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ORIGINAL RESEARCH PAPER

Comparison of methods for obtaining doubled haploids of carrot

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Abstract
Doubled haploid lines of carrot can be obtained through androgenesis in anther cultures and in isolated microspore cultures. The two methods were compared using three carrot cultivars ('Kazan F1', 'Feria F1', and 'Narbonne F1') at the androgenesis induction stage, during plant regeneration from embryos, and during acclimatization of androgenetic plants as well as their characterization. It was found that cultivar was the main factor affecting the efficiency at each stage of plant production in both anther and isolated microspore cultures. The efficiency of androgenesis in anther cultures of 'Feria F1' was considerably higher in comparison with isolated microspore cultures, and more plants were obtained from the embryos of androgenesis-cultured plants. In 'Kazan F1' and 'Narbonne F1', more acclimatized androgenetic plants were produced from anther cultures. Ploidy assessment of acclimatized plants of 'Narbonne F1' showed that the majority of the plants in the population derived from anther cultures had a doubled chromosome (DH) set. On the other hand, the majority of plants obtained from isolated microspore cultures were haploids. When assessing homozygosity, it was found among plants obtained in anther cultures that the percentage of homozygotes for phosphoglucose isomerase (PGI) and aspartate aminotransferase (AAT) depended on the cultivar. In contrast, the majority of plants derived from isolated microspore cultures were homozygous regardless of cultivar.

Keywords
androgenesis; anther culture; *Daucus carota*; haploids; isoenzymes; isolated microspores

Introduction

Haploid plants are used in various studies, including those involving genome mapping, genetic analysis, mutations, transformation, somatic hybridization, and biochemical and physiological analysis, and in the production of artificial seeds [1]. Most often, however, double haploids are used in plant breeding programs. It is estimated that more than 280 varieties worldwide have been obtained from plant materials derived through in vitro androgenesis in anther cultures or isolated microspore cultures [2]. The most advanced work has been conducted with oilseed rape, barley, and rice. Because of the small size of flower buds and anthers in some species such as carrot (*Daucus carota* L.), anther cultures are classified as technically difficult and thus inefficient [3]. A more efficient method of deriving haploids in tissue culture of various plants such as oilseed rape (*Brassica napus* L. ssp. *napus*) involves the technique of isolated microspores [4]. As reported by Babbar et al. [5], isolated microspore cultures of rapeseed are 10-fold more efficient than anther cultures in producing plants. However, the use of this technique has not produced satisfactory results in every tested species. For example, among 20 species of the Apiaceae family, only 10 species underwent androgenesis from isolated microspores [6].

The use of doubled haploid (DH) lines in breeding can significantly shorten the time to derive homozygous parental components. In carrot, in order to obtain homozygous lines it is necessary to conduct self-pollination for at least six generations, and the homozygosity of these lines does not exceed 98% [7].

The first reports on obtaining androgenetic embryos of carrot date back to the 1990s, when Andersen et al. obtained the first embryos using anther cultures [8]. Three years later, Hu et al. [9] obtained 18 carrot plants (16 plants with a chromosome number of $2n = 9$ and two aneuploids) using the same method. Several hundred androgenetic plants were subsequently obtained in anther cultures by Tyukavin et al. [10].

Górecka et al. [3,11], Kiszczak et al. [12], and Krzyżanowska et al. [13] examined and described the influence of various factors, such as genotype, microspore development stage, medium, and condition of the donor plant, on the effectiveness of androgenesis in carrot anther cultures. Kowalska et al. [14] carried out selection of regenerants from embryos obtained through androgenesis in anther cultures to obtain plants tolerant to increased concentrations of copper ions. The following year, Górecka et al. [11] described the formation of secondary embryos and the direct conversion of embryos into plants derived from anther cultures, which accelerated and simplified the procedure for obtaining androgenetic plants. In 2011, Kiszczak et al. [15] analyzed the ploidy and homozygosity in a population of carrot plants obtained through androgenesis in anther cultures. In these experiments, 90% of the carrot plants obtained from anther cultures were DH. The percentage of homozygotic DH plants ranged from 94 to 100% for phosphoglucose isomerase (PGI) and for aspartate aminotransferase (AAT) from 89 to 100%. Matsubara et al. [16] used the technique of isolated microspores for the first time, from which carrot embryos were obtained. Ferrie [6] obtained embryos in isolated microspore cultures of carrot and regenerated plants but did not provide detailed methodological information. In 2010, Górecka et al. [17] experimented with isolated microspore cultures of carrot and developed a complete androgenesis procedure, and Li et al. [18] confirmed the genetic determinants of the capability for producing embryos. A number of factors have been previously tested that affect the most important stages in deriving DH lines of carrot in anther and isolated microspore cultures. These stages include the induction of embryos, regeneration of plants from embryos, acclimatization, and ploidy levels. The goal of this study was to determine which of the two culture methods was more efficient in obtaining DH lines of three carrot cultivars.

Material and methods

Plant material

Three hybrid cultivars of carrot, 'Feria F1' (Rijk Zwan; De Lier, Holland), 'Kazan F1', and 'Narbonne F1' (Bejo Zaden B.V.; Warmenhuizen, Holland), were used in this study.

Donor plants

After harvest, carrot roots of field-grown plants were placed into boxes in alternating layers covered with peat. For vernalization, the carrot roots were placed into a cold chamber at +4°C for 3 months. Afterward, two roots were planted in one plastic container with 10 dm^3 of substrate consisting of 1:2 (v/v) peat and sand. To ensure the proper pH of the substrate, chalk was added at a rate of 8 kg m^{-3} of substrate. The complex fertilizer Azofoska (Inco Veritas, Poland) was used at a rate of 1.2 kg m^{-3} of peat. Planted roots were placed in the greenhouse or in a growth chamber under strictly controlled growth conditions of +20°C during the day and +16°C at night, with a 16-h photoperiod at 30 µmol m^{-2} s^{-1}. One month after planting, the plants in the greenhouse and growth chamber were irrigated at 14-day intervals with a 0.3% solution of Hydrovit 300 liquid fertilizer (Hydrokomplet S.C, Poland). At the end of April or the beginning of May, the roots were planted in the field.

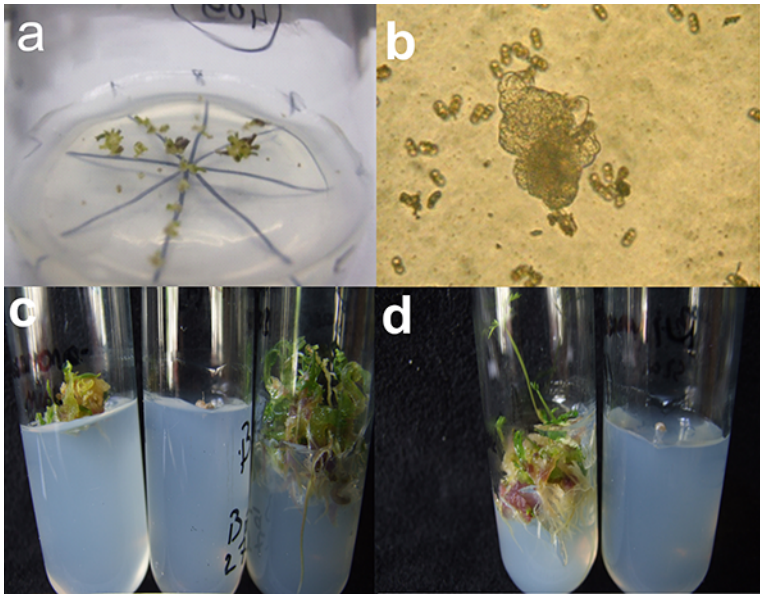

Fig. 1 Anther and isolated microspore cultures of carrot. **a** Androgenetic embryos formed on anthers (flask). **b** Androgenetic embryos formed on microspores (Petri dish). **c** Different types of androgenetic regenerants obtained through the regeneration process (anther culture). **d** Different types of androgenetic regenerants obtained through the regeneration process (microspore culture).

Anther cultures

After the formation of inflorescences by heterozygous donor plants, flower buds at the uninucleate stage of microspore development were collected from developed umbels [8,10]. Buds were then disinfected for 2 min in 70% (v/v) ethanol and rinsed twice with sterile distilled water. Anthers were isolated, and 48 pieces were placed in 100-mL Erlenmeyer flasks containing approximately 30 mL of media for androgenesis induction. The medium consisted of B5 [19] (Tab. 2, Tab. 3), as modified by Keller and Armstrong [20]. This medium was applied by Andersen et al. [8] for carrot anther cultures and contained 0.1 mg L^{-1} each of 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic acid (NAA), 500 mg L^{-1} L glutamine, 100 mg L^{-1} L serine, and 100 g L^{-1} sucrose. Flasks with anthers were placed in the dark at +27°C. After embryo formation (Fig. 1a), the flasks were exposed to continuous light [cool white lamps, L18W/77 Fluora (OSRAM, Germany)] at 30 µmol m^{-2} s^{-1} while maintaining the same temperature.

Isolated microspore cultures

The same donor plants as described above were also used to establish isolated microspore cultures. Approximately 30 of the outermost umbellules were collected from the main umbel. The umbellules contained closed flower buds 0.7 to 1.8 mm in length. Thirty umbellules were examined, and there were 79.3 anthers in each umbellule on average, which means that approximately 2380 anthers were used to establish isolated microspore cultures. The culture protocol was identical to that previously published by Górecka et al. [17]. The microspores were cultured in B5 liquid modified with 0.5 g L^{-1} colchicine (Sigma-Aldrich, USA) for 24 h at +27°C in darkness. The cultures were subsequently rinsed with fresh media without colchicine, centrifuged again and resuspended in the media at a final density of 4×10^4 microspores mL^{-1}.

Regeneration and acclimatization of plants

Plant regeneration and acclimatization were conducted according to the method described by Górecka et al. [21]. When embryos in flasks (Fig. 1a) and in Petri dishes turned green (Fig. 1b), their shape, size, and color were noted; the embryos were then counted and transferred to regeneration media in order to obtain plants. The efficiencies of plant regeneration in anther cultures and isolated microspore cultures were compared on B5-1 media [19] containing plant growth regulators (PGRs) and supplemented with 20 g L^{-1} sucrose, as described by Andersen et al. [8]. Results were collected after 12 weeks of culture.

Regenerated carrot plants were potted in a peat substrate with the addition of the multicomponent complex fertilizer Azofoska (Inco Veritas).

Assessments of ploidy and homozygosity

The ploidy of the acclimatized plants was determined by an indirect method involving measurements of the amount of nuclear DNA with a flow cytometer as described by

Kiszczak et al. [15] and by following a modified version of Galbraith's procedure [22]. The analysis was conducted with the use of a Partec CA-II flow cytometer (Partec GmbH, Germany). The control samples were collected from plants of the 'Narbonne F1' commercial variety that were grown from seed in the greenhouse.

The analysis of homozygosity of the donor plants and DH lines was performed using two isoenzymes, phosphoglucose isomerase (PGI, EC 5.3.1.9) and aspartate aminotransferase (AAT, EC 2.6.1.1), following the extraction protocol described by Kiszczak et al. [15]. Electrophoresis was performed in 10% starch gel as described by Gottlieb [23]. Separation of enzymes was performed according to the methods of Selander et al. [24].

Statistical analyses

The data were subjected to non-parametric analyses, including the Mann–Whitney *U* test and the Kruskal–Wallis test (with Conover–Inman post hoc tests) using Statistica v. 8.0. software for Windows (Statsoft Inc., USA).

Results

In anther cultures, the most embryogenic cultivar was 'Feria F1', in which 338 embryos were obtained from 100 plated anthers. In 'Kazan F1', there were 8.1 embryos, and in 'Narbonne F1' 3.1. In isolated microspores, 'Feria F1' produced only 0.8 embryos (calculated per 100 plated anthers), while 'Kazan F1' generated 8.3 embryos and 'Narbonne F1' 2.0. Regarding the cultivar 'Feria F1', at the induction stage of androgenesis, anther cultures were more effective than microspore cultures, but the differences between the results in these two techniques observed for the other two cultivars were small (Tab. 1).

Tab. 1 Effect of culture type on the effectiveness of androgenesis for three carrot cultivars.

Cultivar	Culture type	No. of anthers plated	No. of embryos obtained	No. of embryos per 100 anthers
Kazan F1	Anther	309	25	8.1 [b]
	Microspore	11900	994	8.3 [b]
Narbonne F1	Anther	621	21	3.1 [a]
	Microspore	11900	241	2.0 [ab]
Feria F1	Anther	782	3036	388.0 [c]
	Microspore	11900	96	0.8 [a]

Numbers followed by the same letter are not statistically different from each other at a significance level of $\alpha = 0.05$ (Mann–Whitney *U* test).

After being transplanted to the regeneration medium from one embryo obtained in anther culture, 1.5 plants, 2.7 rosettes (i.e., unrooted plants), and 0.7 secondary embryos were produced. Embryos obtained from isolated microspore cultures on regeneration media generated an average of 0.7 plants, 1.3 rosettes, 3.5 secondary embryos, and 2.1 compact callus clumps (Tab. 2). Based on these results, we concluded that culture using whole anthers was better with respect to the number of regenerated plants per embryo.

The regenerated plants had a phenotype typical for carrot seedlings: 3–6 leaves that were two-fold or three-fold pinnate and 5–8 cm in length and a normal taproot system with numerous small lateral roots. The plants obtained from anther cultures of

Tab. 2 Effect of culture type on the regeneration of plants, rosettes and secondary embryos in 'Feria F1', 2010. The numbers are per one embryo.

Culture type	Plants (seedling type)	Rosettes	Secondary embryos
Anther	1.5 [b]	2.7 [b]	0.7 [a]
Microspore	0.7 [a]	1.3 [a]	3.5 [b]

Numbers followed by the same letter are not statistically different from each other at a significance level of α = 0.05 (Mann–Whitney *U* test).

Tab. 3 Effect of the method of deriving androgenetic plants on acclimatization in two carrot cultivars.

	Anther cultures			Isolated microspore cultures		
	planted	acclimatized		planted	acclimatized	
Cultivar	No.	No.	%	No.	No.	%
Kazan F1	36	23	63.9	218	128	58.7
Narbonne F1	34	23	67.6	57	22	38.6

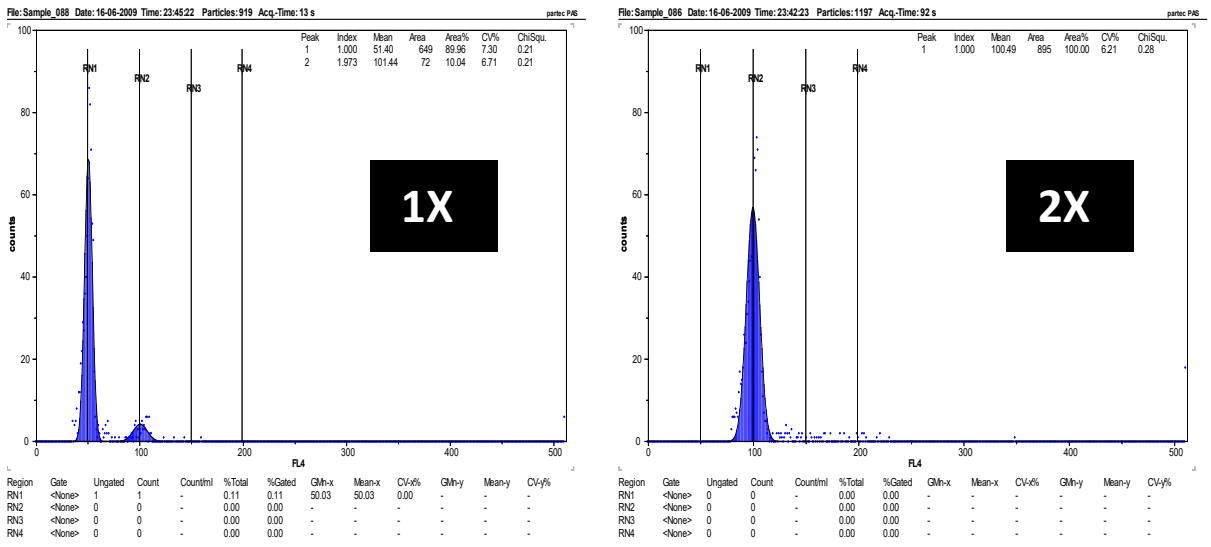

Fig. 2 Histograms of flow cytometric DNA analysis of androgenetic carrot plants derived from anther and isolated microspore cultures of carrot: 1*x* – androgenetic plants; 2*x* – control plants.

'Kazan F1' had acclimatization success rates of 63.9% and of 'Narbonne F1' of 67.6%. Acclimatization success rates for plants from anther cultures was 58.7% for 'Kazan F1' and 38.6% for 'Narbonne F1'. This suggested that androgenetic plants of cultivars derived from anther cultures acclimatized better than plants derived from isolated microspores (Tab. 3).

Among plants derived from anther cultures, plants with a haploid (1*n*) number of chromosomes represented 7.2% of all tested plants, whereas 89.1% of plants had a diploid chromosome number (2*n*) (Fig. 2). There were also 3.6% that were tetraploids. Among plants derived from isolated microspores, 41.8% were haploids, and 38.2% were diploids. More tetraploids, up to 20.0% of all plants, were identified in isolated microspores than in anther cultures (Tab. 4). There was a clear difference in ploidy levels among the androgenetic plants produced using these two techniques.

Tab. 4 Ploidy level of androgenetic plants of the carrot cultivar 'Narbonne F1'.

Ploidy level	Anther cultures		Isolated microspore cultures	
	No. of plants	%	No. of plants	%
1*n*	4	7.2	23	41.8
2*n*	49	89.1	21	38.2
4*n*	2	3.6	11	20.0
Σ	55	100.0	55	100.0

In anther cultures, the percentage of PGI and AAT homozygotes (Fig. 3) depended on the cultivar. For the PGI isoenzyme, the percentage was 94% in 'Narbonne F1' and 13% in 'Kazan F1'. With respect to AAT, 100% of homozygotes were found in 'Narbonne F1' and 89% in 'Kazan F1'.

The majority of plants obtained from isolated microspores were homozygous, regardless of cultivar. In 'Narbonne F1', 92.1% were homozygous for PGI, and 100% were homozygous for AAT. In 'Kazan F1', 97.9% were homozygous for PGI, and 100% were homozygous for AAT.

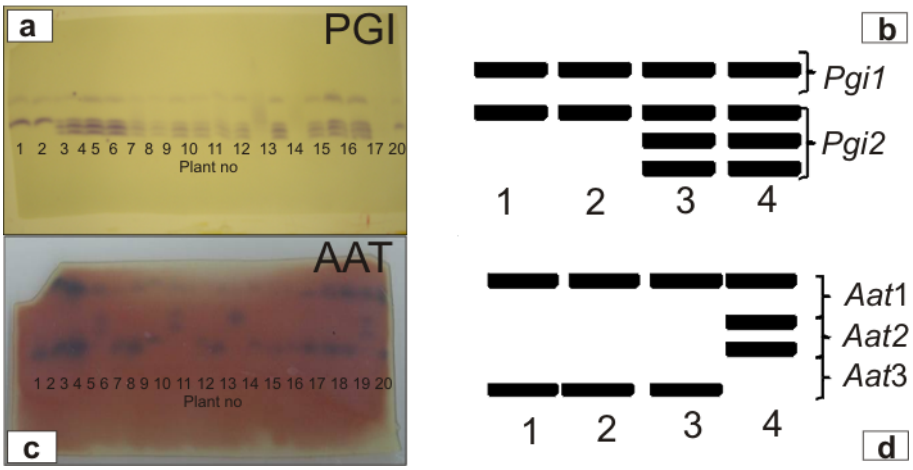

Fig. 3 Zymograms of carrot plants derived from anther and isolated microspore cultures. **a** Example of PGI isoenzyme zymogram with graphic reflection of bands (right side), plant number 1 and 2 – homozygotes, plant number 3 and 4 – heterozygotes. **b** Example of AAT isoenzyme zymogram with graphic reflection of bands (right side), plant number 1, 2, and 3 – homozygotes, plant number 4 – heterozygote.

Discussion

Induction of androgenesis is the first stage in the process of deriving androgenetic plants in anther and isolated microspore cultures. To assess the efficiency of the two methods at this stage, it is necessary to consider the methodological differences. To establish anther cultures, one person can excise on average approximately 240 anthers in 8 hours from a given number of flower buds. However, for isolated microspore cultures, the flower buds are bulked, and the microspores are isolated en masse for a total of approximately 2300 anthers. Aside from the homozygous genetic profile, one of the main reasons for the use of androgenesis to obtain plants is the emergence of new genetic variation that allows selection of DH lines with the desired traits for breeding. The use of a larger number of anthers increases the likelihood that such variation will appear.

In carrot anther cultures, the incubation period for induction of embryos varies considerably. Embryos were observed 2 weeks at the earliest after establishing anther

cultures, but most embryos were produced after approximately 1–3 months. In a study by Andersen et al. [8], the embryos appeared after 2–4 months of anther incubation, whereas Matsubara et al. [16] observed induction of embryos after 4 weeks. In contrast, Tyukavin et al. [10] described in detail embryo formation in the first week of anther culture. However, the majority of embryos were formed after 3–4 weeks of culture. Górecka et al. [21] reported that embryos appeared after 2 weeks from the onset of an experiment, and their formation was observed until the third month. In isolated microspore cultures, the appearance of the first structures was recorded after 2 weeks, but they were not yet visible to the naked eye. Well-developed embryos were distinguishable after 1 month, and their formation lasted up to 6 months. A similar time to obtain initial embryos (from 3 weeks to 6 months) was reported by Górecka et al. [17]. Li et al. [18] confirmed the presence of embryos in isolated microspore cultures after about 1 month. The last embryos were observed after 4 months.

In the present study, the induction of androgenesis and embryo formation in both types of culture was the first stage at which the efficiency of both techniques was compared (Tab. 5). Matsubara et al. [16] had conducted both types of culture with one cultivar but obtained embryos only in anther cultures, at a rate of 2.8 embryos per 100 anthers. Several times more embryos, equal to an amount of 21.4 per 100 anthers, were obtained by Hu et al. [9] on MS medium with 1 mg L^{-1} 2,4-D. In contrast, on the same medium but with 0.2 mg L^{-1} 2,4 D, Tyukavin et al. [10] obtained an average of 156.14 embryos per 100 anthers in anther cultures. In each case, those experiments were conducted with a single cultivar. In our study, the two methods were compared at the induction stage using three cultivars on the same medium. In 'Kazan F1' and 'Narbonne F1', the efficiency of androgenesis was at a similar level regardless of the technique used. In contrast, in 'Feria F1', far more embryos were obtained in anther cultures. An earlier study conducted by Górecka et al. [3,17] had confirmed higher efficiency of that cultivar in anther cultures.

Tab. 5 Efficiencies of the following stages of anther and microspore cultures.

Stages of androgenesis	Culture type*	
	anther	microspore
Induction – No. of embryos per 100 anthers	99.7	11.0
Regeneration – plants (seedling type) per one embryo	1.5	0.7
Acclimatization – % of plants	65.8	48.7
Ploidy – % of 1*n* plants	7.2	41.8
Ploidy – % of 2*n* plants	89.1	38.2
Homozygosity of PGI – %	53.5	96.0
Homozygosity of AAT – %	94.5	98.9
Time from embryo induction to acclimatized plants in weeks	26–46	27–48

* The values are the means of all three cultivars.

When comparing the efficiency of plant regeneration in anther cultures and isolated microspore cultures, differences between the two techniques were not pronounced, as the plant regeneration proceeded methodically and in the same technical manner. Nevertheless, in anther cultures, after the twelfth week of regeneration, twice as many complete plants and three times as many non-rooted rosettes were obtained. By taking advantage of the phenomenon of secondary embryogenesis, Górecka et al. [25] obtained 100 plants from one embryo of 'Feria F1' in anther cultures. In the present study, secondary embryos appeared in both types of cultures, but more embryos were obtained from isolated microspore cultures. Tyukavin et al. [10] had observed the

formation of secondary embryos from androgenetic embryos of carrot that had not yet emerged from anthers and also during regeneration on the regenerating plants. Möllers and Iqbal [26] reported that only very few androgenetic embryos of oilseed rape regenerated directly into plants, and the majority of embryos those plants formed secondary embryos. In isolated microspore cultures, Li et al. [18] obtained between 0.6 and 3.8 plants, depending on the breeding line of carrot. The low end of this range is a value close to the number of plants obtained from secondary embryos in the present study. This suggests that comparison of the two methods at this stage depends on endogenous and exogenous factors that strongly influence the efficiency of androgenesis from season to season.

Assuming the uniformity of these endogenous and exogenous factors, it can be concluded that during a short period of time (4–8 weeks) the anther culture technique is more efficient during the plant regeneration stage. However, during a long period of time (12–24 weeks), a larger number of regenerated plants can be obtained in isolated microspore cultures through secondary embryogenesis.

Andersen et al. [8] planted androgenetic carrot plants in a peat substrate and maintained high humidity levels for 4 weeks. Of the 54 plants of the cultivar 'Nantes Topschoor', 25 survived the acclimatization process. Górecki et al. [27] found that carrot plants that regenerated from androgenetic embryos in two stages (obtaining shoots, rooting plants) were not very effective at adapting. To achieve better adaptation success, Tyukavin [28] used a hydroponic system, but only 14.8% of plants became acclimatized. To acclimatize carrot plants derived from androgenesis in isolated microspore cultures, Górecka et al. [21] used a peat-based substrate, which resulted in 70% plant survival. On the other hand, Li et al. [18] potted two lines of carrot plants in perlite and obtained 76% and 81% survival. Considering the effect of the type of culture on plant acclimatization, our results indicate that a higher percentage of plants obtained from anther cultures survived this stage. No major effect of the cultivar on the process of acclimatization was found in the plants obtained in anther cultures. The plants of the tested cultivars acclimatized at more than 60% success.

Andersen et al. [8] conducted a 3-year study of anther cultures with two carrot cultivars and determined the number of chromosomes of the plants in the regenerated population. They determined that plants with 2*n* chromosomes dominated, constituting 59.5%, 79.1%, and 86.4% of the regenerated plants in each of the 3 years. Haploid plants were also detected at 33.3% but only in one season. Hu et al. [9] obtained 18 plants in anther cultures of carrot; 16 of those plants were haploid, and two were aneuploid.

In our study, 55 androgenetic plants from each type of culture were tested. The distribution of ploidy level depended on the technique used. In anther cultures, 89.1% of the obtained plants were diploid; in isolated microspore cultures, only 38.2% were diploids, while haploids made up 41.8%. In their study with isolated microspore cultures, Li et al. [18] showed that the percentage of diploids is correlated with genotype. They obtained, depending on the line, 81.4%, 57.1%, and 33.3% diploids. During the regeneration of androgenetic carrot embryos into plants, Tyukavin et al. [10] observed changes in ploidy level even under in vitro conditions; the plants became diploid. These researchers observed cells with a variable number of chromosome pairs (from 4 to 18) during their development and also various anomalies in the number of chromosomes during secondary embryogenesis. Germana et al. [2] provided a few reasons for the emergence of non-haploid individuals in a population of androgenetic plants: fusion of nuclei, endomitosis within the pollen grain, or irregular microspores formed by meiotic irregularities. This explains the 20% share of tetraploids in the population of plants obtained in isolated microspore cultures and the 3.6% share in anther cultures. On the basis of ploidy distribution, it can be concluded that isolated microspore cultures are less effective than anther cultures.

In the present study, 94% of the 'Narbonne F1' plants obtained in anther cultures were PGI homozygotes as were 92.1% of 'Narbonne F1' plants obtained from isolated microspore cultures. Regarding 'Kazan F1', 97.9% of the plants obtained from isolated microspore cultures were homozygous for PGI, whereas in the case of anther culture only a 13% share of PGI homozygotes was found in the population of the 'Kazan F1' plants tested. Such a low percentage of homozygotes may result from the action of embryogenesis genes. Foisset et al. [29] obtained a population of androgenetic plants of *Brassica napus* (L.), of which an isoenzymatic analysis for ACO, LP, PGM, and TPI

showed that molecular factors manifesting themselves during in vitro androgenesis interfered with the Mendelian segregation of isoenzymes.

The analysis of 'Narbonne F1' showed that plants from anther cultures and isolated microspore cultures were 100% homozygous for the AAT isoenzyme. In the case of 'Kazan F1', a similar distribution of homozygosity was obtained, with 89% homozygotes observed in anther cultures and 100% in isolated microspore cultures. Such a distribution of PGI and AAT homozygosity is desirable and meets the expectations held for the two techniques, whose main goal is to obtain homozygous plants.

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