to its use as a vegetable, carrot juices, or mixed juices in which carrot is one of the main ingredients, are becoming increasingly popular. Carrot is also a raw material for the pharmaceutical industry.

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Hybrids have become dominant in the carrot market and are replacing traditional cultivars. To breed hybrids, homozygous lines are necessary. However, obtaining these by conventional breeding methods is difficult and very time-consuming. The use of androgenesis has great potential to significantly shorten the production of pure lines and breeding (Bajaj 1990; Wang et al. 2000; Datta 2005). There are very few publications in the literature worldwide on the subject of *in vitro* androgenesis in carrot (see Andersen et al. 1990; Hu et al. 1993; Matsubara et al. 1995; Tyukavin et al. 1999; Ferrie et al. 2005).

At the Institute of Vegetable Crops in Skierniewice, wide-ranging experiments were carried out with the aim of developing a method of obtaining homozygous carrot plants from anther cultures. Detailed results of some of the stages of this work have already been published (Górecka et al. 2005a, b; Kiszczak et al. 2005), other results are in the process of being compiled or published (Górecka et al. in press). This summary article presents the most important and the most interesting results of the whole process of obtaining carrot plants in anther cultures and their subsequent evaluation.

Methodology

Carrot roots harvested in the field were stored in a chilling chamber at about +4°C (±1°C). After 3 months, the roots were placed (in pairs) into 10 L pots containing a mixture of sand and peat (2:1, v/v), with the addition of multi-component complex fertilizer Azofoska and microelements at 1.25 kg m^{-3} and chalk at 8.0 kg m^{-3}. The plants developed from the roots were kept in a glasshouse at about +20°C.

Production of Embryos from Anther Culture

Anthers were obtained from a range of umbel types (from one or more shoots, main shoot or from cut back re-growth) from donor plants grown in the glasshouse or field (Table 2). Flower buds were surface sterilized (70% ethanol, v/v) for 1–2 minutes and then rinsed twice with distilled water. Anthers were picked out under a dissecting microscope (16X) using forceps and needles. Only anthers without filaments were used (cells of the filament were found to form callus in culture). The isolated anthers were placed on modified B_5 medium (Gamborg et al. 1968) with 500 mg L^{-1} glutamine, 100 mg L^{-1} serine, 0.1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} NAA, 100 g L^{-1} sucrose and 6.5 g L^{-1} agar (Andersen et al. 1990). The medium pH was adjusted to 5.8. The anther cultures were kept in the dark at 27°C. The effect of various factors on the process of embryogenesis were studied.

Anthers were crushed and examined under a light microscope at 1,000X magnification. Microspore development was correlated with bud development (Table 1).

Table 1 The influence of microspore development stage and bud length on the effectiveness of androgenesis in two carrot cultivars (Feria F_1 and HCM)

Feria F_1				HCM			
Bud length (mm)	Microspore developmental stage in anther (%)		No. of embryos per 100 anthers	Bud length (mm)	Microspore developmental stage in anther (%)		No. of embryos per 100 anthers
0.7–0.9	Tetrads	25			Tetrads	0	6.9
	Uni-nucleate	64	63.1	0.7–0.9	Uni-nucleate	47	
	Early bi-nucleate	3			Early bi-nucleate	41	
	Late bi-nucleate	8			Late bi-nucleate	10	
	Unidentified	0			Unidentified	2	
1.0–1.3	Tetrads	4	10.2	1.0–1.3	Tetrads	3	2.5
	Uni-nucleate	71			Uni-nucleate	31	
	Early bi-nucleate	19			Early bi-nucleate	41	
	Late bi-nucleate	6			Late bi-nucleate	25	
	Unidentified	0			Unidentified	0	
1.5–1.8	Tetrads	0	17.5	1.5–1.8	Tetrads	0	0.3
	Uni-nucleate	53			Uni-nucleate	0	
	Early bi-nucleate	6			Early bi-nucleate	30	
	Late bi-nucleate	44				70	
	Unidentified	1			Late bi-nucleate	0	
					Unidentified		

Table 2 The effect of donor plant growth conditions and cultivar on androgenesis on carrot

Donor plant growth conditions	Cultivar	No. of cultured anthers	No. of embryos	No. of embryos per 100 anthers	No. of responding anthers	% of responding anthers
Greenhouse	Splendid F_1	833	4	0.5*	3	0.4c*
	HCM	3163	91	2.9b	62	2.0b
	Feria F_1	776	361	46.5a	89	11.5a
Field	Splendid F_1	80	0	0.0c	0.0	0.0c
	HCM	829	2	0.2c	2	0.2c
	Feria F_1	1836	615	33.5a	170	9.3a

*Numbers marked with the same letter within columns do not differ significantly at $\alpha = 0.05$

Anther culture response was compared among 22 cultivars selected by breeders. Individual plants from the same cultivar were also investigated. About 500 anthers from each genotype were cultured.

Two procedures of cultivating anther cultures were used. The first involved transferring anthers laid out on induction media onto fresh media after 2 weeks incubation in the dark at 27°C and placing them under continuous light, but at the same temperature. The second involved leaving the anthers on the first media in the dark at 27°C until embryos had formed.

Donor plants were grown in a greenhouse and in the field.

Once embryos had formed, cultures were transferred into the light, but temperature was maintained at 27°C. On greening up embryos were counted and transferred to regeneration media. First, B_5 medium with 20 mg L^{-1} kinetin and 20 g L^{-1} sucrose was used – the medium which had proved to be the best in our experiments on regenerating head cabbage and Brussels sprouts from androgenetic embryos. Next, MS medium (Murashige and Skoog 1962) containing 1 mg L^{-1} BA, 0.001 mg L^{-1} NAA and 20 g L^{-1} sucrose was used. Since mainly shoots formed on this medium. They were transferred onto rooting medium containing one auxin – IAA, NAA, or IBA, or a combination of two auxins. Other regeneration media were sought and, to this end, media based on B_5 and MS without hormones were introduced while maintaining a reduced concentration of sucrose at 20 g L^{-1}.

Plantlets were acclimatized to none sterile conditions in a glasshouse or growth chamber using various pot substrates: highmoor peat, peat substrate, sand, charcoal, brown coal, mineral wool, superabsorbent Alcosorb 400, Glomus inoculum with the addition of chalk and compound fertilizers. Adaptation was carried out under the conditions of 100% humidity using. small plastic tents.

Plant Evaluation

Ploidy was determined by nuclear DNA content using flow cytometry (Partec CA II) and by chromosome counts from root tip metaphase cells. Homozygosity was determined by isozyme analysis of PGI (phosphoglucose isomerise) and/or AAT (aspartate aminotransferase) which were known to be heterozygous in the donor plants.

Anatomical studies on carrot embryo development were carried out to confirm androgenic origin. Carrots anthers were sampled daily from cultures and embryogenesis monitored (Fig. 2a,b).

Results

Harvested carrot anthers contained microspores at different stages in development, but one stage predominated. The uni-nucleate stage was found to be the most suitable for initiating embryogenesis and this could be determined by bud length (Table 1).

Very large differences were found between cultivars in their ability to form embryos in anther cultures. There were also clear differences in the ability to undergo androgenesis between individual donor plants of the same cultivar.

By comparing the different methods of cultivating anther cultures it was found that transferring them onto a fresh medium 2 weeks after they had been set up and subjecting them to continuous light produced much worse results (a statistically significant difference)

All embryos obtained from transfer onto B_5 medium with 20 mg L^{-1} kinetin and 20 g L^{-1} sucrose, died. On MS medium with cytokinin and at a low concentration of auxin shortened shoots (rosettes) were mainly formed, but very rarely roots. We were thus forced to carry out regeneration of carrot plants in two stages: (1) rosette production and (2) rooting on special media containing one or two auxins. Unfortunately, the rooting process took a long time and only a low percentage of shoots formed roots. In addition plants obtained as a result of the two-stage regeneration often died during the adaptation process despite the different growth substrates used. Work was therefore focused on the regeneration media and on the substrate for adaptation to glasshouse and growth room. On B_5 and MS media without hormones, which were then included in the experiments, secondary embryogenesis was found to take place with a subsequent conversion of embryos into plants (Fig. 1a, b). The conversion occurred faster on B_5 medium. These effects were very beneficial as they made it possible to eliminate the long-lasting and not very effective stage of root formation, and provided several plants from one embryo. Experiment on the effectiveness of regeneration showed that in the case of the most embryogenic cultivar 102 plants were obtained from one embryo over a 12 weeks period using B_5 medium without hormones. Moreover, the plants obtained via conversion survived the adaptation stage better. Over 80% of plants survived the removal from glassware to planting in pots. The best substrate proved to be the one containing larger amounts of highmoor peat and the addition of a compound fertilizer

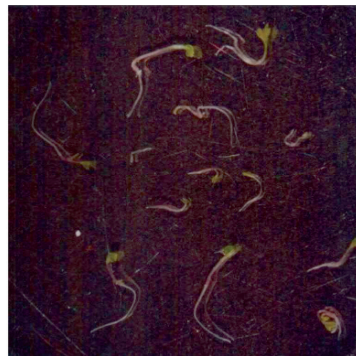

Fig. 1 Regeneration of androgenic plant (**a**) Secondary embryogenesis, (**b**) conversion of secondary embryos

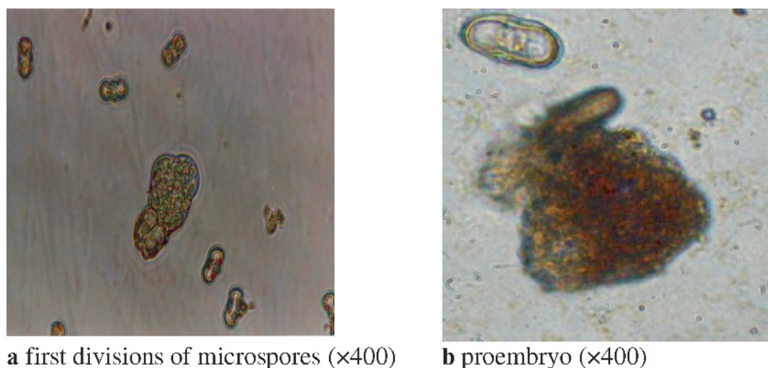

Fig. 2 Anatomical studies of embryo induction form micros pores (phase contrast microscopy) (**a**) First divisions of microspores (×400) (**b**) proembryo (×400)

and chalk. The results of adaptation were also favourably affected by transferring first to a growth chamber rather than a glasshouse.

The counting of chromosomes in the cells of root growth tips and the cytometric analyses both revealed that more than 90% of androgenetic plants had double the number of chromosomes.

The obtained androgenetic plants of the cultivar Narbonne F_1 were in 100% of cases homozygous in respect of the AAT isoenzyme and in 96% for PGI. In the case of the cultivar Kazan F_1, 96% of the androgenetic plants were homozygous for AAT, but only 4% for PGI.

Discussion

Andersen et al. (1990) found responding anthers, i.e. anthers forming callus or embryos, when microspores were cultured at the tetrad stage, either early-uni-nucleate or middle-uni-nucleate. Most success was gained, in both years of the experiments, when microspores were at the middle -uni-nucleate stage. Matsubara et al. (1995) obtained the largest number of embryos when anthers containing tetrads were cultured, which during culture reached the uni-nucleate stage. On the other hand, Tyukavin et al. (1999) were of the opinion that carrot microspores are able to change the path of their development and form either callus or embryos at the tetrad stage or bi-nucleate stage. In our experiments, we obtained the largest number of embryos when uni-nucleate microspores predominated in anthers.

In the experiments by Andersen et al. (1990), pre-embryos and calli, from which embryos formed, were obtained from only 2 out of the 15 cultivars studied. Our experiments also confirmed the effect of genotype on inducing androgenesis. One of the cultivars proved to be highly embryogenic, while five others did not produce any embryos at all. Like Arnison et al. (1990) for broccoli and our team for head cabbage (Górecka and Krzyżanowska 2004) and Brussels sprouts (Krzyżanowska

and Górecka 2004), we found very large differences between individual carrot plants of the same cultivar in their ability to undergo androgenesis.

Tyukavin et al. (1999) laid out whole flower buds on a medium for inducing androgenesis and placed them in the dark for 2–4 weeks. Then they isolated anthers and placed them onto the same medium under continuous light. Andersen et al. (1990) recommend transferring the cultured anthers after 2 weeks onto a fresh medium and exposing them to continuous light. In our experiments, we obtained significantly better results when anthers were left on the same medium in the dark until embryos appeared.

Andersen et al. (1990) and Matsubara et al. (1995) cultivated donor plants in the field, whereas Tyukavin et al. (1999) in a growth chamber. Ferrie et al. (2005) emphasize the importance of donor plant growth conditions for the effectiveness of isolated microspore cultures in the family *Apiaceae*. We compared this effectiveness in anther cultures set up from donor plants cultivated in a glasshouse and an open field. For each of the cultivars involved, we obtained better results from plants grown in glasshouse conditions.

Regeneration of embryos from anther culture was carried out by Tyukavin et al. (1999) by placing embryos on filter paper rafts on liquid MS medium containing 0.1 mg L^{-1} kinetin. Later they transferred the growing rosettes or plants onto liquid MS medium without hormones and again used the filter paper rafts. Initially, we carried out regeneration in two stages. On a medium with cytokinin and auxin, we obtained rosettes which were then placed on special rooting media with the addition of one or two auxins. However, the rooting process took a long time and its efficiency was very low. Later, on B_5 and MS media without hormones, we obtained a significant improvement. Secondary embryogenesis took place followed by embryo conversion. Whole plants were formed, which allowed us to skip the rooting stage. The plants were able to survive adaptation better. Moreover, the secondary embryogenesis made it possible to obtain a greater number of plants from one embryo. Tyukavin et al. (1999) also described the occurrence of secondary embryogenesis.

Andersen et al. (1990), Matsubara et al. (1995), and Tyukavin et al. (1999) all determined ploidy of regenerated plants by counting chromosomes in the cells of root tips. We also used this method, but compared the results with those obtained by the indirect method of determining the amount of nuclear DNA in a flow cytometer. Tyukavin et al. (1999) concluded that ploidy changed from 1n to 2n during secondary embryogenesis, embryo development and plant regeneration. They claimed that the changes in ploidy contributed to better adaptation to open environment. Smykalova et al. (2006) carried out determination of ploidy of caraway plants obtained in anther cultures by means of a flow cytometer. They found that 40% of the plants were haploid. In our experiments, using both methods, we found that more than 90% of carrot plants had a diploid number of chromosomes, which precludes the need for colchicine treatment.

The authors quoted here, who dealt with carrot anther cultures (Andersen et al. 1990; Hu et al. 1993; Matsubara et al. 1995; Tyukavin et al. 1999) did not carry out any studies to confirm homozygosity of the plants they obtained. Ferrie et al. (2005) also did not study this in respect carrot plants obtained by isolated microspore

culture. Smykalova et al. (2006) carried out an analysis of esterase isoenzyme in a population of caraway plants obtained in anther cultures (in order to confirm homozygosity). In our studies, we chose PGI and AAT because Westphal and Wricke (1989) had studied these isozymes in carrot. They investigated the inheritance of these enzyme systems and the linkage relationships among isozyme loci. Androgenic plants of the cultivars Kazan F_1 and Narbonne F_1 obtained from donor plants heterozygous in respect of AAT were found to be homozygous for this locus at a very high percentage. We obtained a similar result for the cultivar Narbonne F_1 for PGI. However, the androgenic plants of the cultivar Kazan F_1 were at a very high percentage of heterozygosity for PGI.

Nitsch and Norreel (1973) while carrying out anatomical studies of microspores during anther culture of *Datura innoxia*, observed pro-embryos. Smykalova et al. (2006) carried out observations of microspore development in caraway anther cultures under fluorescence microscopy. They found that, apart from an analysis of isozymes, this was a good method of confirming the androgenic origin of plants. In our observations, carried out by means of the phase contrast we saw the transformations of microspores into pre-embryos and embryos.

Conclusions

The experiments that we carried out resulted in the development of a technology for obtaining carrot plants by anther cultures. The plants were subsequently evaluated with respect of ploidy and homozygosity. We found that spontaneous doubling of the chromosome complement occurred at a very high rate. With respect to isozymes most of the plants tested were homozygous. The anatomical studies also confirmed that embryos developed from microspores. The plants cultivated by us were handed over to breeders and are in use in breeding.

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