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ORIGINAL PAPER

# Determination of ploidy and homozygosity of carrot plants obtained from anther cultures

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**Abstract** The purpose of the present work was to evaluate carrot plants obtained from anther cultures with respect to their ploidy and homozygosity. Ploidy was determined using flow cytometry. Homozygosity of analyzed plants was determined using isoenzymes, glucose-6-phosphate isomerase (PGI) and aspartate aminotransferase (AAT). The cytometric tests revealed that more than 90% of the carrot plants obtained from anther cultures were doubled haploid. In the initial assessment of polymorphism of the two enzymatic systems in selected androgenetic carrot plants of the cultivars: Berjo, Kazan $F_1$, and Splendid $F_1$, it was proven that 100% of those plants were homozygotes in respect to PGI and also with respect to AAT. In the second experiment, the obtained androgenetic progeny of the heterozygous donor plant of cultivar Narbonne $F_1$ was found to be 94% homozygotic with respect to PGI and 100% homozygotic in the case of AAT. For the androgenetic plants of the cultivar Kazan $F_1$, 89% of them were homozygotic with respect to AAT but PGI enzyme system did not differentiate the homozygotic and heterozygotic androgenetic progeny. These results indicated that enzyme polymorphism depends on carrot genotype, therefore, analysis of some (more than one) isozymes was necessary to confirm homozygosity of plants. PGI and AAT can be a useful tool for determining homozygosity in androgenetic carrot plants.

**Keywords** Androgenetic plant · Carrot (*Daucus carota* L.) · Flow cytometry · Histogram · Homozygosity · Isozyme · Ploidy

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## Introduction

Carrot (*Daucus carota* L.) is a vegetable of great economic importance in both Poland and the world. New carrot cultivars have been bred for various purposes: juices, frozen foods and the pharmaceutical industry. Heterozygous hybrids have dominated the carrot market since the 1970s. Obtaining such hybrids by conventional methods, i.e. self-pollination is difficult and time-consuming, it takes about 7–10 years to achieve homozygosity. The use of androgenesis can significantly shorten the time necessary to obtain complete homozygosity. The world literature contains some information on the use of androgenesis in carrot (Andersen et al. 1990; Hu et al. 1993; Matsubara et al. 1995; Tyukavin et al. 1999; Ferrie 2007) but even less is known about the assessment of carrot plants obtained from anther cultures. Andersen et al. (1990) and Tyukavin et al. (1999) studied the ploidy of plants obtained from anther cultures by counting chromosomes of the meristematic cells of root tips. However, in the literature pertaining to androgenesis in carrot, there is only one example of the use of flow cytometry for determining the ploidy of androgenetic plants (Górecka et al. 2009a). Among the

aforementioned researchers studying androgenesis in carrot, Tyukavin et al. (1999) tested the plants obtained from anther cultures in terms of homozygosity. They used molecular markers for that purpose. However, isoenzymatic markers have not been used before to establish the gametic origin of carrot plants obtained in anther cultures.

For over 20 years now, research in the fields of genetics and plant breeding has employed analyses based on the links between isoenzymatic markers and morphological characteristics. Isoenzymes have a number of advantages in comparison with morphological markers. They are present in various plant organs and tissues throughout the whole life of the plant. This makes it possible to carry out tests at a very early stage in plant development.

Bartošová et al. (2005) applied an analysis of acid phosphatase (E.C. 3.1.3.2) and peroxidase (E.C. 1.11.1.7) isoenzymes to verify the gametic origin of flax plants obtained from anther cultures and ovule cultures of three genotypes: AC Emerson, PRFGL 77 and Viking. Smykalová et al. (2005) used esterase (ESTE E.C. 3.1.1.2) to study homozygosity of caraway plants obtained from anther cultures. Westphal and Wricke (1989) studied the mechanism of inheritance of three enzymatic systems: diaphorase (DIA; E.C. 1.8.1.4), glucosephosphate isomerase (PGI; E.C. 5.3.1.9) and aspartate aminotransferase (AAT; E.C. 2.6.1.1) extracted from the leaves of *Daucus carota* L. ssp. *sativus*, and the relationships between loci of those isoenzymes. Górecka et al. (2009a) showed the use of PGI and AAT isoenzymatic markers for testing the homozygosity of carrot androgenetic plants. PGI and AAT are two important enzymes, which occur in glycolysis and amino acid metabolic pathways. Phosphoglucose isomerase is a housekeeping cytosolic enzyme of sugar metabolism that plays a key role in both glycolysis and gluconeogenesis pathways, catalyzing the interconversion of glucose 6-phosphate and fructose 6-phosphate; extracellularly, it behaves as a cytokine (Funasaka et al. 2005). Several functions have been attributed to aspartate aminotransferase (AAT). Silvente et al. (2003) showed the important role of AAT in catabolism and biosynthesis of aspartate and in assimilation of nitrogen in bean root nodules. This enzyme takes part in the redistribution of nitrogen and carbon pools between plant cell cytoplasm and other compartments, and between microbial symbiont and host cytoplasm. A technology to obtain carrot plants from anther cultures has been developed at the Research Institute of Vegetable Crops in Skierniewice (Górecka et al. 2005a). Embryogenic cultivars have been found (Górecka et al. 2005b), and an effective method of plant regeneration from androgenetic embryos has been developed (Górecka et al. 2009b). The purpose of the presented work was the elaboration of evaluation methods of carrot plants obtained from anther cultures, in relation to their ploidy and homozygosity.

## Materials and methods

The experiments were carried out on three carrot cultivars from Bejo Zaden company: Berjo, Kazan $F_1$, Narbonne $F_1$ and one from Rijk Zwaan company: Splendid $F_1$. Roots harvested from the field were stored in a chilling chamber at about +4°C (±1°C). After 3 months, two roots were planted per 10-l pot containing a mixture of sand and peat (2:1 v/v), with the addition of Azofoska—a multicomponent complex fertilizer containing 13.6% N, 1.9% P, 16.0% K, and microelements such as Mg, Cu, Zn, Mn, B and Mo—1.25 kg/m$^3$, and also chalk at 8.0 kg/m$^3$. The plants developed from the roots were kept in a greenhouse at about +20°C. They were additionally fertilized every 5 days with a liquid fertilizer Hydrovit 300 containing: 2.20% N-$NO_3$, 0.45% P, 2.26% K, 1.32% Ca, 0.49% Mg, and microelements such as Fe, Mn, Cu, Zn, B, Mo (used concentration 0.3% v/v).

Androgenetic embryos were obtained in anther cultures carried out according to the procedure described in a paper by Górecka et al. (2005a), and the process of plant regeneration followed the methodology presented by Górecka et al. (2009b). The assessment of ploidy of 61 plants of three carrot cultivars was carried out using flow cytometry. Samples were prepared in accordance with the recommendations of Galbraith (1984) with slight modifications. Measurement of DNA content in carrot leaves by means of a standard buffer is impossible because carrot leaves contain phenols which inhibit staining of DNA by a fluorochrome. To precipitate the phenols, 0.1% polyvinylpyrrolidone (PVP-40) was used. Plant material (approximately 20 mg of young leaves) was chopped in a Petri dish by means of a razor blade in the presence of 2 ml of cell lysis buffer CyStain DNA (Partec GmbH, Münster, Germany), with the addition of PVP-40, containing DAPI fluorochrome—4′,6-diamidino-2-phenylindole (0.1 mg in 1 ml) The samples were filtered with a filter Partec "Cell Trics" (Partec GmbH, Münster, Germany), 50 µm pore size. Each sample prepared in this way was tested by means of a CAII (Partec GmbH, Münster, Germany) flow cytometer, whose light source was an HBO mercury lamp. The control (diploid, $2n = 2x = 18$) plants were used as an external reference standard (1,000 nuclei were analyzed in each sample). Measurements of the fluorescence emitted by the fluorochrome stained DNA in the nuclei of the cells of the plants being tested were recorded in the form of histograms. Investigated plant ploidy was carried through comparison of position of peak corresponding to $G_1$ nuclei of the androgenetic plant sample with that of a diploid plant, used as the external standard.

The homozygosity of plants was determined by isozyme analysis of two enzyme systems: glucose-6-phosphate isomerase—PGI (E.C. 5.3.1.9), and aspartate

aminotransferase—AAT (E.C. 2.6.1.1). The allozyme polymorphism was observed among plants of two different trials. In the first experiment, an initial assessment was carried out on the polymorphism on the PGI enzymatic system in 12 androgenetic plants of Kazan $F_1$, 5 plants of Berjo, 2 plants of Splendid $F_1$, and on the polymorphism of the AAT enzymatic system in 8 androgenetic plants of Kazan $F_1$ and 5 androgenetic plants of Berjo. In the second experiment, an analysis was carried out on PGI and AAT enzymatic systems of 68 donor plants of Narbonne $F_1$ and 72 donor plants of Kazan $F_1$, which proved to be heterozygous in respect to these two izoenzymes, and of their androgenetic progeny.

The analysis of the enzymatic systems was carried out in the following manner. Extracts for allozyme analysis were obtained by grinding the fresh tissue. 20–30 mg of young leaves from the investigated plants in 0.07 tris-maleate extraction buffer, pH 8.0, containing 10% glycerol (v/v), 10% soluble polyvinylpyrrolidone (PVP-40), 0.5% Triton X-100 and 14 mM 2-mercaptoethanol. Electrophoresis in 10% starch gel was performed as described by Gottlieb (1973). The discontinuous tris-citrate/lithium-borate buffer system (Selander et al. 1971) was used for the separation of isozymes to ensure optimal conditions of separation and for stabilizing the enzymes. Slices were assayed according to Song (2001).

## Results

Flow cytometric measurements revealed that 55 carrot plants obtained from anther cultures (90% of analyzed plants) were diploid. One haploid plant was found, and five plants tested turned out to be tetraploids. The differences between the genotypes in terms of doubling were not stated in presented investigations. In vitro culture conditions can induce ploidy changes of regenerated plants. Examples of the histograms analyzed are shown in Fig. 1.

Because of the electrophoretic analysis of isozymes, in the case of PGI, enzymatic activity was found in two zones, one migrating faster in the electric field—PGI-1, and a slower migrating PGI-2. The PGI-1 activity zone, because of its monomorphic character, was not taken into consideration. In the PGI-2 zone, there were three electrophoretic variants. Two of them were characterized by the occurrence of a single band pattern migrating faster or slower in the electric field. The third variant found was characterized by three band patterns, two of which had the same electrophoretic mobility as the patterns of the single-band variants, and additionally a band pattern with intermediate mobility. The plants with 1 band pattern were classified as homozygotes. Considering the dimeric structure of PGI, the three-band electrophoretic phenotype was typical of a heterozygote consisting of two subunits with different electrophoretic mobilities. Examples of the heterozygote are shown in Fig. 2a. In the case of AAT, there were three activity zones found: AAT-1, AAT-2 and AAT-3. The AAT-1 zone proved to be monomorphic, whereas the other two activity zones exhibited polymorphism. The AAT enzyme has a dimeric structure and therefore single band patterns represent homozygotes, whereas a three-band phenotype signifies a heterozygote, with the central hybrid band pattern typical of a heterozygote of a dimeric enzyme (Fig. 2b).

In the initial assessment of polymorphism of the two enzymatic systems in selected androgenetic carrot plants obtained from anther cultures of the cultivars: Berjo, Kazan $F_1$, and Splendid $F_1$, it was proven that all of analyzed plants were homozygotes in respect of PGI. Visualization of AAT bands turned out to be difficult. There were the instances when obtained image of the bands was not clear. Determination of homozygosity in respect to AAT was not successful in all the plants analyzed in the initial assessment. Out of five plants of cv. Berjo and eight plants of cv. Kazan $F_1$, for which clear AAT bands had been successfully obtained, all were homozygotes (Table 1). In the second experiment, the tests carried out on donor plants of two cultivars: Kazan $F_1$ and Narbonne $F_1$ proved, that intravarietal changes in the number of isozymes patterns occured. In respect of PGI, heterozygotes were found in 39% of the donor plants of the cultivar Kazan $F_1$, and in 35% of the cultivar Narbonne $F_1$ (Table 2). Among the heterozygous donor plants of Narbonne $F_1$ only one plant was embryogenic, while in the cultivar Kazan $F_1$ 3 plants were embryogenic. The obtained androgenetic progeny of the heterozygotic donor plant of the cultivar Narbonne $F_1$ was found to be 100% homozygotic in the case of AAT and in 94% homozygotic with respect to the PGI isozyme (Table 3). According to Fig. 2 and Table 3, with respect to the polymorphic AAT zones, androgenetic progeny of one heterozygotic donor plant of the cultivar Kazan $F_1$ were homozygotic in 89% of cases. On the other hand, the segregation of alleles in the PGI-2 zone showed a pattern indicating homozygosity only in 3% of the androgenetic plants obtained from this donor plant.

## Discussion

Andersen et al. (1990) carried out assessments of the ploidy of carrot plants obtained in anther cultures over a period of 3 years. Each year, diploid plants outnumbered haploids and tetraploids. In the first year, 60% of the plants were diploids, 82% in the second, and 76% in the third year. Tyukavin et al. (1999) studied changes in ploidy during embryo development in carrot anther cultures, and during regeneration of plants from them. They observed cytological instability in

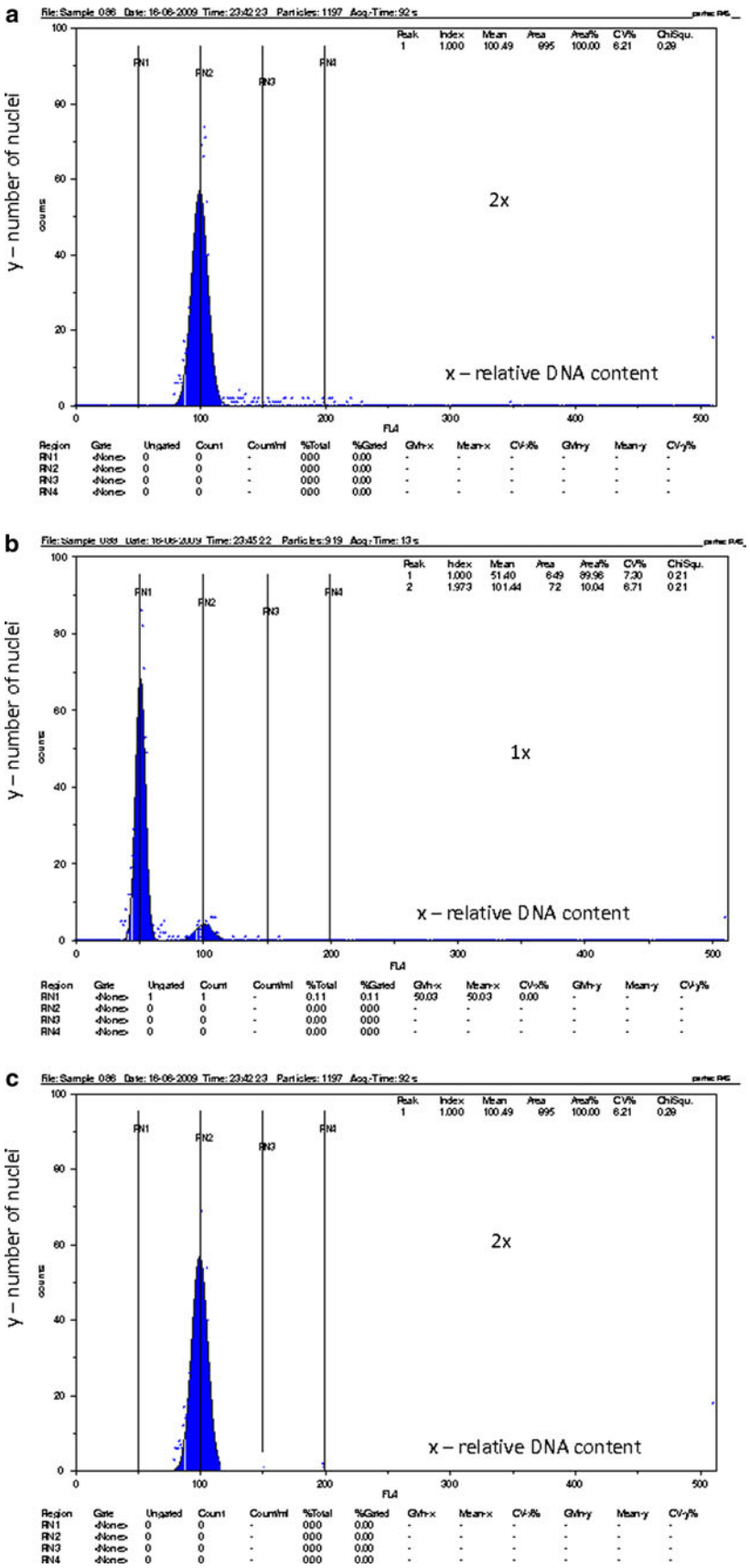


**Fig. 1** Examples of flow-cytometric histograms of relative DAPI fluorescence intensities in nuclei of carrot cv. Narbonne $F_1$ leaves, **a** external standard (2x), **b** haploid plant (1x), **c** doubled haploid plant (2x)

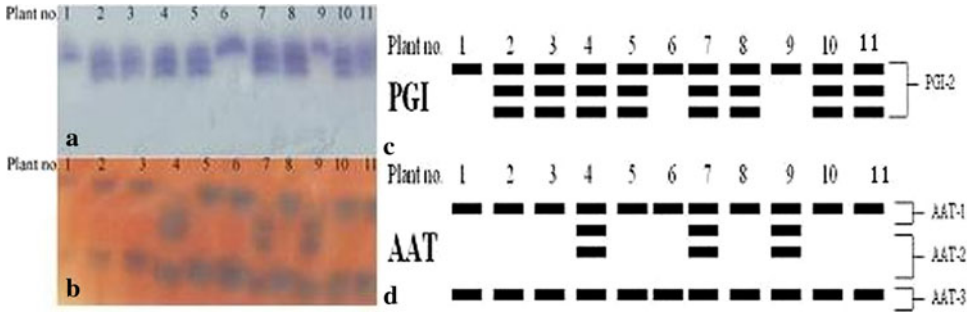


**Fig. 2** **a**, **b** Examples of izoenzyme zymograms of selected plants of cv. Kazan $F_1$. **a**, **b** photos of anodal bands of PGI (*1* homozygotic plant, *2* heterozygotic plant), and AAT (*1* homozygotic plant, *4* heterozygotic plant). **c**, **d** Graphic image of photographed anodal bands

**Table 1** Initial assessment of the homozygosity of carrot plants obtained from anther cultures

| Cultivar | PGI | | | AAT | | |
|---|---|---|---|---|---|---|
| | No. of plants analyzed | No. of homozygotes | No. of heterozygotes | No. of plants analyzed | No. of homozygotes | No. of heterozygotes |
| Kazan $F_1$ | 12 | 12 | 0 | 8 | 8 | 0 |
| Berjo | 5 | 5 | 0 | 5 | 5 | 0 |
| Splendid $F_1$ | 2 | 2 | 0 | 0 | – | – |

**Table 2** Heterozygosity of carrot donor plants in respect to the PGI isozyme

| Cultivar | Number of plants analyzed | No. of heterozygotes | % of heterozygotes | No. of homozygotes | % of homozygotes |
|---|---|---|---|---|---|
| Narbonne $F_1$ | 68 | 24 | 35 | 44 | 65 |
| Kazan $F_1$ | 72 | 28 | 39 | 44 | 61 |

**Table 3** Assessment of the homozygosity of carrot plants obtained from anther cultures from heterozygous donor plants

| Cultivar | Number of plants analyzed | PGI | | | | AAT | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of heterozygotes | Percentage of heterozygotes | No. of homozygotes | Percentage of homozygotes | No. of heterozygotes | Percentage of heterozygotes | No. of homozygotes | Percentage of homozygotes |
| Narbonne $F_1$ | 15 | 1 | 6 | 14 | 94 | 0 | 0 | 15 | 100 |
| Kazan $F_1$ | 78 | 76 | 97 | 2 | 3 | 9 | 11 | 69 | 89 |

androgenetic embryos and plant-regenerating tissues. The changes in ploidy that were taking place in them led to diploidization and, through stabilization on a genetic level, improved the adaptation potential of the plants obtained. In the experiments presented here, 90% of the plants had the normal diploid number of chromosomes, but five plants tested turned out to be tetraploids. Spontaneous duplication of chromosome number in anther or microspore culture in vitro was stated in numerous publications concerning androgenesis in several species (Chen et al. 1984; Cardoso et al. 2004; Höfer et al. 1999). Androgenetic plants of *Datura innoxia* obtained by Sunderland et al. (1974) were 1x, 2x, 3x and 4x. Cardoso et al. (2004) observed, using light microscopy, the initiation of chromosome doubling in soybean microspores, during anther cultures. Obtained data indicated, that in vitro culture conditions induced spontaneous chromosome diploidization. The increase in the ploidy level occurred in the first 20 days of culture. Höfer et al. (1999) in their experiments concerning apple androgenesis detected haploids in early the developmental stages of embryos and were probably eliminated during embryo development. Testillano et al. (2004) stated that maize microspores switched to an embryogenic pathway, diploidization occurs after 5 days of culture. The cells of embryo domain contained two nuclei which fused. The distribution of PGI and AAT enzymatic activity in carrot, obtained in our experiments during the electrophoresis in starch gel, corresponds to the image obtained in *Centrosema* by de Pentado et al. (1997). The image is characteristic of diploid species.

An analysis of the PGI and AAT enzymatic systems in carrot was carried out by Westphal and Wricke (1989). They investigated the inheritance of these systems and the linkage relationships among isozyme loci. They revealed the action of two allelic PGI variants with a single band pattern. The combined allelic action of these two subunits results in the development of a third band pattern with an intermediate velocity of migration. Such an arrangement is characteristic of a heterozygote. In our experiments, the images of band patterns characteristic of both homo- and heterozygotes were obtained for individual donor plants within the cultivars Narbonne $F_1$ and Kazan $F_1$. Zeidler (2000) and Weeden and Gottlieb (1980) stated that allelic forms of different loci must modify the production of isozymes in accordance with Mendel's laws of heredity. It thus proved to be appropriate to study donor plants with the aim of determining heterozygotes that would allow us to generate new genetic variability in anther cultures. Progeny of androgenetic origin should in this case have a homozygotic isoenzymatic profile. Szklarczyk (1996) states that the arrangement in which there are three band patterns is characteristic of a heterozygote encoded by one structural gene with two subunits. Because of that, these subunits, i.e. alleles, are present on homological chromosomes which are divided during the process of meiosis taking place in microsporogenesis. Westphal and Wricke (1989) observed three AAT loci in carrot: *Aat*-1 responsible for the development of band pattern 1 (homodimer), *Aat-2*—band patterns 1 and 5 (homodimers) and band pattern 3 (heterodimer characteristic of a heterozygote), and *Aat3*—band patterns 4, 6 and 7 (two codominating alleles are characteristic of the patterns *Aat 3-2* and *Aat 3-3*. In our work with carrot we found 3 AAT activity zones corresponding to the *Aat-1*, *Aat-2* and *Aat-3* loci, of which *Aat-1* turned out to be monomorphic (band pattern characteristic of a homo- and heterozygotes), whereas the others exhibited polymorphism. The plants, obtained from anther cultures, which were heterozygotes in respect of PGI, produced electrophoretic phenotypes characteristic for AAT homozygotes. In the work described here, all the plants tested in the initial assessment were 100% homozygotes in respect of the PGI isozyme.

The assessment of the populations of donor plants of two hybrid cultivars revealed that not all of them were heterozygotes in respect of PGI and AAT. For that reason, in order to confirm the gametic origin of the plants obtained via anther cultures, the cultures should be set up exclusively with heterozygotes in respect of the analyzed isoenzymes. In the experiment set up in this way, 94% of androgenetic plants of the cultivar Narbonne $F_1$ turned out to be homozygotes in respect of the PGI and 100% of AAT isozymes. The image of PGI band patterns obtained for heterozygotic donor plants and plants regenerated from embryos received from anther cultures indicated the gametic origin of the latter. In the case of the cultivar Kazan $F_1$ only the image of AAT band patterns indicated that the plants from anther cultures were homozygotes. Therefore, it seems appropriate to analyze several enzymatic systems to confirm the homozygosity of the plants obtained in anther cultures. Bartošová et al. (2005) analyzed two isozymes, acid phosphatase (ACP) and peroxidase (PRX), in order to prove the gametic origin and homozygosity of flax plants obtained from anther cultures and by gynogenesis. The patterns of bands produced by electrophoresis confirmed the usefulness of both enzymatic systems for determining homozygosity of regenerants and their gametic origin. The regenerated plants were found to have homozygotic profiles, with one band pattern instead of two or three observed in the donor plant.

At present, the homozygosity of the carrot plants obtained from anther cultures that were carried out in the experiments presented here is being assessed using conventional breeding methods, by selection in the field. By comparing them with the results obtained in those studies it will be possible to establish an optimal enzymatic system for proving the gametic origin of the plants obtained.

## Conclusions

1. In the experiments presented here, 90% of carrot plants obtained from anther cultures were diploid.
2. In the populations of carrot donor plants of two hybrid cultivars homozygotes and heterozygotes were found in respect of PGI, therefore, the selection of heterozygous donor plants using isozyme analyze is indispensable.
3. For confirmation of gametic origin carrot plants obtained from anther cultures, it is necessary to analyze at least two isozymes.
4. Obtained in presented investigation carrot plants were doubled haploids, their homozygosity was proved by isozyme analyze.

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