

## **Evaluation of phenotypic uniformity of androgenic R<sub>1</sub> population of carrot derived by anther culture technique**

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### ABSTRACT

Phenotypic uniformity of carrot androgenic lines of R<sub>1</sub> generations was assessed in respect to several morphological traits in vegetative and generative stages. Seeds of R<sub>1</sub> populations were obtained by self pollination of R<sub>0</sub> androgenic plants derived by the use of anther culture technique from three donor cultivars: 'HCM', 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>'. R<sub>1</sub> plants of donor cultivar 'HCM' were obtained from different embryo-like structures (excluding two lines) and plants of cultivars 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' were developed within the same embryo-like structure. The level of interline uniformity of individual R<sub>1</sub> genotypes was very diversified depending on donor cultivar and some morphological features of leaf and root of carrot plants. The highest intraline variation, according to the most of the investigated traits, was recorded for the clones derived from donor cultivars 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' but the lowest was observed for lines obtained from donor cultivar 'HCM'. Generally, there was a higher intraline

diversity of  $R_1$  lines in marketable traits than in measured traits. Androgenic  $R_1$  populations varied also in the level of fertility. All lines derived from donor open pollinated cultivar 'HCM' were fertile, in contrast to the clones originated from donor  $F_1$  hybrids 'Cx9900  $F_1$ ' and 'Narbonne  $F_1$ ' that segregated on fertile and sterile plants. Phenotypic characteristics and segregation in fertility particularly, may show genetic diversity, but they could also be influenced by some environmental factors.

## INTRODUCTION

Breeding of carrot hybrids, very pronounced in commercial production, is based on the development of inbred lines and incorporation of cytoplasmic male sterility lines crossed with male fertile pollinators. Inbred lines of carrot can be obtained with the use of traditional breeding or biotechnological methods, that may speed up and improve the process of deriving the source material for breeding.

Androgenesis is a biotechnological method used for generating doubled haploid (DH) homozygous lines as potential parental components for producing  $F_1$  hybrids for many crops. An advantage of anther technique over traditional breeding methods is the shortening of the breeding period by skipping steps of inbreeding for several generations. It might be important for crops such as carrot, with strong inbreeding depression, biennial habit and long vernalization period.

The androgenesis technique is used with success in obtaining DH plants for many crops. Homozygous eggplant specimens were produced by *in vitro* anther cultures in three years, less than half the time normally required (Borgel and Arnaud 1986). DH lines of head cabbage were uniform and stable in consecutive generations (Dore and Boulidard 1988, Kamiński et al. 2005). Doubled haploid lines of carrot were obtained by Andersen et al. (1990) using the anther culture technique. Recently, Adamus and Michalik (2003) demonstrated that the anther culture might not be effective or useful for carrot breeding as only 1% of regenerants resulted from androgenesis. In the Research Institute of Vegetable Crops (RIVC) in Skierniewice the anther culture technique has also been applied (Górecka et al. 2005) to yield androgenic carrot plants.

Androgenic plants from *Daucus carota* derived by the use of anther culture usually contained diploid individuals due to spontaneous polyploidisation during the culture. Because of that the use of chromosome count and flow-cytometry analysis can not be effective and were not used in our studies. Also, the isozyme glucose-6-phosphate isomerase test (PGI) (Adamus and Michalik 2003) was not performed to assess the level of homozygosity of  $R_0$  generation.

Therefore, in this study, evaluation of phenotypic uniformity of androgenic lines of  $R_1$  generation obtained from selfed  $R_0$  plants of carrot in both the

vegetative and generative stage, in respect to several morphological traits, were made to assess their level of homozygosity at the RIVC, Skierniewice, Poland. Simultaneously, molecular analyses including RAPD (Random Amplified of Polymorphism DNA) technique were performed to confirm or deny the level of homozygosity of R<sub>1</sub> populations (Staniaszek and Habdas 2006).

## MATERIAL AND METHODS

Seeds of R<sub>1</sub> generation were obtained by self pollination of R<sub>0</sub> androgenic plants derived by the use of anther culture technique in The Tissue Culture Laboratory, RIVC, Skierniewice, Poland (Górecka et al. 1999). The R<sub>0</sub> seedlings were transplanted into 5 litre plastic pots and grown in a greenhouse. After three months, plants were vernalised for 8 weeks at 2°C to induce seed stalk development. Plants with umbels were isolated in a cloth pollination cage and self-pollinated by houseflies. Androgenic carrot plants which set a sufficient amount of seeds of the R<sub>1</sub> generations were obtained from the three donor cultivars: 'HCM', 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' and were represented by 9, 4, and 3 lines respectively (Table 1). R<sub>1</sub> plants of donor cultivar 'HCM' were derived from different embryo-like structures (excluding two lines obtained from one embryo-like structure). Plants of cultivars 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' were developed within the same embryos, thus they should be named the clones. Donor cultivar 'HCM' was an open pollinated cultivar and two others ('CxC9900' and 'Narbonne') were F<sub>1</sub> hybrids.

Table 1. Breeding material used in the studies

Donor cultivar	No. of embryo	Line/clone
'HCM'	23.6	AA 50
	29.6	AA 59
	39.4	AA 62
	26.4	AA 78
	40.3	AA 90
	40.3	AA 92
	45.3	AA 94
	61.12	AA 97
	32.1	AA 119
'CxC9900 F <sub>1</sub> '	44.1	BB 2
	44.1	BB 3
	44.1	BB 4
	44.1	BB 12
'Narbonne F <sub>1</sub> '	3.10	EE 5
	3.10	EE 6
	3.10	EE 19

Phenotypic evaluation of uniformity of androgenic lines was performed in vegetative and generative phase. The experiments were conducted in 2003 (vegetative phase in the field) and 2004 (generative phase in a greenhouse) at the RIVC in Skierniewice. The soil type was a pseudopodsolic over loamy sand (1.15% organic mater, pH 6.5). Seeds of  $R_1$  populations were sown on May 10 in the field in 3 cm density between plants in one row and with 67.5 cm distance between the rows. The experiments were set up according to a randomised complete block design with three replication of 14 m<sup>2</sup> plot area. Fertilisation and plant protection against pests and diseases were provided according to current recommendations for carrot. At vegetative stage androgenic carrot  $R_1$  population were estimated in respect of leaf size (width, length) and leaf division (classes: 1 – very coarse, 5 – very fine). Carrot roots were harvested in October, when they reached maturity. Mass, length and width of ten carrot roots from each plot were measured. Morphological features of root (surface, shape of root and tip of root) were also evaluated. Morphological traits were classified using the multigrade scale of the International Union for the Protection of New Cultivars of Plants, Geneva, Switzerland (U.P.O.V.).

To asses the uniformity of androgenic  $R_1$  lines, variation coefficient (V%) was calculated. All of the harvested androgenic plants of carrot were vernalised at 2°C through December 2003 to February 2004 to induce generative phase. Then roots of carrot were planted into 5 litre plastic pots in the greenhouse and were self-pollinated under cloth pollination cages. At flowering, the level of fertility/sterility of plants was estimated by macroscopic observation of flowers, based on the number of plants with cytoplasmic male sterility (*cms*) within each population.

## RESULTS AND DISCUSSION

The aim of this study was to evaluate phenotypic uniformity of androgenic  $R_1$  populations of carrot obtained from three donor cultivars in both vegetative and generative stage in respect to several morphological traits. The level of uniformity of individual  $R_1$  genotypes was very diversified depending on donor cultivar and some morphological features of leaf and root of carrot plants. The highest intraline variation according to the most of investigated traits was recorded for the clones derived from donor cultivars 'CxC9900 F1' and 'Narbonne F1' and the lowest was observed for lines obtained from donor cultivar 'HCM'. Generally, there was a higher intraline variation of  $R_1$  lines in marketable traits than in the measured traits.

Low values of variation coefficient for measured traits (except for root weight) reflects high uniformity within each tested lines/clones derived from all donor cultivars (Tables 2 and 3). For most traits and lines, the values of variation coefficient were low and did not exceed 27%. However, the biggest intraline variation was observed in root weight for almost all tested  $R_1$  lines/clones. In that case variation coefficient was higher and reached 30-80%.

Table 2. Morphological features of carrot leaf of R<sub>1</sub> populations

Donor cultivar	Line/ clone	Leaf length		Petiole length		Leaf width		Leaf division**
		mean (cm)	V (%)*	mean (cm)	V (%)*	mean (cm)	V (%)*	
'HCM'	AA 50	32.0	7.9	12.0	18.3	15.7	14.1	1-2
	AA 59	35.0	8.8	14.0	23.1	18.6	20.3	1-2
	AA 62	33.0	12.3	12.0	20.6	17.0	18.4	2-3
	AA 78	30.0	9.8	13.0	15.5	14.0	20.5	1-2
	AA 90	35.0	9.6	13.0	18.2	17.2	18.7	1-2
	AA 92	34.0	12.2	14.0	20.2	16.3	22.0	1-2
	AA 94	38.0	8.2	15.0	20.7	17.1	21.4	1-2
	AA 97	30.0	15.1	12.0	30.5	14.0	15.4	1-2
	AA 119	34.0	6.3	12.0	18.4	18.4	13.6	1-2
'CxC 9900 F <sub>1</sub> '	BB 2	30.5	11.2	13.1	16.3	12.5	9.4	3-5
	BB 3	26.0	8.3	10.6	18.4	15.8	12.6	3-5
	BB 4	30.0	6.2	13.0	21.4	13.6	24.6	3-5
	BB 12	26.0	11.1	9.5	14.2	13.6	11.6	4-5
'Narbonne F <sub>1</sub> '	EE 5	35.0	10.2	17.0	21.7	16.1	13.8	2-4
	EE 6	35.0	10.2	15.0	17.1	15.0	14.4	2-4
	EE 19	18.5	11.2	9.0	17.4	12.5	14.2	3-5

\*V (%) – variation coefficient

\*\* Leaf division 1 – 5 (1 – very coarse, 5 – very fine)

Table 3. Morphological features of carrot root of R<sub>1</sub> populations

Donor cultivar	Line/clone	Root length		Root width		Root weight		Root surface**		Root shape***		Root tip****	
		mean (cm)	V (%)*	mean (cm)	V (%)*	mean (cm)	V (%)*	mean	range	mean	range	mean	range
'HCM'	AA 50	18.9	23.9	3.5	11.7	0.11	27.9	4.8	4-5	5.0	5	3.0	3
	AA 59	18.6	27.0	4.1	12.3	0.21	44.4	3.3	2-4	3.4	3-4	3.0	3
	AA 62	21.7	9.3	3.4	13.5	0.13	37.5	3.5	3-5	4.3	4-5	3.0	3
	AA 78	21.1	10.6	4.1	10.8	0.16	32.9	5.0	5	3.4	3-4	2.8	2-3
	AA 90	19.4	11.7	4.0	9.3	0.15	25.0	4.0	3-5	4.0	4	2.8	2-3
	AA 92	21.3	13.5	3.9	17.9	0.16	40.8	5.0	5	3.8	3-4	2.9	2-3
	AA 94	21.1	8.5	4.3	9.8	0.15	15.1	3.4	3-5	4.0	4	2.4	2-3
	AA 97	21.9	10.3	4.1	10.1	0.15	36.6	5.0	5	4.0	4	3.0	3
	AA 119	22.4	15.5	3.7	18.2	0.16	38.7	5.0	5	4.0	4	3.0	3
	'CxC 9900 F <sub>1</sub> '	BB 2	14.4	14.3	2.9	11.8	0.15	39.3	1.8	1-3	2.5	2-4	1.6
BB 3		13.6	21.1	2.8	18.9	0.17	59.1	1.7	1-3	3.6	3-4	1.8	1-3
BB 4		21.2	19.1	4.1	17.9	0.25	39.9	2.5	1-3	2.9	2-4	1.3	1-2
BB 12		17.7	15.5	4.1	12.6	0.17	40.6	2.0	1-3	3.7	2-4	1.8	1-3
'Narbonne F <sub>1</sub> '	EE 5	18.3	18.6	4.2	19.9	0.20	32.9	2.9	2-4	3.3	2-4	2.1	1-3
	EE 6	17.4	23.6	5.0	24.9	0.28	48.4	2.1	2-4	2.9	2-4	1.8	1-3
	EE 19	18.8	10.2	4.5	11.9	0.20	49.6	3.8	2-5	3.6	2-5	2.5	1-3

\*V (%) – variation coefficient

\*\*Root surface 1-5 (1-almost smooth, 5-rough)

\*\*\*Root shape 1-5 (1-splidle-shaped, 5-strongly conical)

\*\*\*\*Root tip 1-3 (1-circular, 3-pointed)

In contrast to the measured traits, clones originated from donor cultivars 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' showed large intraline diversity according to some investigated features of leaf and root of carrot. Leaf division, surface, shape of root and tip of root clearly diversified the tested populations. Three or four types of leaf division (Table 2), shape of root and tip of root (Table 3) were observed among the clones derived from donor cultivar 'CxC9900 F<sub>1</sub>'. A similar variation including three classes was noticed for the same features in the most clones obtained from cultivar 'Narbonne F<sub>1</sub>'.

In contrast to cultivar 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>', all lines from donor cultivar 'HCM' seemed to be uniform according to most leaf and root marketable morphological traits. A stronger diversity was observed only in the type of root surface and leaf division among some androgenic lines (Tables 2 and 3).

The phenotypic differences observed appeared to correlate well with the divergence seen between androgenic R<sub>1</sub> populations derived from a single embryo. It is possible but not likely that such diversity between clones may be a result of somatic embryogenesis.

The intraline R<sub>1</sub> diversity of the analysed traits of leaf and root may reflect genetic differences, particularly for populations derived from 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' donor cultivars. It could also be influenced by some environmental factors such as lack in soil uniformity and its conditions, plant stand and micro-climatic variation (Rubatzky et al. 1999). Also Andersen et al. (1990) demonstrated that DH lines were not completely uniform in the field condition. Therefore, an evaluation of R<sub>1</sub> generation based only on morphological characteristics is not an effective method to evaluate their uniformity and consequently to estimate their homozygosity.

Additionally, androgenic R<sub>1</sub> populations varied also in the level of fertility (Table 4). All lines derived from donor open pollinated cultivar 'HCM', as expected, were fertile and developed typical for carrot flowers with five stamens. In contrast to the material obtained from cultivar 'HCM', all clones originated from donor F<sub>1</sub> hybrids 'CxC9900' and 'Narbonne F<sub>1</sub>' segregated on fertile and *cms* sterile plants with additional five petals replaced the five stamens. The highest number of sterile plants was observed in the clone BB 3 (46.7%) and the lowest in the clone BB 12 (26.7%). Moreover, sterile plants of most of the investigated clones from 'CxC9900 F<sub>1</sub>' and 'Narbonne F<sub>1</sub>' segregated in petal colour and stamen transformation (data not shown). The petals and stamens transformations were white, white-green and green. Different shapes of petaloids were also observed as complete transformation or the stamen were transformed to spoon like structures and filamentous. The diversity of tested androgenic plants of R<sub>1</sub> generation in regard of the analysed traits showed their genetic differences.

Table 4. Level of fertility/sterility of R<sub>1</sub> populations

Donor cultivar	Line/clone	Number of plants (%)	
		fertile	sterile
'HCM'	AA 50	100	0
	AA 59	100	0
	AA 62	100	0
	AA 78	100	0
	AA 90	100	0
	AA 92	100	0
	AA 94	100	0
	AA 97	100	0
	AA 119	100	0
'CxC9900 F <sub>1</sub> '	BB 2	58.8	41.2
	BB 3	53.3	46.7
	BB 4	62.8	37.2
	BB 12	83.3	26.7
'Narbonne F <sub>1</sub> '	EE 5	71.8	28.2
	EE 6	62.5	37.5
	EE 19	60.0	40.0

In contrast to morphological traits investigated in the vegetative stage, segregation in fertility/sterility estimated in the generative phase, can be rather an effect of genetic diversity than environmental factors. Additionally, segregation on fertile and sterile plants within some clones showed the evidence of heterozygosity in regard to the sex expression. Such segregation in R<sub>1</sub> populations obtained from donor cultivars being F<sub>1</sub> hybrids constituted a potential proof of genetic differentiation and not uniformity of R<sub>1</sub> generation.

Diversity in phenotypic characteristics and segregation in fertility especially may show genetic diversity but molecular analysis could provide final information on the origin (somatic or gametic) of all tested populations obtained by the use of anther culture technique. Performed RAPD analyses of 30 lines/clones of R<sub>1</sub> generation derived by the use of anther culture technique demonstrate that all tested R<sub>1</sub> populations are not homozygous and should not be named DH lines (Staniaszek and Habdas 2006). Our results are consistent with the work performed by Adamus and Michalik (2003), where only 1% of regenerants resulted from androgenesis.

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## CONCLUSION

Observations on morphological characters in both, vegetative and generative phase and especially RAPD analysis as well of carrot R<sub>1</sub> generations indicate that anther culture is not an effective method for the development of DH lines in carrot. Final conclusion can be made that the applied anther culture technique is not efficient in raising DH lines in carrot.

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## OCENA FENOTYPOWEGO WYRÓWNANIA ANDROGENICZNYCH POPULACJI R<sub>1</sub> MARCHWI UZYSKANYCH METODĄ KULTUR PYLNIKOWYCH

**Streszczenie:** Celem badań była ocena wyrównania populacji R<sub>1</sub> pod względem cech morfologicznych zarówno w fazie wegetatywnej jak i generatywnej. Nasiona populacji R<sub>1</sub> otrzymano w wyniku samozapylenia roślin pokolenia R<sub>0</sub>, uzyskanych techniką kultur pylnikowych z trzech donorowych odmian: 'HCM', 'CxC9900 F<sub>1</sub>' i 'Narbonne F<sub>1</sub>'. Badane populacje R<sub>1</sub> odmiany 'HCM' pochodziły z różnych zarodków (z wyjątkiem dwóch linii), natomiast z odmian 'CxC9900 F<sub>1</sub>' i 'Narbonne F<sub>1</sub>' z tego samego zarodka. Badania przeprowadzono w latach 2003 (faza wegetatywna) i 2004 (faza generatywna). W czasie wegetacji dokonano obserwacji najważniejszych cech morfologicznych liścia (długość i szerokość, długość ogonka liściowego, podział blaszki liściowej) i korzenia (masa, długość i szerokość, kształt korzenia i jego zakończenia oraz gładkość skórki). W fazie generatywnej oceniono poziom płodności/sterylności roślin badanych populacji. Poziom wyrównania populacji R<sub>1</sub>, ocenianych pod względem morfologicznych cech zarówno w fazie wegetatywnej, jak i generatywnej, był zróżnicowany w zależności od odmiany donorowej. Najmniejszym wyrównaniem pod względem większości badanych cech morfologicznych liścia i korzenia charakteryzowały się linie pochodzące z odmian 'CxC9900 F<sub>1</sub>' i 'Narbonne F<sub>1</sub>', które jednocześnie segregowały na rośliny sterylne i płodne. Natomiast największy stopień wyrównania stwierdzono u linii z odmiany 'HCM'. Fenotypowe zróżnicowanie wewnątrzliniowe badanych populacji R<sub>1</sub> zarówno w fazie wegetatywnej jak i generatywnej może mieć podłoże genetyczne, jak również wynikać z wpływu warunków środowiska.

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