ISSN 1062-3590, Biology Bulletin, 2015, Vol. 42, No. 4, pp. 302–309. © Pleiades Publishing, Inc., 2015.
Original Russian Text © D.V. Shumilina, N.A. Shmykova, L.L. Bondareva, T.P. Suprunova, 2015, published in Izvestiya Akademii Nauk, Seriya Biologicheskaya, 2015, No. 4, pp. 368–375.

GENETICS

Effect of Genotype and Medium Culture Content on Microspore-Derived Embryo Formation in Chinese Cabbage (*Brassica rapa* ssp. *chinensis*) cv. Lastochka

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Received May 12, 2014

Abstract—The influence of different factors on microspore embryogenesis in Chinese cabbage (*B. rapa* ssp. *chinensis*) was studied. A genotype dependence for embryo formation was observed. The majority of embryos and plants were obtained from microspores isolated from flower buds (2–2.9 mm in length) and cultured in the NLN liquid medium with 13% sucrose (w/v) supplemented with 24-epibrassinolide and 1% activated charcoal. Embryos cultured on the 1/2 Murashige-Skoog culture medium with 2% sucrose (w/v), 0.1 mg/L benzylaminopurine, and 3 g/L Phytagel stimulated the formation of secondary embryos that resulted in development of large number of doubled haploid plants.

DOI: 10.1134/S1062359015040135

INTRODUCTION

Haploid plants are of great importance for quantitative genetic analysis, investigation of the interaction of genes and genetic variation, determination of linkage groups, mapping populations, and breeding for producing of pure lines. The basis for obtaining haploids is in vitro microspore cultivation, which is the leader in breeding programs dedicated to accelerated production of highly productive hybrids and varieties of agricultural plants. Isolated microspores under certain conditions can be switched from normal gametophytic development to sporophytic. As the result of this process, embryos that develop into haploid (Hs) or doubled haploid plants (DH) are produced.

The Chinese cabbage *Brassica rapa* ssp. *chinensis* is one of the major vegetable crops in Asia and leaves forms of nappa cabbage and pak choi are becoming more and more popular in Russia. Breeding of Chinese cabbage is mainly aimed at the production of F1 hybrids, requiring constant lines with high combining ability. Typically, these lines are produced by inbreeding within 6–8 generations; therefore, the development of methods of obtaining of homozygous lines through microspores cultures for one regeneration is an important task. Despite the fact that articles about the regeneration from isolated microspores in *B. rapa* ssp. *oleifera* (Lichter, 1989; Baillie et al., 1992; Burnett et al., 1992; Ferrie et al., 1995; Guo and Pulli, 1996), *B. rapa* ssp. *pekinensis* (Sato et al., 1989; Kuginuki et al., 1997), *B. rapa* ssp. *chinensis* (Cao et al., 1994) and *B. rapa* ssp. *parachinensis* (Wong et al., 1996) have already been published, an effective universal technology for obtaining doubled haploid plants (DH-technology) does not exist. The literature data show that a subspecies, *B. rapa* ssp. *chinensis*, is the most recalcitrant to microspore culture among members of the species *B. rapa*.

One of the tasks of DH-technology is the production of the highest possible number of haploid plants, since it allows the most comprehensive coverage of the range of genetic recombinant forms, including those with recessive traits. The most important element of the DH-technology allowing an increase in the yield of regenerated plants is the increased induction of microspore embryogenesis. Some parameters of DH-technology (growing donor plants at low temperatures, heat treatment of microspores during the first days of cultivation (Lo and Pauls, 1992; Cegielska-Taras et al., 2002), and the use of media with a high sucrose content (Baillie et al., 1992; Ilic-Grubor et al., 1998; Ferrie et al., 1999; Lionneton et al., 2001) are universal for the *Brassica* genus. According to published data, the efficiency of embryogenesis depends on factors such as the plant genotype, the developmental stage of microspores, the type of pretreatment of buds and microspores, the composition of culture media, and culture conditions (Ferrie et al., 1995).

The goal of this study is investigation of the effect of the genotype of individual plants, microspore developmental phase, 24-epibrassinolide, and activated charcoal on the number of embryos and the yield of normal *B. rapa* ssp. *chinensis* regenerated plants.

MATERIALS AND METHODS

Chinese cabbage *B. rapa* ssp. *chinensis* cv. Lastochka breeding VNIISSOK was used. Donor plants were grown in a climate chamber at 15°C around the clock, 16L8D, where L is light time (day) and D is dark, and the light intensity was 9000 lx. A cytology study of developmental stages of microspores was performed during bud selection. Visualization of microspores and pollen (Alexander, 1969) was performed using the differential staining technique and an A2 Axio Imager microscope (Zeiss, Germany). The correlation between the size of the bud and developmental stage of microspores was determined using these techniques.

Buds for microspore culture were collected from plants at the initial stage of flowering, and sterilized for 30 s in 96% ethanol, then for 5 min in a 50% aqueous solution of the "Belizna" (Russia) commercial preparation with the addition of Tween 20 (Panreac, Spain) 1 drop per 100 mL solution, followed by washing three times with sterile distilled water.

Sterile buds were placed in a half-strength of Lichter medium (1/2 NLN), pH 5.8 (Lichter, 1982), with 13% sucrose (30 buds per 6 mL of medium) and homogenized using a magnetic stirrer. The microspore suspension was filtered through a nylon filter with a mesh size of 40 microns and pelleted for 5 min using an Eppendorf 5804R centrifuge (Germany) at 125 *g*. The pellet with microspores was resuspended in 1/2 NLN medium, then centrifugation was repeated. Microspore washing was performed twice.

After isolation and washing, microspores from 10 buds were placed in a Petri dish (6 cm in diameter) with 5 mL medium mentioned above and incubated at 32°C in the dark for 2 days. Further incubation was at 25°C in the dark until embryos were formed.

To determine the optimal stage of bud and microspore development, the microspore suspension was prepared from buds with a length of 2–2.9 or 3–4 mm. Experiments were performed twice, with four replications for each variant.

Buds were individually collected from three plants of Chinese cabbage cv. Lastochka for investigation of the effect of the plant genotype on the induction of embryogenesis in the microspore culture. The microspore suspension was prepared from buds with a length of 2–2.9 mm. The experiment was performed twice, with four replications for each variant.

Buds with a length of 2–2.9 mm, collected from 10 plants, were used to study the effect of 24-epibrassinolide (EpB) and activated charcoal (AC) on the development of embryos in the microspore culture. Microspore culturing was done using 1/2 NLN with 13% sucrose. In AC experiments, 250 μL of autoclaved 1% AC suspension in 0.5% agarose was added to each Petri dish. In experiments with brassinosteroid (BR), 1 mM EpB stock solution (Duchefa, Czech Republic) in dimethylsulfoxide was used. This solution was added directly to the culture medium in the Petri dish to a final concentration of 0.4×10^{-7} M.

The experiment comprised the following treatments options: control (1/2 NLN with 13% sucrose without additives); addition of AC on the first day of culturing; addition of EpB on the first day of culturing; addition of EpB and AC on the first day of culturing; addition of EpB on the third day of culturing; addition of EpB and AC on the third day of culturing.

Formed embryos at the stage of large globules and at the torpedo or heart-shaped stages of development were placed in Petri dishes on the Gamborg medium (B5) (Gamborg, 1968), containing 0.5% sucrose, 0.5% glucose, and 3 g/L Phytagel. Explants were transferred to 1/2 MS medium (Murashige and Skoog, 1962) with 2% sucrose, 0.1 mg/L benzylaminopurine (BAP), and 3 g/L Phytagel for the formation of secondary embryos. The resulting embryos and shoots were separated and transferred to 1/2 MS medium, containing 2% sucrose and 3 g/L Phytagel. Culturing was carried out on shelves with fluorescent lamps at 25°C and a photoperiod of 14 h light and light intensity 2500 lx.

Plants with leaves and a normally developed root system were transferred to pots filled with a mixture of peat and perlite (7 : 3), covered with perforated plastic cups for the adaptation of the plants to in vivo conditions. Regenerated plants were grown under the same conditions as donor plants.

Determination of ploidy of regenerated plants was performed by counting chloroplasts in guard cells of stomata, because the number of plant chloroplasts correlates with the chromosome number; i.e., in diploid plants the chloroplast number is approximately two times lower than that of the haploid (Monahos et al., 2014). The epidermal cell layer was removed from the underside of the leaves and washed in distilled water, then it was placed on a mount slide in a drop of water, covered, and viewed under an A2 Axio Imager fluorescence microscope (filter bank BR 490 and 515). The pictures of at least 10 pairs of guard cells of each plant were taken, and chloroplasts were counted.

Statistical analysis was performed using ANOVA (Gomez, K. and Gomez, A., 1984)—One way ANOVA, Factorial ANOVA, and Fisher's Exact Test.

RESULTS AND DISCUSSION

Success in obtaining haploid embryos through in vitro culture critically depends on the stage of development of microspores. It is known that the microspores of the late unicellular stage and pollen grains of early bicellular developmental stage of plants of the genus *Brassica* can switch from gametophytic to sporophytic pathway (Pechan and Keller, 1988; Baillie et al., 1992; Telmer et al, 1992; Kott, 1998).

The cytological analysis performed demonstrated that the best embryogenic microspores came from

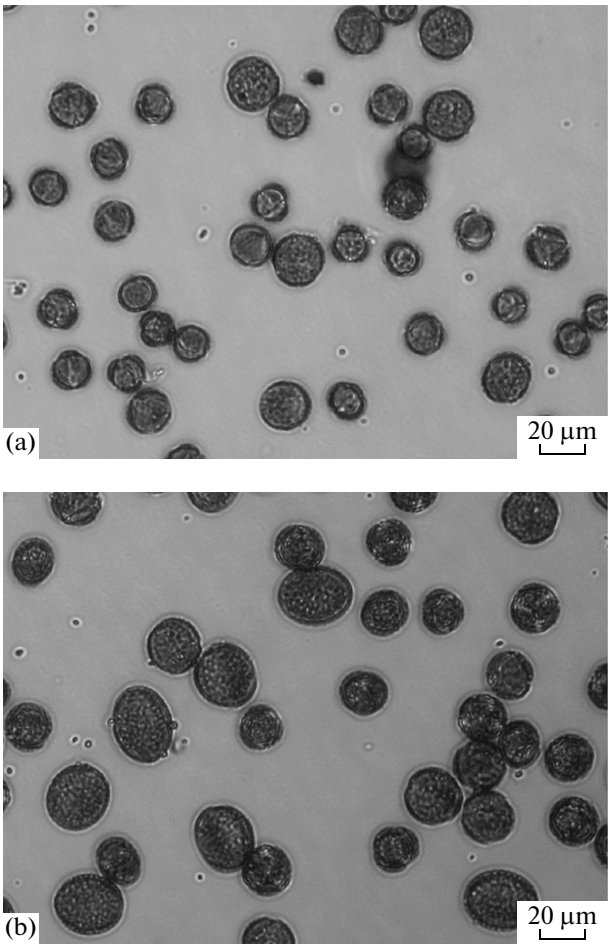

Fig. 1. Microspores of Chinese cabbage cv. Lastochka isolated from the bud with a length of 2–2.9 (a) and 3–4 mm (b).

flower buds of 2–3 mm length (Fig. 1). The formation of different suspensor-like structures was observed during the early stages of embryo development when flower buds of 2–3 mm length were used. In these cases, after 5 days of culture, microspores underwent division into two unequal cells (Fig. 2a), one of the cells formed a suspensor-like structure at the end of which an embryo was formed (Figs. 2b–2d). Typically, the cells of suspensor-like structures had a rich cytoplasm and were filled with starch grains. The suspensor-like structures were preserved for a long time in embryos, up to the heart stage.

During zygotic embryo development, suspensors perform the function of storage and transport of nutrients to the embryo (Yeung, 1980; Nagl, 1990). Plant hormones such as gibberellic acid, auxin, cytokinins, and abscisic acid were identified in suspensors of different plant species (Yeung and Meinke 1993; Friml, 2003). Thus, suspensors can be a site of synthesis and a supplier of plant hormones. Most likely, in the microspores culture, suspensorlike structures perform a similar role supplying the embryo with storage compounds and the regulation of its growth. In our experiments, embryos containing a suspensor developed more slowly than embryos without a suspensor. On the 15th day of culture, the first embryos reached the globular stage, while the second embryos developed to the torpedo stage (Fig. 2e), which is consistent with the data of other authors obtained during investigation of a *B. napus* microspore culture (Supena, 2004).

In our experiments, the highest number of embryos and regenerated plants were obtained using 2- to 3-mm-long flower buds. Single root-like embryos, which subsequently died, were developed from microspores from flower buds of larger size (Table 1, Fig. 2f).

The differences in the response of the individual genotypes of Chinese cabbage to microspore culture were clearly detected in our experiment. The yield of embryos and normally developed regenerated plants in the microspore culture of individual genotypes varied more than tenfold (Table 2). The most responsive was plant no. 6, for which more than seven embryos were obtained for each Petri dish.

In practice, many scientists faced weak responsiveness of individual cultivars and individual plants (Keller and Armstrong, 1983; Ockendon, 1988). Differences in the responsiveness of individual plants within the same cultivar can be explained by the peculiarities of the conventional breeding process, which is usually directed to the selection of genotypes based on certain economically valuable traits, and usually responsiveness to haploid embryogenesis is not considered. Implementation of DH-technology in the breeding process will provide more stable and straight varieties, also by the responsiveness to induction of haploid embryogenesis. However, today the development of DH-technologies for specific varieties and investigation of the influence of various factors on embryogenesis require the use of a large number of buds collected from several plants, for leveling differences in the responsiveness of genotypes to embryogenesis.

EpB and AC were used in order to increase the yield of embryos in the microspore culture. The effect of EpB on embryogenesis was positive for both the additional first and third days of incubation of microspores, but a significant difference compared to the control was observed only for variants with an addition on the first day of culture (Table 3). Most likely, microspores on the first day of incubation are most susceptible to hormonal induction. Unfortunately, abnormally developed embryos were observed among formed embryos and regenerated plants were not obtained from these embryos. The positive effect of epibrassinosteroides on embryogenesis in the *B. napus* and *B. juncea* microspore culture and the

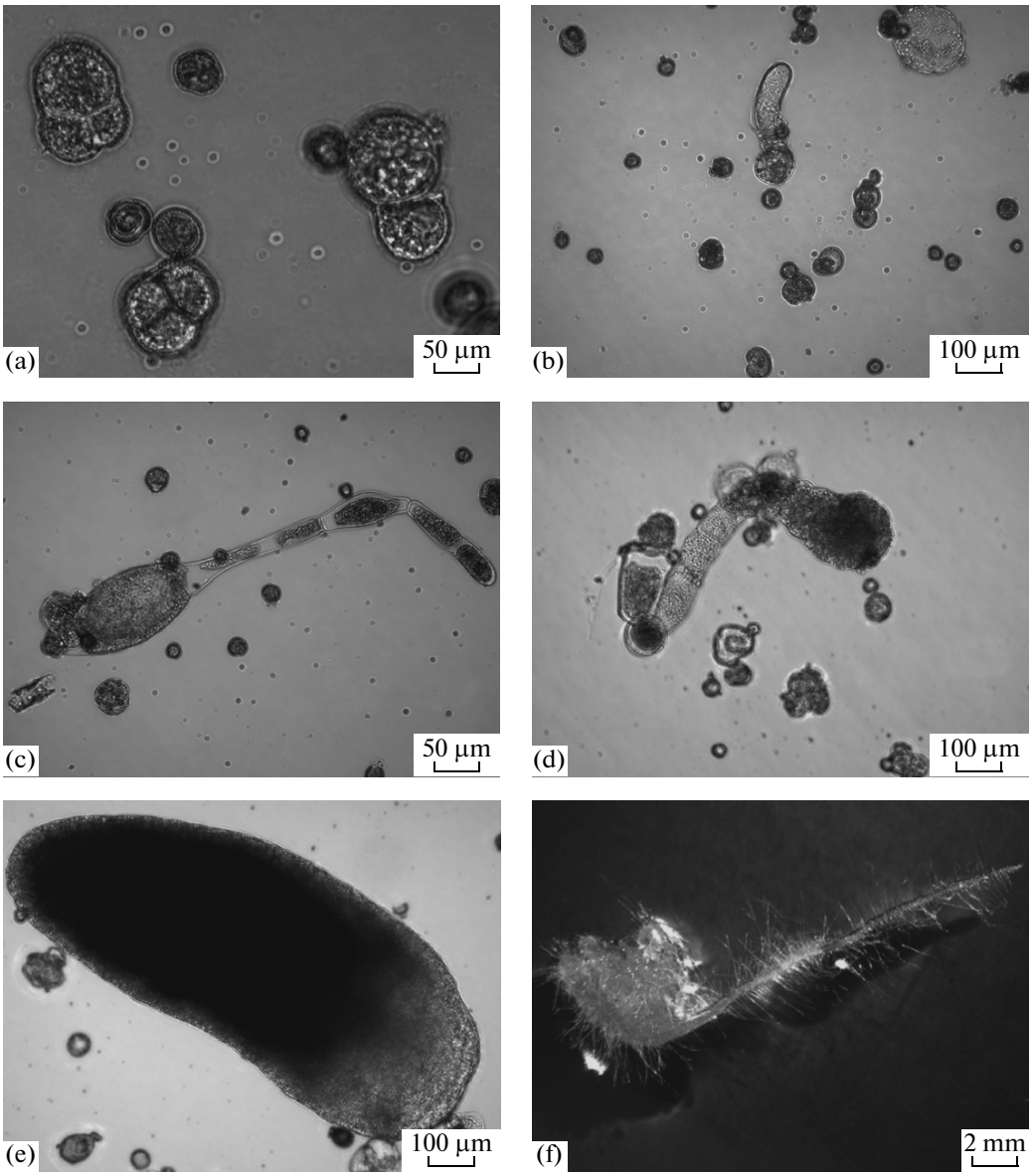

Fig. 2. The development of embryos in the microspore culture of Chinese cabbage cv. Lastochka at 1/2 NLN medium with 13% sucrose. (a) Unequal divisions of microspores cells; (b–d) development of suspensor-like structures; (e) torpedo-like embryo; (f) root-like embryo.

absence of this effect on the *B. rapa* microspore culture was shown previously (Ferrie et al., 2005).

BR efficiency in the microspore culture may be due to the fact that their highest concentration was detected in the reproductive organs (Grove et al., 1979; Moore, 1989). BR in very small concentrations affects the growth and development of plants in terms of extension, division, and differentiation of cells (Nakajima et al., 1996; Brosa, 1999; Sasaki, 2002; Lu et al., 2003). Furthermore, it is known that this group of compounds is involved in mechanisms of resistance to abiotic and biotic stresses (Wilen et al., 1995).

Since elevated culture temperature is required to switch the microspores from gametophytic to sporophytic pathway of different *Brassica* species, the addition of Br on the first day of incubation could reduce the damaging effects of heat treatment, thereby increasing the number of normal embryos.

Application of AC without growth regulators did not have a significant effect on embryogenesis. Application of AC with EpB on the first day of culture caused a significant decrease in the total number of embryos formed in comparison with that of the variant when only EpB was used (Table 3), but the rate of nor-

Table 1. Development of embryos in a microspore culture of Chinese cabbage cv. Lastochka isolated from buds of different sizes

Bud size, mm	The number of embryos in Petri dish, pcs		Number of DH-plants on a Petri dish, pcs
	sum	including root-like	
2–2.9	43[a]*	5[a]	38[a]
3–4	8[b]	8[b]	0[b]
LSD	0.91	0.58	0.41

LSD (least significant difference) is the least significant difference between the compared values.

* Variants marked with the same letter do not have a significant difference with a probability of 95%; i.e., all values marked with the letter "a" are not statistically different from each other, but differ from the values marked with the letter "b"; for Tables 1–3.

Table 2. Development of embryos in a microspore culture of Chinese cabbage cv. Lastochka isolated from buds of individual plants

No. of plant	The number of embryos in Petri dish, pcs		Number of DH-plants on a Petri dish, pcs
	sum	including abnormal	
4	2[a]	0[a]	0[a]
6	34.3[b]	12[b]	6.7[b]
7	1.8[a]	0.3[a]	1.5[c]
LSD	1.06	1.06	0.91

Table 3. Development of embryos in a microspore culture of Chinese cabbage cv. Lastochka with addition of activated charcoal and brassinosteroid to the culture medium

Variant of the experiment	The number of embryos in Petri dish, pcs		Number of DH plants per Petri dish, pcs
	sum	including abnormal	
Control	4.7[a]*	0.7[a]	4[a]
AC on the 1st day	7[ab]	2.3[ab]	4.7[a]
EpB on the 1st day	39.3[c]	31.3[c]	8[ab]
EpB + AC on the 1st day	19[d]	7.7[b]	11.3[b]
EpB on 3rd day	5.7[ab]	1. 7[ab]	4[a]
EPB + AC on the 3rd day	12[bd]	6.3[ab]	5.3[a]
LSD	2.3	2.24	1.35

mally developed plants increased. The addition of EpB simultaneously with AC on the first day of culture was the best option, allowing us to obtain the maximal number of normal plants compared to the control.

Earlier, the positive effect of AC on microspore embryogenesis in the genus *Brassica* was demonstrated (Margale and Chevre, 1991; Dias, 1999; Prem et al., 2008). However, some researchers found a negative effect of AC on the development of embryos from microspores (Prem et al., 2005; Takahashi et al., 2012). This contradiction can be explained by the fact that, in the first case, the researchers used AC with agarose, preventing the adhesion of microspores to AC particles, and in the second case, AC powder was added to the culture medium. AC cleans the medium from toxic substances but it is not a selective adsorbent and the AC probably blocks the intake of nutrients from the medium. Therefore, the intake of nutrients can be impaired in microspores located on the surface of AC particles.

Thus, the use of AC and agarose and the addition of EpB on the first day of culture is efficient for an increased yield of Chinese cabbage DH-plants in a microspore culture. Among the *Brassica* species, the highest level of abnormal development of embryos, followed by secondary embryogenesis, was observed in microspore culture of Chinese cabbage (Palmer and Keller, 1999). This was also shown in our experiments. Embryos obtained in the microspore culture, usually after transferring from a liquid medium to the solid medium are grown without direct formation of plants (Fig. 3).

In order to obtain normal plants, in our experiments the developed embryos were transferred onto 1/2 MS agar medium with 0.1 mg/L BAP; after culturing on this medium, we observed the formation of secondary embryos. Afterwards the embryos were separated and transferred to the hormone-free medium for rooting (Fig. 3).

The are other approaches for obtaining of regenerated plant of the genus *Brassica* described by different authors. For direct plant regeneration, some researchers suggested performing pretreatments of embryos by low temperatures, abscisic acid, and drying (Kott and Beversdorf, 1990; Huang et al., 1991; Wakui et al., 1994; Zhang et al., 2006). Culturing of embryos on filter paper placed on the top of an agar medium and/or on the surface of the medium with a high content of agar increases the regeneration efficiency (Takahata and Keller, 1991; Peng et al., 1994; Takahashi et al., 2012).

We observed the advantage of growing regenerated plants through secondary embryogenesis because the obtained plants were doubled haploids, which did not require further treatment with colchicine and easily formed seeds by self-pollination. In addition, few plants were obtained from each embryo and it allowed

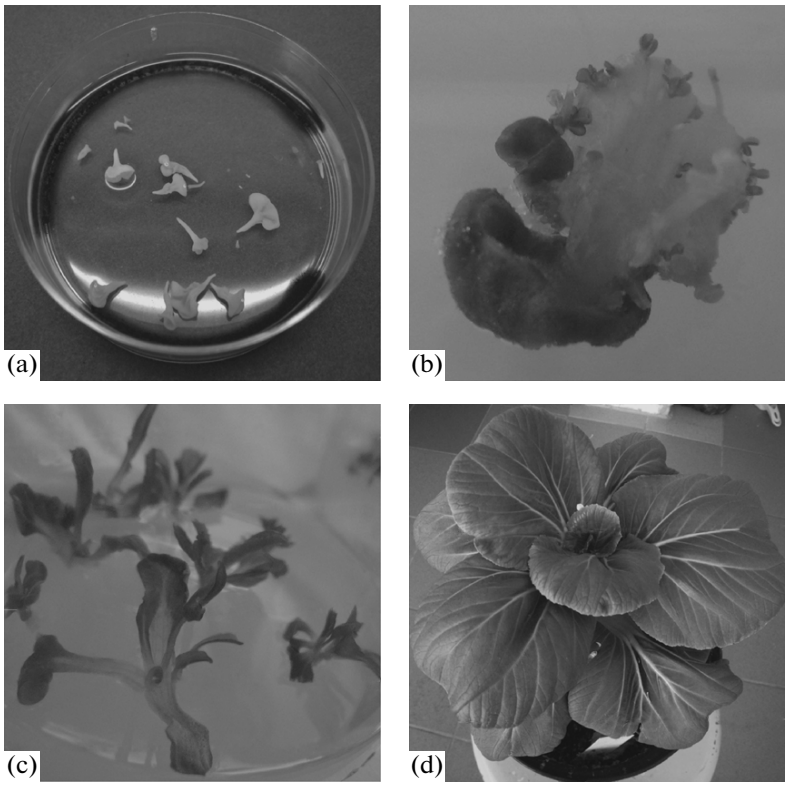

Fig. 3. The development of plants obtained in a microspore culture of Chinese cabbage cv. Lastochka. (a) Embryos in Petri dish with 1/2 NLN medium with 13% sucrose and AC; (b) the formation of secondary embryos on an 1/2 MS medium with 2% sucrose, 0.1 mg/L benzylaminopurine, and 3 g/L Phytagel; (c) rooting of separated shoots, developed on primary embryos on 1/2 MS medium with 2% sucrose and 3 g/L Phytagel; (d) DH-plant.

an estimation of plants already in the first generation and the collection of a large number of seeds.

Thus, our research demonstrated that the optimal size of the flower bud for the isolation of microspores from Chinese cabbage cv. Lastochka was 2–3 mm, which corresponds to the late single cell or early two-cell developmental stage of microspores. During culturing of microspores of Chinese cabbage, two types of development of haploid embryos were detected, one of which was accompanied by the formation of suspensor-like structures.

In order to increase the effectiveness of the methodology, EpB and AC together with agarose should be added to the culture medium. The number of donor plants should be maximized for leveling the significant differences in the responsiveness of individual genotypes to microspore embryogenesis. The use of secondary embryogenesis on hormonal medium increases the proportion of doubled haploids without the application of colchicine.

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Translated by V. Mittova