AGRICULTURAL BIOLOGY,
2016, V. 51, № 5, pp. 636-644
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Plant breeding: tissue cultures, molecular markers

UDC 633.63:631.52:581.3

doi: 10.15389/agrobiology.2016.5.636rus
doi: 10.15389/agrobiology.2016.5.636eng

SUGAR BEET (*Beta vulgaris* L.) HAPLOID PARTHENOGENESIS in vitro: FACTORS AND DIAGNOSTIC CHARACTERS

T.P. ZHUZHZHALOVA, O.A. PODVIGINA, V.V. ZNAMENSKAYA, E.N. VASIL'CHENKO, N.A. KARPECHENKO, O.A. ZEMLYANUKHINA

A.L. Mazlumov All-Russian Research Institute of Sugar Beet and Sugar, Federal Agency of Scientific Organizations, 86, VNIISS, Ramon Region, Voronezh Province, 396030 Russia, e-mail biotechnogiya@mail.ru

Acknowledgements:
Supported by the grant of the President of the Russian Federation for the Leading Scientific Schools of Russia (HIII=5282.2014.4 — project «Development of plant reproduction theory as based on integrity and reliability of biosystems. Polyvalent morphogenesis programs and their effective natural and artificial models»)
Received June 30, 2015

Abstract

Traditional obtaining of inbred lines and hybrids in sugar beet breeding requires a long time and is labour-consuming because of 2-year cycle of plant development, self- and cross-incompatibility, and inbreeding depression. To induce genotypic diversity in initial population, biotechnology methods including haploid parthenogenesis are promising. We have shown that, when inducing sugar beet (*Beta vulgaris* L.) haploids in vitro, express-diagnostics using phenotypic and embryological characters that are representative of periods of flowering shoots, organs and buds development, and stages of embryo sac, ovule and pollen grains formation is effective. The regenerative activity is observed in ovules of bud 1 to bud 25 located on ear part of pleochasium upward from the open flower. The nuclei and cells of female gametophyte of isolated ovules under in vitro conditions are capable of neoplasm at all stages of development, but the 8-nuclear or 7-celled embryo sacs are the most appropriate to morphogenesis and switching of development program from gametophyte to sporophyte type. Critical period of embryo sac development has been beforehand determined from the accompanying embryological characters — the presence of single-nuclear and two-three-celled pollen grains of anthers located in the same bud as ovules. The results we obtained indicate that hormonal composition of the Gamborg's B-5 (B5) medium is an important factor that effectively regulates direction of morphogenetic development in isolated ovules through direct regeneration (embryoidogenesis) or via callus (hemorhizogenesis) that is the evidence of totipotency of both sexual and somatic cells in the explant. The obtained data on in vitro reproduction of haploid regenerants add to available scientific notion of morphogenetic potential specificity in sugar beet plants. Stabilizing selection used to produce double haploids promotes detection of valuable morphological features of the regenerants. Determination of chromosome and chloroplast numbers in stomata guard cells as well as isozyme electrophoretic mobility (for *Adh-1*, *Mdh-1*, *Mdh-2*, *Me-1*, *Idh-1*, Idh-2, *Gdh-1* loci) can serve as markers when inducing haploidy and producing homozygous restitution lines of sugar beet. Efficiency of RFLP-analysis method using Hind III restrictase that has allowed for the first time to identify haploid microclones according cytoplasm type is shown. Molecular markers have indicated that regenerants with normal cytoplasm (N) have one PCR-product of 800 bp in length not digested by Hind III. Two fragments (320 bp and 480 bp) of 800 bp product digestion are found in cytoplasmic male sterile (CMS) forms (S) that reflects combination of recessive and dominant genes. Obtaining haploid regenerants with sterile cytoplasm from initial population is of great theoretical and practical importance for sugar beet breeding thus facilitating the problem of producing homozygous lines with CMS and high-productive hybrids on the sterile basis.

Keywords: sugar beet (*Beta vulgaris* L.) haploid parthenogenesis, female gametophyte, doubled haploid, embryoids, organogenesis, isozymes, RFLP-analysis

The modern domestic gene pool of sugar beet is sufficient enough to create varieties with the desired properties, however, traditional obtaining of inbred lines and hybrids requires a long time, and is labor-consuming, due to a 2-year cycle of plant development, self- and cross-incompatibility, and inbreeding depression [1]. A biotechnology method of haploidy, involving the in vitro cultivation of plants [2], may be effectively used to induce genotypic diversity in the

initial population, although its use is limited due to the narrow specificity of these conditions not only for different genotypes of the same species, but also depending on the cultivation stage. The variation observed when cultivating reproductive organs of plants may significantly extend the limits of variability and facilitate the derivation of forms for breeding purposes (including those obtained based on the doubled haploids) [3].

To improve the method of haploid parthenogenesis in sugar beet, the conditions for inducing unfertilized ovules and cultivating haploid regenerants with valuable genetic recombinations are optimized, and techniques of including restitution lines in the selection process are practiced [4-8). The most challenging problem is the hormonal composition of the growth media for the in vitro cultivation (of note, the principle of empirical choosing conditions for the induction of haploidy remains dominant) [9-10]. Several methods appear to be of interest, such as the use of liquid media, which significantly increases the output of haploid regenerants [11), as well as spontaneous haploid diploidization in long-term (over 1 year) cultivation [12], pretreatment of ovules with different agents (e.g. colchicine, trifluralin, etc.) that promote the antimitotic activity of the female gametophyte cells and increase the frequency of haploid regeneration [13]. The comparison between morphology of haploids and doubled haploid regenerants revealed the advantages of the first ones in the rate of shoot development and propagation in vitro. Based on this, it is proposed to use the sugar beet haploids both to produce doubled haploids and in specialized projects on genomic analysis, or in genetic transformation, when haploid tissues serve as the starting material [14].

In sugar beet, in vitro cultivation of unfertilized ovules and obtaining haploids from them allowed to directionally produce homozygous breeding lines [9, 15]. However, the commercial application of these technologies is limited by a low output of haploid regenerants and the lack of methodical research in regard to a) the critical periods in the development of the embryo sac and the establishment of the morphogenetic competence of its elements in order to switch from a gametophyte to sporophyte development program, and b) biochemical and molecular genetic evaluation of the produced homozygous lines.

For the first time, we analyzed the traits associated with the morphogenesis and in vitro reproduction of regenerated plants of sugar beet in case of haploid parthenogenesis; also, we demonstrated the possibility of their use as a diagnostic tool to assess the critical periods in the development of the female gametophyte during the transition to a sporophytic morphogenesis. Furthermore, the application of the RFLP analysis using the Hind III restriction enzyme allowed to identify for the first time haploid microclones from sugar beet variety populations based on the cytoplasm type (N or S), thus facilitating the problem of producing lines with the cytoplasmic male sterility (CMS).

Our objective was to identify traits that improve the efficiency of in vitro haploid parthenogenesis in sugar beet, involving biochemical and molecular markers.

Technique. The organ and tissue explants of *Beta vulgaris* L. hybrids (the collection of A.L. Mazlumov All-Russian Research Institute of Sugar Beet and Sugar) were used for plant tissue cultures. A conventional technique for sterilization and preparation of growth media was as described by R.G. Butenko [2].

The activity of the NADP isocitrate dehydrogenase enzyme was determined in plant tissue homogenates [16]. Electrophoresis of isoenzymes in the starch gel and PAGE was performed as described [17].

A Hind III RFLP (restriction fragment length polymorphism) [18] was used to identify sterile and fertile haploid regenerants. The products were separated by 1 % agarose gel electrophoresis with ethidium bromide.

Ploidy levels were determined by flow cytometry on a Ploidy Analyser

PA-2 (Partec GmbH, Germany) [11].

The cytoembryological data was processed using the coefficient of variation *Cv* [19].

Results. The in vitro cultivation of sugar beet unfertilized ovules revealed a unique ability in embryogenic cells to implement the developmental potential influenced by exogenous factors [20], which enabled to develop a technology for producing homozygous lines. This technique allows to obtain genetically and morphologically diverse material from donor plants without repeated self-pollination. The proposed technique provides an output of up to 38 haploid regenerants from a single donor plant, with overall production of homozygous sugar beet plants over 2-3 years (instead of 8-10 years with traditional methods). Currently, more than 100 of such homozygous lines are used in breeding programs [20]. Different experiments revealed a number of morphological, cytoembryological, biochemical and molecular genetic features that enhanced the efficiency of haploid detection when inducing in vitro haploid parthenogenesis, thus accelerating the creation of homozygous lines.

When introducing ovules into the culture, a period of flowering shoot development in donor plants is among critical factors affecting the efficiency of haploid regeneration. In sugar beet, budding at the end of an early generative stage of inflorescences (VII and VIII steps of the organogenesis) is an optimal phase [21]. Buds with ovules, more capable of inducing haploidy and most suitable for tissue culture, are located in the central stem of the pleiochasium raceme. Regeneration activity is observed in ovules from buds 1 to 25 (located on the spicate raceme upward from the opened flower).

For a successful ovule culture, it is also required to select the optimum stage of the embryo sac development. Cytoembryological observations indicate that nuclei and cells of the female gametophyte in sugar beet are capable of in vitro neoplastic processes at all stages of development. This appears to be related to the peculiarities of the female gametophyte, primarily to its well-defined polarity, differentiating divisions and totipotency of its elements, that together promotes, under in vitro conditions, the initiation of the program of haploid regenerant formation. Our findings demonstrated that the polarization started in the mononuclear embryo sac which, with its development, increased in size and stretched out toward the micropyle. As a result of the next three rounds of mitotic divisions, an eight-nucleus embryo sac is formed. After the last round of mitosis, one polar nucleus leaves each pole and migrates toward the center. Around the nuclei, cytoplasm is localized and cytokinesis occurs, which ends with the formation of the seven cells of the embryo sac [22].

Culture of isolated ovules, containing eight-nucleus (or 7-cell, but eight-nucleus) embryo sacs, is considered the most favorable for the induction of haploidy and regeneration [8]. One can assume that the stringent polarity of the embryo sac in this period, as well as totipotency of its nuclei and non-specialized cells of female gametophyte, ensure the greatest capacity for morphogenesis. This is a crucial factor for switching to sporophytic type of development.

Determining stages of the embryo sac development is complicated and long. In this case, embryological traits, such as the presence of mononuclear microspores and 2- or 3-cell pollen grains of anthers located in one bud with ovules are used to indicate a critical period. Depending on the weather conditions at flowering, these traits can be observed 1-5 days before the opening of buds.

Anomalies of the male and female gametophytes, which were apparent in some genotypes, resulted from self-pollination and polyploidy, and in the CMS hybrids with the apomixis frequency up to 40 %, may also serve as the diagnostic signs. Recessive genes, involved in the apomixis control [23], including in the

haploid parthenogenesis, may possibly accumulate in different ways during the selection. Genotypes with the greatest anomalies are more likely to induce haploidy in vitro.

A significant disadvantage of the in vitro haploidy technology is a low rate of regeneration (1.7-10.5 %). It may be increased by stress factors, e.g. pretreatment of ovules with cold (4-6 °C, 2-4 days) or X-rays (1000-5000 P, 50 min) [24]. In sugar beet ovules (similar to wheat microspores) [25], under in vitro conditions, low positive temperatures, altering metabolism, may delay the development of the embryo sac and induce sporophytic morphogenesis.

Hormonal composition of the growth media when culturing ovules was also a key factor in determining the type of morphogenesis, i.e. through regeneration (embryoidogenesis) or via a callus (gemmorhizogenesis) [26].

In our experiments, induction and morphogenesis of the haploid regenerants toward sporophyte were limited by Gamborg (B5) medium supplemented with vitamins by White and various hormones [26]. For example, the addition of gibberellin (2 mg/l) induced the embryoidogenesis of the haploid regenerants. The cell division in the egg apparatus was observed in the ovule embryo sac from the first days, with subsequent development of a multicellular (5-8 tiers of cells) proembryo on day 3. By day 5, the embryo took the form of a ball, and increased in size due to divisions transversely and longitudinally, filling all the space of the embryo sac by days 8-12 (while the haustorial outgrowth in the nucellus did not increase). The central cell which did not started dividing, remained until the days 5-8, and then degenerated. Sometimes several endosperm nuclei were formed, which also degenerated. The starch in the cells of the nucellus was not accumulated, perisperm and seed coat were not formed. On day 28, the embryo ruptured the integuments, and a seedling appeared on the surface of the ovule, with subsequent formation of small cotyledonary leaves, hypocotyl and the primordial root. This indicates the similarity between haploid embryoidogenesis and embryogenesis of the zygotic embryo, although an embryoid is formed using only mineral salts from growth medium (without nutrients from the endosperm which does not form under in vitro conditions).

Auxins stimulated the growth of callus along with gynogenetic embryos. Gibberellin (2 mg/l), 6-benzylaminopurine (0.1 mg/l) and indolyl-3-butyric acid (0.1 mg/l) induced haploid embryoids and then secondary regenerates from hypocotyl callus tissues that increased 6- to 10-fold the output of haploids [20]. As totipotency of the heterogeneous callus cells varies, two major types of callus may probably form, i.e. morphogenic and non-morphogenic. A non-morphogenic callus consists of parenchymal cells and can not regenerate. Root formation (rhizogenesis) or gemmogenesis (the appearance of the buds), or gemmorhizogenesis occurred in morphogenic callus which had more dense fine grain structure. Gemmorhizogenesis began in the subepidermal layer. In vitro, initial cells appeared having a thickened cell membrane and a larger nucleus, from which meristem zones developed de novo as pimples. Following the periclinal divisions on the outside of the pimples, an apex (sickle-shaped fold) and the first leaf primordium (the growth bud) were formed. Root apexes were set in the lateral or basal part of the callus. A conducting bundle was formed between the root and the bud. Plant reproduction via callus occurred under gemmorhizogenesis or gemmogenesis, followed by root induction in 3-4 weeks.

Culture of unfertilized ovules in liquid medium resulted in the increased proliferation of the female gametophyte cells and maintained their viability for 4-6 months. The transfer of these explants onto the agar medium of the same composition stimulated regeneration (13.7 and 18.9 % for calluso- and embryoidogenesis, respectively). Note that the callus is a system of indirect organogen-

esis which extends the time of regenerant formation, therefore, the direct in vitro regeneration is a more promising for breeding.

The stage of stabilization involves the selection of normally developed haploid regenerated plants [27] with a high ability to form adventitious shoots and micropropagation. To improve the efficiency, we used morphological and cytological characteristics at this stage. Accordingly, the height and size of organs in haploid regenerants are usually lower than in diploids. Depending on the genotype, normally developed haploid forms have more narrow leaf blades with long petioles or, on the contrary, broad leaves with wavy edge and short petioles. A typical cytological trait of haploids is 9 chromosomes, which are revealed through microscopic examination or cytophotometry based on the content of nuclear DNA [28]. The latter method is faster, more reliable and can be widely used for identifying plants with altered ploidy. The presence of 8-10 chloroplasts in the stomata guard cells, among which there are abnormal or non-forming chloroplasts, or those having one bean-like cell, should be considered cytoembryological features. As cytoembryological signs in haploids varied only slightly, they can be used as morphological markers for identification and selection of haploid regenerants. This allows to make an assessment at the earliest stages of the regenerated plant development, when the analysis of the chromosome set is an extremely difficult and leads to the death of the explants.

Colchicination of haploids [27] resulted in polyploidization with the formation of diploids, triploids and tetraploids. With increasing ploidy level, the number of chloroplasts in a pair of the stomata guard cells also increased, e.g. from 9-11 in haploids to 12-14, 15-17 and 18-21, respectively, in di-, tri- and tetraploids. The ratio of the area of guard cells in haploids vs. di-, tri- and tetraploid forms was also modified (1:1.56; 1:1.87; 1:2.68, respectively) (Table 1).

1. Cytomorphological characteristics of pairs of stomata guard cells in sugar beet (*Beta vulgaris* L.) plants at different ploidy levels

Ploidy	Pairs of cells, examined	Number of chloroplasts, pcs.		*Cv*, %	Area, μm^2	*Cv*, %
		mean	min-max			
Haploid (×)	250	10.2	9.4-10.9	9.3	0.95	0.9
Dihaploid (2×)	250	13.2	11.8-14.5	9.7	1.48	1.6
Triploid (3×)	250	15.5	14.4-16.6	5.8	1.78	1.8
Tetraploid (4×)	250	19.7	18.5-20.9	6.4	2.55	1.3

It should be noted that some doubled haploids during the reproduction (both in vitro and in soil) recovered their haploid state, which may probably arise from a shorter mitotic cycle in haploid regenerants. Therefore, colchicination and intracellular selection may contribute to an increased proportion of haploid cells in the mixoploid meristem of the regenerants which adversely affects the diploidization. To increase the effect of diploidization in haploids in vitro, we modified the hormonal composition of the growth medium by adding cytokinins and gibberellin, which inhibit the growth of haploid tissues. Kinetin, when added at 0.25 mg/l, demonstrated its high ability to stimulate cell division in diploids or to slow it down in haploids, and that allowed to produce up to 90 % of regenerated plants with a constant diploid set of chromosomes.

A comparison of isozyme spectra is a convenient tool for identifying haploid regenerates of sugar beet. When studying the electrophoretic mobility of the isoforms of NADP-isocitrate dehydrogenase (NADP-IDH, Rf 1.1.1.42), we revealed differences between haploid regenerants and the original diploid plant. Previously, we demonstrated the presence of two isoforms of NADP-IDH, such as cytoplasmic (~ 95 % activity) and mitochondrial (~ 5 % activity), in leaves of control plants, which was consistent with the results of electrophoresis: the control plants had two isoforms of NADP-IDH (R_f 0.31 and 0.39), with bands of equal

intensity, while in haploids the enzyme was represented by two areas of activity, i.e. monomorphic (R_f 0.37) and polymorphic (R_f 0.26 and 0.31).

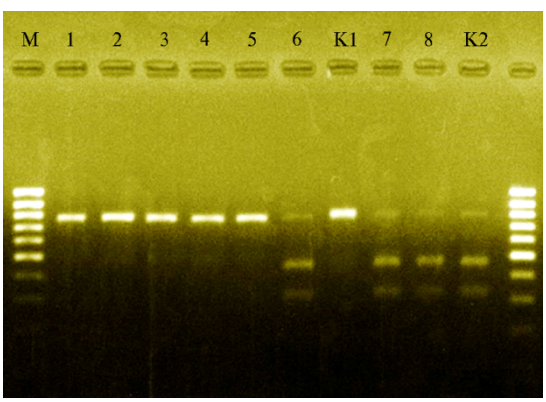
The isozyme analysis can also be used to determine the degree of homozygosity of the restitution lines obtained as a result of the stabilizing selection. Theoretically, all the lines of doubled haploids must be homozygous. However, on average 4 % heterozygous loci are revealed in the restitution lines produced via treatment with colchicine [29]. It cannot be ruled out that such polymorphism can be explained by epigenetic variability in the doubled haploids of sugar beet [30, 31]. The index of isozyme homozygosity (Iiz) in the tested lines, defined as the average percentage of homozygosity by seven isozyme loci, ranged from 0.81 to 1 [29] and averaged to 0.96 (Table 2). Homozygosity of these restitution lines is probably quite high, despite the possible epigenetic changes.

2. Indicators of isozyme homozygosity in restitution lines of sugar beet (*Beta vulgaris* L.)

Line	The proportion of homozygotes based on isozyme loci, %							Index of isozyme homozygosity (Iiz)
	Adh-1	*Mdh-1*	*Mdh-2*	*Me-1*	*Idh-1*	*Idh-2*	*Gdh-1*	
1	100	87	93	88	100	100	100	0.95
2	100	97	100	68	100	100	96	0.94
3	100	40	93	42	100	100	93	0.81
4	100	87	93	100	100	100	100	0.97
5	100	100	100	100	100	100	100	1.00
6	100	100	100	96	100	96	100	0.99
7	100	100	100	96	93	96	100	0.98
8	97	79	84	96	100	100	100	0.94
Mean	99	86	95	98	99	99	99	0.96

Molecular markers are neutral with respect to the phenotype, they are not tissue-specific, and can be detected at any stage of plant development. They allow to control the genetic transfer from the donor plants and screen for the desired trait, such as CMS [32, 33]. It is known that there are plants with normal (N) and sterile (S) cytoplasm in the sugar beet populations. Pollen is viable and fertile in N-plants, while in S-plants it can be either fertile or sterile, depending on the interaction between sterile (S) cytoplasm and recessive nuclear genes rf_1 and rf_2. The sterility of cytoplasm in sugar beet is caused by a change in the nucleotide sequence in the mitochondrial and chloroplast genomes [34, 35].

Our studies showed that the PCR and Hind III RFLP analysis enabled to identify the type of cytoplasm in the produced haploids by the restriction fragment pattern. In haploid microclones with normal and sterile cytoplasm, a single fragment (800 bps) was amplified, which was digested only in sterile (S) forms (see Fig.; two restriction fragments and the residues of the 800 bps fragment can be observed).



An electrophoregram of restriction digests of the amplified DNA fragments (Hind III RFLP analysis**) in haploid regenerated plants of sugar beet (*Beta* vulgaris L.):** K1 — control fertile plants; K2 — control sterile plants; 1-5 — forms with normal (N) cytoplasm, 6-8 — forms with sterile (S) cytoplasm; M — molecular weight markers (MassRuler™ DNA ladder, 80-1031 bps, SM0383, Thermo Scientific, USA).

Haploids, in which this fragment was not digested with Hind III, were represented by completely fertile forms with normal cytoplasm (N) and nuclear genes in the recessive state (*rf*). In the remaining samples, polymorphism of

fragments was observed which appeared to imply that corresponding haploid forms had the sterile cytoplasm (S) and various combinations of recessive and dominant alleles of the Rf_1/rf_1 and Rf_2/rf_2 nuclear genes. Note that the PCR profiles of all sterile regenerants (both haploids and doubled haploids) are identical. Therefore, the identification of regenerated plants with sterile cytoplasm at the different stages of cultivation is of great interest for the sugar beet breeding, thus facilitating the production of lines with the CMS and highly productive sterile-based hybrids.

Thus, we demonstrated the efficiency of a combination of diagnostic features for improving the technique of in vitro haploid parthenogenesis in sugar beet. The identified phenotypic, morphological, cytoembryological markers reliably characterize the critical periods for the development of generative organs in donor plants and the induction of haploidy, as well as the stage of in vitro morphogenesis, which are considered the most favorable during the stabilizing selections of haploids and doubled haploids. Determination of the isozyme electrophoretic mobility and molecular marking make it possible to evaluate the degree of homozygosity in the produced lines. RFLP analysis provides an opportunity to select the haploid regenerants possessing a desired trait, such as the cytoplasmic male sterility (CMS), and produce lines homozygous for the CMS.

REFERENCES

1. Burenin V.I. *Geneticheskie resursy roda Beta L. (Svekla)* [Genetic resources of genus *Beta* L. (beet)]. St. Petersburg, 2007 (in Russ.).
2. Butenko R.G. *Biologiya kletok vysshikh rastenii in vitro i biotekhnologii na ikh osnove* [Plant cell biology in vitro and cell cultures]. Moscow, 1999 (in Russ.).
3. Baenziger P.S., Russell W.K., Groef G.L., Campbell B.T. Improving lives: 50 years of crop breeding genetics and cytology. *Crop Sci.*, 2006, 46: 2230-2244 (doi: 10.2135/cropsci2005.11.0404gas).
4. Atanassov A.I. Method for continuous bud for mation in tissne culture of sugar beet (*Beta vulgaris* L.). *Pflazenzuchtg*, 1980, 84: 23-29.
5. Slavova J. Effective method for sugar beet haploids obtained from unpollinated ovules. *Biotechnology & Biotechnological Equipment*, 2014, 7(2): 34-35 (doi: 10.1080/13102818.1993.10818689).
6. Chen J.F., Cui L., Malik A.A., Mbira K.G. In vitro haploid and diploid production via unfertilized ovule culture. *Plant Cell Tiss. Organ Cult.*, 2011, 104(3): 311-319 (doi: 10.1007/s11240-010-9874-6).
7. Murovec J., Bohanec B. Haploids and doubled haploids in plant breeding. In: *Plant breeding*. I. Abdurakhmonov (ed.). InTech d.o.o., Croatia, 2012: 87-106 (doi: 10.5772/1389).
8. Znamenskaya V.V., Zhuzhzhalova T.P., Podvigina O.A. *Materialy Rossiiskogo simpoziuma «Novye metody po biotekhnologii rastenii»* [Proc. Russian Symp. «New methods in plant biotechnology»]. Pushchino, 1993: 111 (in Russ.).
9. Podvigina O.A. *Sakharnaya svekla*, 2003, 10: 22-24 (in Russ.).
10. Nagl N., Mezei S., Kovačev L., Vasić D., Čačić N. Induction and micropropagation potential of sugar beet haploids. *Genetica*, 2004, 36(3): 187-194 (doi: 10.2298/GENSR0403187N).
11. Sliwinska E., Pedersen H. Flow cytometric analsis of nuclear replication stages during germination of sugar-beet seeds different in vigour. *Electronic Journal of Polish Agricultural Universities*, 1999, 2(1).
12. Svirshchevskaya A.M., Doležel J. Production and performance of gynogenetic sugarbeet lines. *Journal of Sugar Beet Research*, 2000, 37(4): 117-133 (doi: 10.5274/jsbr.37.4.117).
13. Gürel S., Gürel E., Kaya Z., Erdal M., Güler E. Effects of antimitotic agents on haploid plant production from unpollinated ovules of sugar beet (*Beta vulgaris* L.). *Biotechnology & Biotechnological Equipment*, 2003, 17(2): 97-101 (doi: 10.1080/13102818.2003.10817065).
14. Klimek-Chodacka M., Baranski R. Comparison of haploid and doubled haploid sugar beet clones in their ability to micropropagate and regenerate. *Electron. J. Biotechn.*, 2013, 16(2): 1-12 (doi: 10.2225/vol16-issue2-fulltext-3).
15. Svirshchevskaya A.M. *Vestsi natsyyanal'nai Akademii navuk Belarusi*, 2008, 2: 48-55 (in Russ.).
16. Zemlyanukhin A.A., Zemlyanukhin L.A. *Metabolizm organicheskikh kislot rastenii* [Organic acid metabolism in plants]. Voronezh, 1995 (in Russ.).
17. Levites E.V., Svirshchevskaya A.M., Kirikovich A.A., Mil'ko L.V. *Informatsionnyi vestnik VOGIS*, 2005, 9(4): 512-517 (in Russ.).
18. Beckmann J.S., Soller M. Restriction fragment length polymorphisms in genetic im-

provement: methodologies, mapping and costs. *Theor. Appl. Genet.*, 1983, 67: 35-43 (doi: 10.1007/BF00303919).
19. Zaitsev G.N. *Metodika biometricheskikh raschetov* [Mathematical statistics in biology]. Moscow, 1973 (in Russ.).
20. Podvigina O.A. V sbornike: *Entsiklopediya roda Veta. Biologiya, genetika i selektsiya svekly (nauch. tr. Instituta tsitologii i genetiki SO RAN)* [In: Encyclopedia of beet Veta genus: biology, genetics and breeding]. Novosibirsk, 2010: 455-465(in Russ.).
21. Zhuzhzhalova T.P., Fomenko N.R. *Sakharnaya svekla*, 2003, 7: 26-27 (in Russ.).
22. Zhuzhzhalova T.P., Znamenskaya V.V., Podvigina O.A., Yarmolyuk G.I. *Reproduktivnaya biologiya sakharnoi svekly* [Reproductive biology of sugar beet]. Voronezh, 2007 (in Russ.).
23. Petrov D.F. *Geneticheskie osnovy apomiksisa* [Genetic control of apomixis]. Novosibirsk, 1979 (in Russ.).
24. Taratonov N.A. *Vliyanie ekzogennykh faktorov na rost i razvitie regenerantov v protsesse formirovaniya digaploidnykh form sakharnoi svekly. Kandidatskaya dissertatsiya* [Effect of exogenous factors on the growth and development of the regenerated plants under sugar beet dihaploidization. PhD Thesis]. Ramon', 1999 (in Russ.).
25. Batygina T.B., Kruglova N.N., Gorbunova V.Yu., Titova G.E., Sel'dimirova O.A. *Ot mikrospory — k sortu* [From microspore to variety]. Moscow, 2010 (in Russ.).
26. Zhuzhzhalova T.P., Podvigina O.A., Znamenskaya V.V. V sbornike: *Entsiklopediya roda Veta. Biologiya, genetika i selektsiya svekly (nauchnye trudy Instituta tsitologii i genetiki SO RAN)* [In: Encyclopedia of beet Veta genus: biology, genetics and breeding]. Novosibirsk, 2010: 403-419 (in Russ.).
27. Znamenskaya V.V. V sbornike: *Entsiklopediya roda Beta. Biologiya, genetika i selektsiya svekly (nauchnye trudy Instituta tsitologii i genetiki SO RAN)* [In: Encyclopedia of beet *Beta* genus: biology, genetics and breeding]. Novosibirsk, 2010: 420-437 (in Russ.).
28. Vasil'chenko E.N., Zhuzhzhalova T.P. *Sakharnaya svekla*, 2011, 1: 18-21 (in Russ.).
29. Fedulova T.P. *Teoreticheskie i prakticheskie aspekty molekulyarno-geneticheskogo markirovaniya v selektsii sakharnoi svekly (Beta vulgaris L.). Doktorskaya dissertatsiya* [Theoretical and practical aspects of molecular marking in sugar beet (*Beta vulgaris* L.) breeding. DSci Thesis]. Ramon', 2005 (in Russ.).
30. Kirikovich S.S., Svirshchevskaya A.M., Levites E.V. Variation at isozyme loci in seed offspring of sugar beet gynogenetic lines. *Sugar Tech.*, 2003, 5(4): 289-292 (doi: 10.1007/BF02942487).
31. Levites E.V., Svirshchevskaya A.M., Kirikovich S.S., Mil'ko L.V. Variation at isozyme loci in cultured in vitro sugar beet regenerants of gynogenetic origin. *Sugar Tech.*, 2005, 7(1): 71-75 (doi: 10.1007/BF02942421).
32. Nishizawa S., Kubo T., Mikami T. Variable number of tandem repeat loci in the mitochondrial genomes of beets. *Curr. Genet.*, 2000, 37: 34-38 (doi: 10.1007/s002940050005).
33. Nishizawa S., Mikami T., Kubo T. Mitochondrial DNA phylogeny of cultivated and wild beets: relationships among cytoplasmic male-sterility-inducing and nonsterilizing cytoplasms. *Genetics*, 2007, 177: 1703-1712 (doi: 10.1534/genetics.107.076380).
34. Cheng D., Kitazaki K., Xu D., Mikami T., Kubo T. The distribution of normal and male-sterile cytoplasms in Chinese sugar-beet germplasm. *Euphytica*, 2009, 165: 345-354 (doi: 10.1007/s10681-008-9796-0).
35. Moritani M., Taguchi K., Kitazaki K., Matsuhira H., Katsuyama T., Mikami T., Kubo T. Identification of the predominant nonrestoring allele for Owen-type cytoplasmic male sterility in sugar beet (*Beta vulgaris* L.): development of molecular markers for the maintainer genotype. *Mol. Breeding*, 2013, 32: 91-100 (doi: 10.1007/s11032-013-9854-8).