UDC 635.132 : 631.527 : 631.147 : 635.132

REGENERATION OF PLANTS FROM GYNOGENETIC CARROT CALLUSES

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Received on September 5, 2015

Aim. To optimize the regeneration and adaptation stages of the methods of obtaining dihaploid carrot plants via gynogenesis in the culture *in vitro*, namely embryogenesis from gynogenetic callus, and to obtain normally developed regenerant plants and regenerant roots. **Methods**. Common biotechnological methods were applied. The gynogenetic carrot callus, line 345, was obtained from non-fertilized ovaries using the method, invented by the authors. **Results.** The culture medium, in which the yield of embryos from one gynogenetic callus increases 4-fold, and the yield of normal plants – 12-fold, was invented. While growing roots using regenerant plants in unprotected soil the mulching of soil with white agrofiber ensures the survival of regenerants at the level of 86.5 % which is 16 % higher than the control (without mulching) and the increase in the average weight of roots up to 92.8 g (38.5 g more compared to the control). **Conclusions.** The regenerating and adaptive stages of obtaining dihaploid regenerant plants from gynogenetic carrot calluses, induced from non-fertilized ovaries in culture *in vitro* were optimized. The efficient culture medium for embryogenesis from gynogenetic calluses was elaborated. It was established that soil mulching using white agrofiber increases the survival of gynogenetic plants in unprotected soil considerably and ensures the increase in the average weight of roots. It allows refusing from greenhouses or tunnels while growing regenerant roots of gynogenetic origin.

Keywords: carrots, experimental haploidy *in vitro*, non-fertilized ovaries, callus, embryogenesis, regenerant plants.

DOI: 10.15407/agrisp2.03.049

INTRODUCTION

In two recent decades the artificial selection of domestic plants witnesses ever wider application of the experimental haploidy method – obtaining plants with a single set of chromosomes. In nature haploids may occur spontaneously with the frequency of one haploid per 10^5–10^6 plants. Haploid plants are remarkable for a smaller stalk, late ripeness, and, as a rule, sterility [1]. To restore the diploid set of chromosomes haploids are treated with colchicine, which allows obtaining fertile plants, completely homozygous by all the allele genes. Spontaneous restoration of ploidy degree is also possible (for instance, for carrots).

The application of haploids in the selection of plants allows solving the following tasks:

– fast obtaining of genetically stable non segregating lines, homozygous for all the loci. It shortens the duration of the breeding procedure for self-pollinated plants by 3–4 years, and for cross-pollinated plants – by 5–6 years. This approach is very efficient for heterosis breeding, when the production of inbred lines lasts for 5–6 years;

– the efficient selection of genotypes due to phenotype manifestation of recessive genes in haploids and dihaploids. There is elimination of organisms with lethal and semilethal genes;

– the obtaining of monosomic lines for their application in the genetic analysis and chromosomal engineering.

Two *in vitro* methods are widely used to obtain haploids (or diploids):

androgenesis – the development of haploid plants using microspores grown aseptically on the nutrient medium, which is applied for over 250 species of plants, including wheat, rice, corn, barley, flax, alfalfa, grapes, apples, carrots, *etc*.;

gynogenesis – the development of haploid plants using the isolated ovaries grown aseptically on the nutrient medium, which is less common compared to androgenesis, but efficient in the production of haploids of sugar beets, sunflower, potatoes, rice, wheat, corn, barley, cotton, *etc*.

The regenerants, obtained using one of the above-mentioned methods, differ among themselves by genotype, *i. e.* they are remarkable for gamete-clonal variability which may be enhanced using hybrids F_1 as initial material. Due to recombinogenesis the gametes of hybrids F_1 carry different combinations of parental genes. The transfer of haploids to diploid level using colchicine allows obtaining a wide spectrum of variability for dihaploids in a short period of time.

Experimental haploidy *in vitro* is widely used in the breeding procedure. For instance, new high-yield varieties of rice and wheat were produce China in short time using the ovary culture; four-way hybrids of corn were obtained in the USA. There are on-going studies in experimental haploidy using wheat in Russia (Saratov State University); using corn – in Moldova (Institute of Genetics, AS of Moldova). In Belarus haploids are studied in the Institute of Genetics and Cytology (triticale, sekalotritikum, wheat, flax, potatoes, sugar beets) and in SIC NAAS of Belarus for Agriculture (rape, barley).

The gynogenesis method was first applied in France in 1976, when haploid plants were obtained from non-fertilized barley ovaries [2]. Since then haploids of similar origin were obtained from such crops as barley, wheat, rice, tobacco, gerbera, hevea, sugar beets, corn, sunflower, cotton and potatoes [3].

Andersen *et al.* used the method of ovary culture to obtain embryoids from two carrot samples. The average efficiency of androgenesis is 0.8 % [4]. Dihaploid lines, involved in the breeding process, were created using embryoids. In Russia Tyukavin *et al.* elaborated the methods of androgenesis and gynogenesis for carrots [5], which were used to obtain isogenic strains. In general the haploidy induction method *in vitro* for carrots is remarkable for low efficiency, dependent on the genotype, the stage of gamete development, culture medium and climatic conditions of growing donor plants.

To obtain dihaploid plants of carrots the method of inducing neoplasms in the culture of carrot ovaries *in vitro* was developed (Pat. No. 30285 dated February 25, 2008 [17]), which allows obtaining gynogenetic callus from non-fertilized ovaries [6]. However, the regenerating ability of this callus and the viability of plants, received from it, were found to be very low. For instance, the average yield of embryoids from gynogenetic calluses was 3.1 ± 1.2 per one callus, whereas the percentage of the formation of normal plants from these embryoids did not exceed 6.3 % [7]. A number of factors, participating in the regulation of embryogenesis *in vitro* have been established. These are nitrogen compounds (ammonium form), potassium, calcium, osmotic force of the solution and the concentrations of carbohydrates, vitamins, amino acids and growth regulators [8]. There are data, proving that $CaCl_2$, the inhibitor of ethylene synthesis, causes three-fold increase in the yield of embryoids in the suspension culture [9]. Some authors [10, 11] came to the conclusion on active participation of calcium ions in the initiation of the program of embryonic development of sporophytes of plants.

There is confirmed positive effect of calcium chloride on embryogenesis from carrot calluses, originating from somatic tissues [6]. Murashige and Scoog (MS) basal medium was used to develop the culture medium for the induction of somatic embryoids of carrots, which contains $CaCl_2$ in the increased concentration [12].

Taking into consideration the fact that due to haploid status the calluses of gynogenetic origin require specific conditions for the manifestation of embryogenic ability, the aim of this work was to optimize the regenerative and adaptive stages of the method of obtaining dihaploid plants by the gynogenesis *in vitro*.

MATERIALS AND METHODS

The experiments were conducted in laboratory and field conditions. Common methods were applied to cultivate carrot tissues and cells *in vitro* [13–16].

The productive line 345 of hybrid origin, Shantene variety, elaborated in the Institute (number in the catalog of biotechnological samples of IVMG NAAS) was used. The initial plants were selected by optimal parameters of the seeded cluster in the field selection seed-plot. The inflorescences of orders I and II were cut at the stage of opening the last row of flowers.

The inflorescences were disinfected with 0.1 % water solution of $HgCl_2$ with the exposition for 20 min, and washed with distilled water five times. The ovaries were extracted from the closed buds to obtain a callus therefrom, using the method of neoplasm induction, developed in the Institute for the culture of carrot ovaries *in vitro* [17]. The gynogenetic calluses obtained were cloned on the medium for callusogenesis during several passages [11].

In order to select the variant of medium, efficient for the formation of embryoids, the gynogenetic calluses were cultivated in the following conditions:

1. MS − control [18];
2. MS + 999 mg/l $CaCl_2$ (MS + $CaCl_2$);
3. MS + 90 g/l sucrose (MS + sucrose).

25 calluses were used in each variant. The yield of embryoids from calluses was estimated 30 days later. The embryoids over 3 mm were subjected to estimation. The regeneration of seedlings from the obtained initial embryoids was conducted on the MS medium with 0.1 mg kinetin [19]. The completion of growing and root development of regenerant plants was conducted on the hormone-free medium of ½ MS [19]. The number of plants, obtained from embryoids, was estimated 40 days later. Plants of normal structure with 2–4 leaves, 5 cm high, and with developed root system were registered.

Plants in tubes were adjusted to *in vivo* conditions in the coconut pills under the film in the cultural room, where they were cultivated at the temperature of 20 °C and illumination of 10,000 lx. The nutrition with Knop's solution was performed every 7 days [16].

The regenerant plants were planted into the open soil in the phase of 3–5 actual leaves in the first decade of July 2014.

In order to determine the optimal method of cultivating gynogenetic regenerant plants in the field and of obtaining roots from them, three kinds of material for soil mulching were studied: black film; transparent film, white agrofiber; control – no mulching [16].

The regenerant plants were planted into the cuts in the mulching material using the 45 × 20 cm scheme. Each variant was presented with three repeats, each repeat – with 20 plants.

The collection and biometric estimation of regenerant roots was conducted during the first decade of October. Regenerant roots of standard size (DSTU 7035:2009) were selected for storing in winter.

The statistical analysis of the results obtained was performed using common methods [20, 21].

RESULTS AND DISCUSSION

During the experiment in elaborating the optimal culture medium for embryogenesis from gynogenetic calluses the callus, obtained from non-fertilized ovaries of genotype 345, was cultivated on two modifications of the culture medium MS. After 30 days of cultivation (passage I) the yield of embryoids in all the variants of the medium was found to be very low (Table 1). They were also abnormal and it was impossible to obtain normally developed plants from them. Further cultivation (passage II) on the same media allowed increasing the level of embryogenic ability of calluses and obtaining up to 8.9 ± 2.1 embryoids from one callus in the variant MS + $CaCl_2$, which was considerably higher compared to the control (2.1 ± 0.7 of embryo/callus). The cultivation of calluses on the medium MS + sucrose did not affect the embryogenic ability of gynogenetic calluses.

The aftereffect of inductive media was observed at the stage of plant regeneration from embryoids in pas-

Table 1. The effect of modification of the culture medium on embryogenesis and the yield of normally developed regenerant carrot plants from gynogenetic callus 345, 2014

Variant of medium	Yield of embryoids from one callus, it.		Yield of normal plants from one callus, it.	Share of living plants after the adaptation, %
	I passage	II passage	III passage	
MS (control)	0.3 ± 0.1	2.1 ± 0.7	0.6 ± 0.3	10.0
MS + $CaCl_2$	1.1 ± 0.3	8.9 ± 2.1	7.3 ± 2.0	56.0
MS + sucrose	0	0.4 ± 0.1	0.1 ± 0.02	0
LSD_{05}	0.4	1.2	1.1	6.8

sage III. On average 7.3 ± 2.0 of normal plants from one callus were obtained from embryoids, cultivated on the medium MS + $CaCl_2$, which was considerably higher compared to the control, where the number of normally developed plants was 0.6 ± 0.2 it. (Table 1).

While growing in the coconut substrate *ex vitro* in the cultural room the regenerant plants, obtained on the medium MS + $CaCl_2$, survived better than regenerants in the control (56 and 10 % of planted plants respectively).

The cultivation of carrot regenerants in the field is more complicated as weather conditions, soil overheating and overdrying affect them, decreasing the survival of plants and the quality of roots. In order to optimize the conditions of obtaining roots from gynogenetic regenerants in the unprotected field conditions the effect of different mulching materials on the development of regenerant plants was studied.

It was established that mulching with black and transparent films had negative effect on the survival of plants, decreasing it considerably, compared to the control (15.0; 42.5 and 70.5 %) (Table 2). This fact may be explained by the increase (due mulching with films) in the soil temperature compared to the control which has negative effect on the development of the root system of the plant, as the biological specificity of carrots is the suspension of growth processes in the root system at the soil temperature over 25 °C.

A considerably higher number of surviving plants compared to the control was obtained while using white agrofiber when this index was 86.5 %. It is evident that this material prevents overheating of soil and promotes the establishment of plants.

The results on biometric analysis of plants in the phase of technical ripeness demonstrated that all the variants of mulching had positive effect on the growth of the heart of leaves, thus increasing its average height. The excess of the control by this index in the variants with black film and agrofiber was insignificant (23.0 ± ± 1.2 cm, 23.1 ± 1.3 cm, 20.4 ± 1.0 cm respectively), whereas the variant with a transparent film was exceeding the control significally (by 5.6 cm). Evidently, the mulching with a transparent film promotes better provision of plants with moisture.

The average number of leaves increased for the plants in the variants with soil mulching was compared to the control. A significant increase was noted for the variant with a black film, where the number of leaves was 18.2 ± 2.0 per one plant, whereas in the control it was 13.1 ± 1.1. This phenomenon may be explained by specific response of carrot plants to the conditions of increased temperature and humidity of soil, created by the black film.

The average weight of roots in all the test variants was considerably higher than that for the control due to the improvement of water balance. The largest weight of roots was registered in the variants with the transparent film (108.8 ± 7.4 g) and white agrofiber (92.8 ± 6.5 g). The increase in the weight of regenerant roots is a positive phenomenon which promotes the improvement in their quality and ensures preservation capacity due to the decrease in weight during long-term storing.

The results of the studies were 96 regenerant roots, the application of which is planned to obtain generation R_1 and to perform further selection work in creating combination-capable lines.

CONCLUSIONS

The regenerating and adaptive stages of obtaining dihaploid regenerant plants from gynogenetic carrot calluses, induced from non-fertilized ovaries *in vitro* were optimized. The culture medium MS, modified with the increased content of $CaCl_2$, the cultivation on which leads to 4-fold increase in the number of

Table 2. The effect of different types of soil mulching on the viability and growth of carrot regenerant plants of gynogenetic origin from callus 345, 2014

Type of mulching material	Establishment of regenerants in soil, %	Height of heart, cm	Number of leaves per one plant	Root weight, g
No mulch (control)	70.5	20.4 ± 1.0	13.1 ± 1.1	54.3 ± 4.7
Black film	15.0	23.0 ± 1.2	18.2 ± 2.0	74.3 ± 6.7
Transparent film	42.5	26.0 ± 1.5	14.0 ± 1.6	108.8 ± 7.4
White agrofiber	86.5	23.1 ± 1.3	14.2 ± 1.7	92.8 ± 6.5
LSD_{05}	8.1	3.2	2.4	11.2

embryoids, formed from gynogenetic calluses, compared to the control (8.9 ± 2.1 and 2.1 ± 0.7 respectively) and 12-fold increase in the yield of normal plants from one callus (7.3 ± 2.0 and 0.6 ± 0.3), was developed.

While growing roots using regenerant plants in the field the mulching of soil with white agrofiber ensures the survival of regenerants at the level of 86.5 % which is 16 % higher than the control (without mulching) and the increase in the average weight of roots up to 92.8 g (38.5 g more compared to the control). It allows refusing from greenhouses or tunnels while growing regenerant roots of gynogenetic origin.

Регенерація рослин з гіногенних калюсів моркви

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Мета. Оптимізація регенераційного та адаптивного етапів способу отримання дигаплоїдних рослин моркви з використанням гіногенезу у культурі *in vitro*, а саме: ембріогенезу з гіногенного калюсу та одержання нормально розвинених рослин-регенерантів і коренеплодів-регенерантів. **Методи.** Застосовано загальноприйняті біотехнологічні методи. Гіногенний калюс лінії моркви 345 отримано з незапліднених насінних зачатків *in vitro* за винайденим авторами способом. **Результати.** Розроблено поживне середовище, на якому вихід ембріоїдів з одного гіногенного калюсу збільшується у 4 рази, а вихід нормальних рослин − у 12 разів. При вирощуванні коренеплодів з рослин-регенерантів у незахищеному ґрунті мульчування ґрунту білим агроволокном забезпечує приживлення регенерантів на рівні 86,5 %, що на 16 % вище, ніж у контролі (без мульчування), та збільшення середньої маси коренеплодів до 92,8 г (на 38,5 г більше порівняно з контролем). **Висновки.** Оптимізовано регенераційний та адаптивний етапи способу отримання дигаплоїдних рослин-регенерантів з гіногенних калюсів моркви, індукованих з незапліднених насінних зачатків у культурі *in vitro*. Розроблено ефективне поживне середовище для ембріогенезу з гіногенних калюсів та встановлено, що мульчування ґрунту білим агроволокном істотно підвищує приживлення гіногенних рослин у незахищеному ґрунті і забезпечує збільшення середньої маси коренеплодів. Це дозволяє не використовувати теплиці чи тунельні укриття при вирощуванні коренеплодів-регенерантів гіногенного походження.

Ключові слова: морква, експериментальна гаплоїдія *in vitro*, незапліднені насінні зачатки, калюс, ембріогенез, рослини-регенеранти.

Регенерация растений из гиногенных каллусов моркови

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Цель. Оптимизация регенерационного и адаптивного этапов способа получения дигаплоидных растений моркови с использованием гиногенеза в культуре *in vitro*, а именно: эмбриогенеза из гиногенного каллуса, получения растений-регенерантов и корнеплодов-регенерантов. **Методы.** Применены общепринятые биотехнологические методы. Гиногенный каллус линии моркови 345 получен из неоплодотворенных семяпочек *in vitro* по изобретенному авторами способу. **Результаты.** Разработана питательная среда, на которой выход эмбриоидов из одного гиногенного каллуса увеличивается в 4 раза, а выход нормальных растений − в 12 раз. При выращивании корнеплодов из растений-регенерантов в незащищенной почве мульчирование почвы белым агроволокном обеспечивает приживаемость регенерантов на уровне 86,5 %, что на 16 % выше, чем в контроле (без мульчирования), и увеличивает среднюю массу корнеплодов до 92,8 г (на 38,5 г больше по сравнению с контролем). **Выводы.** Оптимизированы регенерационный и адаптивный этапы способа получения дигаплоидных растений-регенерантов из гиногенных каллусов моркови, индуцированных из неоплодотворенных семяпочек в культуре *in vitro*. Разработана эффективная питательная среда для эмбриогенеза из гиногенных каллусов и установлено, что мульчирование почвы белым агроволокном существенно повышает приживаемость гиногенных растений в незащищенной почве и обеспечивает увеличение средней массы корнеплодов. Это позволяет не использовать теплицы или туннельные укрытия при выращивании корнеплодов-регенерантов гиногенного происхождения.

Ключевые слова: морковь, экспериментальная гаплоидия *in vitro*, неоплодотворенные семяпочки, каллус, эмбриогенез, растения-регенеранты.

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