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Effect of cultivation factors on embryogenesis in isolated microspore culture of carrot (*Daucus carota* L.)

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Abstract: Using doubled haploid technologies inbreeding can significantly reduce the time to obtain homozygous parental lines required for the production of F1-hybrid of vegetable crops. This study aims to investigate the influence of factors on the efficiency of carrot embryogenesis in isolated microspore culture to optimise the elements of protocol for producing doubled haploids. Microspores were isolated from inflorescences of 21 genotypes and incubated in NLN13 medium supplemented with 0.1 mg·dm^{-3} 2,4-dichlorophenoxyacetic acids, 0.1 mg·dm^{-3} 1-naphthyl acetic acids, 130 g·dm^{-3} sucrose, and 400 mg·dm^{-3} casein hydrolysate and its modifications. Embryoids and their groups were formed after 2–6 months, in some cases after 12 months of cultivation. Depending on the variant, the embryogenesis efficiency averaged from 0 to 4.9 embryoids or groups of embryoids per Petri dish (10 cm^3). Embryoids within the group were formed from different microspores. No significant effects of inflorescence position on the plant (branching order), sucrose, and casein hydrolysate concentration in the medium were observed. Significant advantages ($p \geq 0.05$) for some genotypes were shown: 1) microspore suspension density 4·10^4 cells·cm^{-3} (5.0 embryoids per Petri dish were formed at a microspore suspension density of 4·10^4 cells·cm^{-3}, 0.0 embryoids per Petri dish at a density of 8·10^4 cells·cm^{-3}); 2) cultivating microspores of tetrad and early mononuclear stage (4.9 ±3.1 embryoids per Petri dish were obtained by culturing tetrads and early mononuclear microspores, while 0.6 ±0.7 embryoids per Petri dish were obtained by culturing of later developmental stages); 3) high-temperature treatment duration of five days (4.9 ±2.1 embryoids per Petri dish were obtained after five days of high-temperature treatment, 2.7 ±2.6 embryoids per Petri dish formed after two days of high-temperature treatment; 9.8 ±4.7, 10.1 ±6.1, 0.0 ±0.0 embryoids per Petri dish formed after two, five and eight days of high-temperature treatment respectively); 4) adding colchicine 0.5 mg·dm^{-3} to the nutrient medium for two days of high-temperature treatment, followed by medium replacement (3.3 ±2.6 embryoids per Petri dish were obtained by using a nutrient medium with colchicine, while 1.7 ±1.5 embryoids per Petri dish were obtained by culturing in the reference variant).

Keywords: carrot (*Daucus carota* L.), doubled haploids, embryogenesis, isolated microspore culture, male sterility

INTRODUCTION

Doubled haploid production *in vitro* is a biotechnological method that allows only the first one to be accelerated, but even this can make the work of breeders easier and quicker. By selecting plant material from the best F1-hybrid seeds with a complex of economically valuable traits, the probability of obtaining highly productive lines increases. Since carrot seed production is based on nuclear-cytoplasmic male sterility, it is especially valuable and promising to obtain doubled haploids, i.e., homozygotes, for nuclear sterility genes with normal cytoplasm [Chistova 2018]. In order for the microspore to switch from the gametophytic to the sporophytic developmental pathway, i.e., to form the embryoid instead of pollen grain, a number of factors needs to be combined

at the right time. Studies are known to describe numerous changes, ranging from general morphology to gene expression, which result in the ability of microspores to realise totipotency. It is assumed that different stresses induce a similar signalling response in plants. Only combinations of conditions are required for different crops [Kiszczak *et al.* 2015; 2018; Seguí-Simarro, Nuez 2008].

Sangwan and Sangwan-Norreel [1987] researched the cytological mechanism of microspore transition from gametophytic to sporophytic pathway in carrot anther culture and revealed that this process is associated with disruption of differentiating mitosis, which normally should lead to division of mononuclear microspore nucleus into generative and vegetative. The generative nucleus is more compact and better stained, while the vegetative nucleus is several times larger. However, during *in vitro* cultivation of anthers, Sangwan and Sangwan-Norreel [1987] observed the division of microspores into cells morphologically similar to meristematic cells with centrally located identical nuclei. Subsequently, embryoids could be developed from the both of cells or from one of them.

It is thought that exposure of microspores to stress factors causes disruption of the microtubules that determine asymmetric division of the nucleus. One or both nuclei may continue to divide, forming a multinucleated cell that later becomes a multicellular haploid structure. In other cases, the two nuclei may fuse after postmeiotic mitosis to form a single 2n-nucleus. It may continue to divide; the multi nucleus cell was becoming a multicellular haploid structure, resulting in the formation of a plant as a doubled haploid. Shim *et al.* [2006] incubated barley microspores and observed this process of membrane fusion of two nuclei. Chromosome doubling can occur not only through fusion of haploid nuclei membranes but also through endomitosis (doubling the number of chromosomes without subsequent separation). Endomitosis can occur spontaneously or artificially by colchicine treatment [Shim *et al.* 2006]. Chromosome doubling can also be induced by treatment of microspores with mannitol, which induces nuclear fusion after the first mitotic division [Kasha *et al.* 2001]. Researchers have published a sufficient number of papers reporting the successful cultivation of isolated carrot microspores [Górecka *et al.* 2010; Li *et al.* 2013]. They obtained from five to hundreds of embryoids per Petri dish depending on the genotype. The process seems to be well understood, but it requires optimisation for mass production of doubled haploids for breeding purposes. Additional detailed research of the effect caused by various factors on the isolated carrot microspore culture is also important because the results published by researchers do not always coincide. Thus, the aim of this work was to study the effect of a number of factors on the efficiency of embryogenesis, and also to optimise incubation technology elements for isolated carrot microspores.

MATERIALS AND METHODS

The work was carried out in 2018 at the N.N. Timofeev Breeding Station Ltd. and at the Laboratory of Genetics, Breeding and Biotechnology for Vegetable Crops, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy.

Carrot plants of F1 hybrids and inbred lines were used as plant material. Carrot seeds were sown in June 2017 in the field conditions. The root crops were stored from October to January 2018 and then planted in a heated outdoor greenhouse. DNA was isolated from the regrown young leaves according to the Murray and Thompson [1980] with minor modifications. Molecular genetic analysis was then carried out to differentiate plants according to the "petaloid" and normal types of cytoplasm. The polymerase chain reaction was performed according to the protocol by Bach *et al.* [2002].

Young flat inflorescences of 4–6 cm in diameter were sampled in the morning (8:30–9:30), placed in a container on a water-moistened filter paper, transferred to the laboratory for 15 min and used for microspore isolation. The predominant stage of microspore development was determined by cytological analysis. Several buds were crushed on a glass slide with a dissecting needle and large pieces of tissue were removed. Microspores were fixed on the glass slide with 0.02 cm^3 of a 3:1 mixture (3 parts of 96% alcohol and 1 part of glacial acetic acid) for 10–12 min, then stained with 0.012 cm^3 DAPI for 10–15 min. The preparations were covered with a coverslip and microscopically examined. It should be noted that the microspores were isolated and incubated according to the methods described by Li *et al.* [2013]. The factors studied in the current study, and the number of replications (Petri dishes) per experiment variant, are shown in Table 1.

Table 1. Number of replications to study the influence of factors on embryogenesis efficiency in the isolated microspores culture of carrot

Factor	Variant	Number of Petri dishes
Branching order (location of inflorescence on the plant)	central axis	9
	1st order	9
	2nd order	8
Dominant stage of microspore development	tetrads, early mononuclear	11
	middle and late mononuclear	11
Heat shock	two days (reference)	40
	five days	33
	eight days	11
Addition of colchicine to the growth medium	NLN13mk without colchicine (reference) [Lighter 1989]	15
	2 days on NLN13mk medium supplemented with 0.5 $mg \cdot dm^{-3}$ colchicine followed by a change of medium to NLN13mk	14
Density of the microspore in suspension	$4 \cdot 10^4$ (reference)	16
	$8 \cdot 10^4$	12
Sucrose content in the growth medium	130 $g \cdot dm^{-3}$ (reference)	19
	100 $g \cdot dm^{-3}$	16
The concentration of casein hydrolysate in the nutrient medium	400 $mg \cdot dm^{-3}$ (reference)	10
	800 $mg \cdot dm^{-3}$	10

Source: own elaboration.

RESULTS AND DISCUSSION

In this work, microspores of 21 genotypes were incubated, 7 of which showed responsiveness (Nakton 2, Nakton 3, SKN 6, Vilmorin 1, Vilmorin 2, Vilmorin 3, Volkano 1). In addition, single embryoids of the other four genotypes (Nakton 1, Nakton 4, Mercurio 2, SKN 4) were obtained, but these were not considered in the overall data analysis.

Depending on their genotype, carrot embryoids can be seen at eye level after 2 and 6 months of incubation. In this work, the formation of both single embryoids and groups of embryoids was observed. The individual embryoids within the groups were formed from different microspore cells. Consequently, plants derived from different embryos of the same group can have different genotypes. When incubated on a shaker, groups of embryoids did not disintegrate and continued to develop together. When a single embryoid reached the torpedo stage, active secondary embryogenesis was observed. To compare the embryogenesis efficiency, the number of single embryoids or groups of embryoids formed in a Petri dish was counted.

Embryogenesis in different replications (Petri dishes) within each variant was very uneven. Thus, the number of embryoid groups in different replicates of the same variant often varied from several tens to zero. The highest number of embryoids per Petri dish was 45, while the average values ranged from 0 to 4.9. Huge values for the standard deviation and thus for the confidence interval are related to this. Comparison of embryogenesis efficiency in microspores of Nacton 2 and Volcano 1 genotypes with a predominance of 1) tetrad and early mononuclear stage of development and 2) medium and late mononuclear stage of development showed no significant differences. However, the Nacton genotype revealed a significant increase ($p \geq 0.05$) in the number of embryoids while incubating cell suspension with the predominance of tetrads and early mononuclear microspores (4.9 embryoids per Petri dish), compared with the incubation of microspores of later developmental stages (0.6 embryoids per Petri dish) – see Table 2.

Table 2. The average number of embryoids formed by incubating microspores of different developmental stages (embryoids per Petri dish)

Predominant stage of microspore development	The genotype of the donor plant		
	Nakton 3	Nakton 2	Volkano 1
Tetrads and early mononuclear	4.9 ±3.1a	2.0 ±2.0a	4.3 ±4.6a
Medium and late mononuclear	0.6 ±0.7b	1.0 ±1.0a	0.0 ±0.0a

Explanations: values marked with the same letters have no significant difference when $\alpha = 0.05$ ($p \leq 0.05$).
Source: own study.

The effectiveness of increasing the high-temperature treatment duration to 8 days was researched; the study was carried out on three genotypes selected from the Vilmorin plant population: Vilmorin 1, Vilmorin 2, and Vilmorin 4. No statistically significant differences were found in the number of formed embryoids for all genotypes when the heat treatment duration was 2 and 5 days. In the case of 8-days heat treatment of Vilmorin 1 genotype microspores, reliably fewer embryoids were formed (0 pcs per Petri dish) compared with the 2-days (9.8 pcs per Petri dish) and 5-days (10.1 pcs per Petri dish) incubation at 33°C. In general, increasing the high-temperature treatment duration for microspores had no significant effect on the number of forming embryoids of most genotypes studied. However, a 5-day high-temperature treatment leads to a slight increase in embryogenesis efficiency, which makes this variant recommendable for use (see Tab. 3).

Table 3. The average number of embryoids formed after high-temperature (33°C) treatments of different durations (embryoids per Petri dish)

Year	Genotype	Number after		
		2 days	5 days	8 days
2018	Nakton 2	2.7 ±2.6a	4.9 ±2.1b	–
	Nakton 3	1.3 ±2.5a	3.2 ±2.6a	–
	SKN 6	0.0 ±0.0a	0.7 ±0.7a	–
2019	Vilmorin 1	9.8 ±4.7a	10.1 ±6.1a	0.0 ±0.0b
	Vilmorin 2	8.4 ±10.5a	7.6 ±5.3a	8.5 ±14.8a
	Vilmorin 4	7.8 ±9.4a	9.6 ±7.6a	8.5 ±7.0a

Explanations as in Tab. 2.
Source: own study.

Incubating the microspores on nutrient medium with colchicine added during the high-temperature treatment followed by a change of nutrient medium showed an increase in the number of embryoids per Petri dish, but only for the Nacton 2 genotype; this increase was statistically significant (Tab. 4).

Table 4. The average number of embryoids formed depending on the presence of colchicine in the nutrient medium during the high-temperature treatment followed by medium replacement (embryoids per Petri dish)

The genotype of the donor plant	Number at nutrition solution	
	NLN13mk	NLN13mk + colchicine
Nakton 2	1.7 ±1.5a	3.3 ±2.6b
Nakton 3	1.1 ±2.2a	2.6 ±3.3a

Explanations as in Tab. 2.
Source: own study.

For the Volcano genotype, significant differences in the number of embryoids were found at the 0.1 significance level (5.0 embryoids per Petri dish at a microspore suspension density of $4 \cdot 10^4$ cells·cm^{-3}, 0.0 embryoids per Petri dish were formed at a density of $8 \cdot 10^4$ cells·cm^{-3}). After transfer to a solid B5 nutrient medium without growth regulators, the embryoids germinated into seedling-like plants with a normal root system and few leaves. Secondary embryogenesis continued in some cases. The efficiency of acclimatisation of those plants to soil conditions varied from 23.3 to 100% depending on greenhouse conditions (temperature, humidity). Ploidy analysis by indirect method

(number of chloroplasts in stomata guard cells and direct chromosome counting [Kirov *et al*. 2014] in anthers of produced plants revealed diploid plants.

CONCLUSIONS

This paper showed that to obtain carrot doubled haploids by isolated microspores, the plant material in the tetrad and early mononuclear stage of development should be selected; the microspores should be isolated using B5 medium supplemented with 130 g·dm^{-3} sucrose and 50 g·dm^{-3} mannitol, and incubated on NLN13 medium supplemented with 0.1 mg·dm^{-3} 2,4-D, 0.1 mg·dm^{-3} NAA, 400 mg·dm^{-3} casein hydrolysate and 130 g·dm^{-3} sucrose. The microspore suspension density should be 4·10^{4} cells·cm^{-3}; the recommended period of high-temperature treatment at 33°C should be increased to 5 days. Embryogenesis efficiency was 0–4.9 embryoids per Petri dish (10 cm^{3} microspore suspension) and depended on the genotype. The highest number of embryoids per Petri dish was 45. Adding 0.5 mg·dm^{-3} colchicine to the nutrient medium during high-temperature treatment followed by medium replacement increased the embryoid yield, and this effect was observed only in one of the genotypes studied.

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