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THE METHODS FOR ISOLATION OF HAPLOID AND DIHAPLOID PEPPER PLANTS BY THE USE OF ANTHER AND MICROSPORES CULTURE (review)

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S u m m a r y

The role of haploid and dihaploid lines in the breeding and the studying of genotypes of plants from *Capsicum* genus is discussed. The factors are presented, which has an effect on embryogenesis efficiency in the culture of pepper microspores and anthers, the most effective method are described. At present the productivity of such methods is rather low, and their development has practical importance.

Keywords: *Capsicum*, haploid plants, microspores, anthers.

Pepper is a common vegetable crop used both as fresh and in cooking spices. According to the Food and Agriculture Organization (FAO), in 2007 pepper was grown on more than 1,7 million hectares, while the annual world commodity production of pepper exceeds 27 million tons.

All varieties of pepper belong to the family *Solanaceae* Juss., the genus *Capsicum* (1). This genus includes about 27 species including 5 domesticated ones - *C. annuum* L., *C. baccatum* L., *C. chinense* J., *C. frutescens* L. and *C. pubescens* Ruiz & Pav. Diploid set of chromosomes consists of 24 chromosomes in all cultivated pepper species and in most of wild forms; some wild species have 26 chromosomes (*C. campylopodium*, *C. villosum*, etc.). Most of pepper species are to varying degrees susceptible to phytopathogenic fungi, bacteria, viruses, insects and other pests. Extreme fluctuations of abiotic factors (temperature, humidity, light, nutrient content, pH, chemical composition of air, and pesticides) can adversely affect the yield of this crop as well. Modern varieties of pepper must correspond to high standards in both taste properties of products and resistance to adverse abiotic and biotic environmental factors. Currently, almost all new varieties of pepper have been designed with the use of dihaploid lines.

Haploid and dihaploid plants are the important material for breeding work revealing rare recessive mutations and unique recombinations. Haploids have the only copy of allele of each locus, which allows identification of recessive mutations masked in normal diploid plants by non-mutant dominant alleles. Doubling of chromosomes in haploid plants provides rapid formation of dihaploids homozygous for all genes for further obtaining hybrids (2). In addition, the use of F1 hybrids in anther culture provides isolation of best genotypes and their conservation by doubling the chromosomes.

The unified genetic linkage map of pepper (*C. annuum* L.) including mainly RFLPs (restriction fragment length polymorphism) and RAPDs (random amplified polymorphic DNAs) markers has been created by comparing three inter-species linkage maps designed upon the analysis of dihaploids' progeny (3). Using haploid and dihaploid plants allows gene labeling and determination of genetic components for resistance to biotic and abiotic factors (4).

Haploid plants can be obtained experimentally from unfertilized ovules or microspores. Formation of haploids in culture of microspores or anthers can be induced by irradiation, radioisotopes, heat shock, distant hybridization, pollination with sterile pollen, treating flowers with chemical substances (plant regulators, nitrous oxide, etc.), chromosome elimination during the cultivation of young embryoids, parthenogenesis in vitro. In nature, this process occurs spontaneously. Spontaneous formation of haploids has been observed in different species of *Capsicum* (5-7). Usually they develop in the seed containing two twin embryos (in 1-10 per 10 000 germinated seeds). To obtain spontaneous haploids of pepper through parthenogenesis, there should be selected and propagated genotypes with a high degree of haploids in progeny, as well treating flowers with nitrous oxide (7). Doubling the number of chromosomes in haploid plants is often performed by treating sprouts with 0,3% colchicine solution. Anther culture still remains the most common technique of obtaining haploids and dihaploids of pepper.

The factors switching on the gametophytic pathway instead of sporophytic development during the incubation of microspores are high or low temperature, composition of a culture medium, and content of plant hormones. K. Kristiansen and S.B. Andersen studied the effects of temperature and photoperiod on formation of embryoids in anther culture of *C. annuum* among F_1 hybrids of cultivars Jetta, Parma and Trophy (8). Plants were grown in a greenhouse at temperatures ranging from 16 to 30 °C and at a photoperiod of 11-19 h. The anthers were collected from individual plants during 5-9 weeks of growing and cultured using the method of R. Dumas de Vaulx et al. (9). Embryoids were obtained from the source material collected from plants grown at all temperature regimes with a growth optimum at 26,4 °C. Photoperiod didn't affect the formation of embryoids, while the number of embryoids significantly decreased with increasing age of the donor plant. Thus, embryoids didn't develop from the anthers collected from maternal plants aged 12-14 weeks. Similar results were reported by J. Mityko et al. (10), who tested four breeding lines (*C. annuum* L.), 7 varieties and 4 F_1 hybrids using donor plants grown in a greenhouse. These authors recorded the increased yield of embryoids in anther culture due to the fact that the used flower buds had been collected for only 4 weeks from the first flower appearance.

Another important factor influencing the formation of embryoids is a stage of microspores' development. Most researchers agree that microspores of later stages are the most responsive (11, 12). As a rule, anthers containing such microspores have partial anthocyanin coloration and they are located in flower buds whose corolla is slightly larger than sepals (10, 13-16).

Along with it, some researchers describe the formation of pepper haploids in culture of anthers and microspores as dependant on species, hybrid, variety and genotypes of individual plants (10, 14, 17-19).

Pre-cultivation in vitro treatment of anthers (8) or flower buds is often used to increase the yield of haploids (9, 14, 15). This can be done by using both low (4 °C) (14, 15, 20, 21) and high (35 °C) temperature (9). Particular varieties of *C. annum* of Russian

selection were found to be more responsive to incubation of flower buds at low temperature (4-10 °C). Anthers are most often cultivated on Murashige-Skoog medium (MS) (22) or modified MS-medium. Some researchers also use the media LS (23), NT (24), B5 (25) and SI (15).

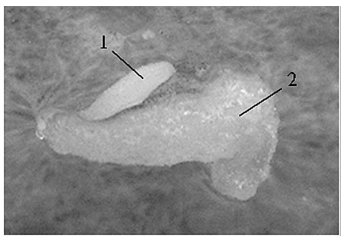

Fig. 1. The development of embryoids from microspores of pepper *Capsicum annum* L. cv Zdorov'e: 1 – torpedo-shaped embryoids, 2 – near completely shaped embryoids with primordial cotyledons.

Regulators of plant growth and development are usually applied to improve the formation of embryoids. The most effective is the combination of auxins and cytokines: kinetin + β-indolyl-3-acetic acid (IAA) (11), Kin + α-naphthaleneacetic acid (NAA) (26), 2,4-dichlorphenoxyacetic acid (2,4-D) + Kin (9, 27). M. Sibi et al. (15) increased the yield of haploid plants by adding vitamin B_{12} to a medium inducing the development of embryoids. For this purpose other researchers used coconut milk (26), yeast extract, casein (11), carrot extract and activated charcoal (17). At the same time, the own authors' data indicate that some species (Mazurka, Zdorov'e) demonstrate the best yield of haploids in anther culture on hormone-free media (Fig. 1). In the case of a hormone-free medium, there is more confidence of androgenic origin of the obtained plants along with a significant proportion of resulting embryoids developed into viable plants (Fig. 2) (unpublished experimental data).

Fig. 2. Normally developed embryoid with two cotyledons (A) **and abnormally developing embryoid with one cotyledon, root and without apical bud** (B)**, obtained in anther culture of pepper *Capsicum annum* L. cv Zdorov'e.**

Y.Y. Wang et al. were the first who published the technique for obtaining haploid plantlets in anther culture of hot pepper (28). They used anthers of the cultivar Yeo Hsien Small Red pepper with microspores having parietal nucleus. Anthers were cultured on MS-medium modified in respect of some trace elements and vitamins, with the addition of 4,65 mM Kin; 5,37 mM NAA and 4,52 mM 2,4-D. Green shoots were obtained from the anthers after 33 days of cultivation. On the medium containing NAA the efficiency of formation of calli and shoots was, respectively, 28,6 and 4,8%; on the medium with 2,4-D – 23,5 and 2,6%. The results of cytological analysis of root apexes showed the haploid nature of regenerants carrying 12 chromosomes. J.S. Kuo et al. (29) described the factors influencing the development of haploid pepper from anther culture. The anthers were cultured on NT and MS media with hormones added (late stage of microspores, parietal located nucleus). L. George and S. Narayanaswamy (11) in experiments with *C. annuum* var. *grossum* cultured immature anthers with mononuclear microspores immediately after the stage of tetrads on LS-medium keeping 2% sucrose and FeEDTA as iron source. Auxins, cytokines, coconut milk, yeast extract and casein hydrolysate (CH) at various combinations and concentrations were the inducers of embryoid development. The process was carried out under constant artificial light at a temperature of 25±2 °C. Divisions of microspores were induced by cultivation on media containing the following combinations of active substances: 1,39 mM Kin + 17,12 mM IAA; 13,9 mM Kin + 5,71 mM IAA; 2,85 mM IAA + CH (400 mg/l) + inositol (100 mg/l). Dedifferentiation of microspores and formation of multicellular conglomerates similar to pro-embryogenic structures were observed in 1% microspores. Then the anthers were transferred on a media containing 2,85 mM IAA + CH (400 mg/l) + myo-inositol (100 mg/l) to provide the further development of embryoids. Shoots were obtained only from 0,1% of hundreds of anthers. Acetic-carmine staining of root apexes showed the presence in their cells of 12 chromosomes confirming haploidy of these plants.

Today, the most effective protocol of anther culture of pepper has been developed by R. Dumas de Vaulx et al. (9). It is used in its original form as well as with slight modifications (8, 10, 17, 18, 27).

R. Dumas de Vaulx studied the effects of heat treatment on androgenesis in hot pepper culture in vitro (*C. annuum* L.). The anthers were incubated for 2 or 8 days at 35 °C on the culture medium added with various concentrations of 2,4-D and Kin. Anther treatment for 2 days resulted in a lower regeneration than the 8-days treatment. When adding to the medium of 2,4-D (0,45; 4,52 and 9,00 mM) and Kin (9,3 mM), the best result was obtained in the anthers incubated at 35 °C for 8 days at presence of 0,45 mM 2,4-D and 9,3 mM Kin (regeneration rate - 5%).The efficiency of regeneration increased (up to 12%) in anthers pre-incubated for 8 days at 35 °C on the medium containing 0,04 mM 2,4-D.

R.A. Morrison et al. (16) derived haploid plantlets in anther culture of a hybrid Emerald Giant (*C. annuum*) ½ CA4 (*C. chinense*) using a two-layer medium. The hybrid was not responsive on the medium proposed by R. Dumas de Vaulx et al. (30), so the authors used the medium described by M. Sibi et al. (15) added with FeEDTA and sucrose according to the prescription of T. Murashige and F. Skoog (22). C medium (6% sucrose and 0,01 mM 2,4-D) was used during the first 12 days of culturing (30), R medium (3% sucrose; no growth regulators) – at the end of cultivation. In both media C and R, 1% agarose was added instead of agar. Dual media were prepared upon the base of R medium with activated charcoal (2%) and agarose (1%). Flower buds were harvested from a maternal plant and incubated for 100 h at 4 °C. Then the anthers were isolated and incubated on C medium for 8 days at 35 °C and for 4 days at 25 °C in the dark, then transferred on the two-layer medium with activated charcoal. Embryoids were detected in the anthers after 2-4 weeks and developed into plants in 2-3 weeks of cultivation on R medium. All the obtained plants were diploid and homozygous for a number of loci. Electrophoretic analysis has proved their gametic origin. The technique using the two-layer medium was shown to be also effective for cultivars Calwonder, Yolo Wonder, Emerald Giant.

In 2006 E.D.J. Supena et al. (21) suggested the technique for obtaining haploid pepper plants which still remains probably the most effective. These authors highlight several main factors: using the flower buds containing at least 50% microspores at late stages of development; pre-treatment of the flower buds for 1 day at 4 °C; cultivation of anthers on the two-layer medium during 7

days at 4 °C, then – at 28 °C days in the dark until the formation of embryoids. The used two-layer medium was Nitch medium added with 2% maltose and 1% activated charcoal + 0,6% Plant Agar (for solid phase) and 2,5 uM zeatin + 5 uM IAA (for the upper liquid layer). Embryoids were transferred on the medium $^1/_2$ MS with sucrose (2%), 0,1 uM 6-benzylaminopurin (6-BAP) and 0,6% Plant Agar. Cultivation was carried out in Petri dishes 6 cm diameter at 25 °C under 16 h illumination. After 3-4 weeks, the seedlings with first true leaves were placed in vessels with vermiculite, liquid $^1/_2$MS and sucrose (1%); after the formation of 4-6 leaves, the plants were replanted into soil. All the 10 tested genotypes of hot pepper have demonstrated high yield of haploid plants – seven plants per one flower bud were obtained in the anther culture of cv Galaxy.

Haploid pepper plants can be obtained from microspore culture as well. M. Kim et al. (31) performed this on hot pepper (*Capsicum annuum* L.) using a modified NLN medium (NLNS). Globular and heart-shaped embryoids were formed after 3 weeks of culturing microspores. After 4 weeks, the embryoids at the stage of cotyledons were transferred on B5 agar medium. There have been also tested various media for pre-treatment of microspores with different contents of carbohydrates. Heat shock was found to provide higher effects against the carbohydrate-free medium. It has been also shown the effectiveness of embryoid formation on the medium containing 9% sucrose.

C. Lantos et al. (12) optimized the technique for obtaining haploid hot pepper in the microspore culture by joint culturing of microspores and ovules of pepper and wheat. The cultivated anthers contained 80% microspores at the late developmental stage and 20% early-stage pollen. Isolated microspores were cultured in the liquid medium W14 (32) with 9% maltose, glutamine (1 g/l), Kin (0,5 mg/l), 2,4-D (0,5 mg/l) and cefotaxime (200 mg/l). Using wheat ovules as a nurse culture was found to be more effective than using ovules of pepper. The efficiency of embryoid yield in this case was dependant on the used cultivars and genotypes; some of resulting haploid plants manifested a mutation – leaf rosettes.

Thus, at present time there are various techniques allowing to obtain haploid plants of pepper varieties. However, the efficiency of these techniques is still low and it largely depends on the responsiveness of used pepper cultivars or species. A large proportion of embryoids can't regenerate into viable normal plants. Development of a universal technique providing large yields of haploid pepper plants of different species and varieties is a subject of great practical importance for breeding, study of genotypes and effects of individual genes.

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