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CONTENTS

AGRICULTURAL BIOLOGY,
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FEATURES OF RICE (*Oryza sativa* L.) VARIETIES FOR ORGANIC FARMING IN CONNECTION WITH MARKER ASSISTED BREEDING

(review)

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Abstract

Organic agriculture is actively developing worldwide with a 30 % annual increase (S.Y. Dhurai et al., 2014). Today, the market for organic products reaches more than $200 billion a year. Products grown by organic farming technologies cost 20 % and sometimes 100 % higher. However, decrease in crop yields in organic farming largely eliminates the cost advantage (G.N. Fadkin et al., 2015). The use of specialized varieties should increase the profitability of organic farming (V. Seufert et al., 2012). However, there is still no clear separation in generating breeding material for these technologies. Purpose of this work is to review characteristics that must be selected when creating rice varieties for organic farming and effective working methods. Variety for this technology should possess a number of characteristics, i.e. high adaptability to biotic and abiotic stresses, competitiveness of the genotype, efficiency of mineral nutrition and photosynthesis (T. Vanaja et al., 2013). Note, all of these traits are complex and largely interconnected. So, the competitiveness of the genotype is ensured by a number of features, i.e., high growth rate; effective tillering; morphotype with minimal shading in dense crops; high efficiency of photosynthesis for the full use of solar energy; high root absorption (E.T. van Bueren et al., 2011; J.K. Goncharova et al., 2018). Increasing specific adaptability to a complex of stresses requires more effort and does not guarantee a result due to a significant decrease in the effect of individual genes resulted from intralocus and intergenic interactions. In nature, a complex of factors acts on the plant, which depreciates specific adaptability. Specific resistance to pathogens, as a rule, is overcome in a very short time (A.H. Bruggen, 1995). The great promise of increasing the overall adaptability of plant due to non-specific adaptability is shown. The most polymorphic loci of the Russian rice varieties for non-specific adaptability associated with the efficiency of genetic systems providing the growth rate, photosynthesis, mineral nutrition are summarized (L. Huang et al., 2016). Intensive growth, high photosynthetic activity and the effectiveness of mineral nutrition increase the vitality, allow plants to pass stress sensitive phases as quickly as possible, which reduces the likelihood of damage caused by extreme temperatures or other factors that reduce viability, including during organic farming. In Russian rice varieties, Microsatellite markers RM154, RM600, RM550, RM347, RM240, RM154, and RM509 are associated with loci for the efficiency of photosynthesis, RM261, RM6314, RM126, RM463, RM405, RM509, RM242 are associated with loci for mineral nutrition, RM463, RM245, RM242, RM3276, RM5508, RM574, RM542 are associated with salt resistance, and RM261, RM405, RM463, RM242, RM6314 are linked to loci for seedling growth rates. The markers identified by us are located in the same chromosome regions as the genes that determine the germination energy, drought resistance, tolerance to low temperatures, the morphotype and size of the root system, the ratio of the aboveground to the underground part of the plant, the stability of cell membranes under stress conditions, and the photosynthetic potential of the variety (G.A. Manjunatha et al., 2017; J. Ali et al., 2018).

Keywords: rice, adaptability, abiotic stresses, drought, salinization, non-specific resistance, mineral nutrition, organic farming

Mineral fertilizers and pesticides allowed a significant increase in crop yields during a certain period of use. However, over time, negative consequences (soil erosion, environmental problems, and an increase in people morbidity) also arose [1-3], thence, organic farming systems for crop cultivation [4-6] with the use of organic fertilizers, biologicals and biological plant protection methods [7-9] are topical. Organic farms confirm the possibility to obtain stable yields, especially when using crops capable of nitrogen fixation [10-12]. Initially, the rejection of mineral fertilizers often leads to a decrease in yield [13-15]. It can be especially significant (up to 60%) in the first year of application of environmentally friendly technologies [16-18]. Soil fertility is restored in the next 3-4 years, during which the yield approaches the original (19-21). At this time, the availability of nutrients of organic fertilizers for plants increases due to humus formation [22-24].

The advantage of organic farming systems (OFS) is the provision of sustainable crop yields under stressful conditions (drought, salinity, temperatures outside the variety's response range) [25-27]. The use of OFS for intensive varieties is often not reasonable, since their yield decreases by more than 30% if the level of mineral nutrition is low [28-30]. This necessitates to develop a novel approach to breeding varieties for OFS technologies.

In this review we consider the characteristics for which breeding is necessary when creating rice varieties for organic farming, and the appropriate methodological approaches and breeding technologies.

Characterization of varieties for OFS. The traits that a variety for OFS should have are still debated [31-33]; however, they undoubtedly include high adaptability under biotic and abiotic stresses, genotype competitiveness, effective mineral nutrition and photosynthesis [34-36). All these traits are complex and largely interrelated. Thus, the high efficiency of mineral nutrition and photosynthesis ensures high adaptability to all stress factors and the competitiveness of the genotype [37-39].

Genotype competitiveness. The high competitiveness of the genotype is one of the main traits that allows the variety to be used in organic farming. The components of the competitiveness of rice plants include a high growth rate, effective tillering, a morphotype that provides minimal shading in dense sowing, high efficiency of photosynthesis, which allows the most complete use of solar energy even with shading, formation of a root system with high absorbing capacity [40-42].

The adaptability to stress can be influenced by increasing either the specific adaptability to each stress, or non-specific adaptability, which simultaneously enhances resistance to various stressors. Changing specific adaptability requires great efforts and does not guarantee the result, since all traits are polygenic, therefore, both intralocus and intergenic gene interactions can largely neutralize the effects of individual genes [43-45]. In nature, a plant is influenced by a complex of factors, which devalues one-way adaptability. Specific resistance to pathogens, as a rule, is overcome by them in a very short time. Therefore, from our point of view, the second strategy is more promising when an increase in general adaptability (non-specific resistance) occurs due to the functioning of several genetic systems that provide effective photosynthesis and mineral nutrition, a high rate of growth and development, and resistance to salinity.

Microsatellite markers associated with loci that determine plant photosynthesis efficiency of Russian rice varieties. Molecular markers linked to gene loci (quantitative traits loci, QTL), affecting the efficiency of photosynthesis and other traits in rice varieties of different geographic origin, is shown at http://www.gramene.org. However, their applicability for assessing Russian

breeding material must be confirmed. It is necessary to confirm reliable separation of contrasting groups with the use of these markers [46, 47].

The study of allelic polymorphism of intragenic SSR markers and markers linked to genes that determine the photosynthetic potential of Russian rice cultivars revealed polymorphic loci (Fig. 1) [47].

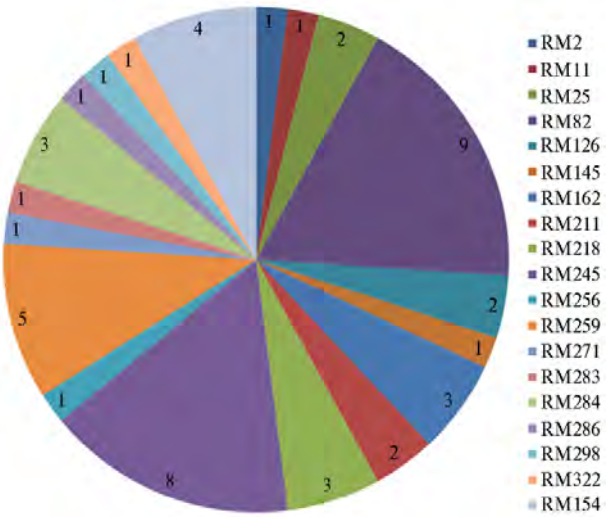

Fig. 1. Allelic polymorphism of markers (the number of alleles is indicated) **associated with photosynthesis efficiency of Russian rice varieties [47].**

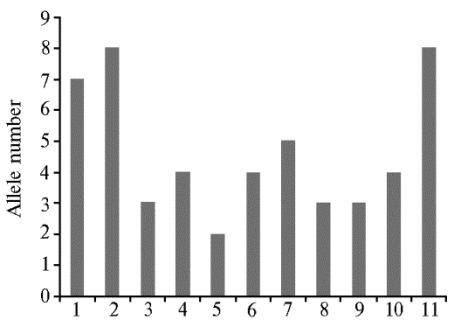

Fig. 2. Microsatellite markers linked to loci for photosynthesis efficiency of Russian rice varieties: 1 — RM600, 2 — RM5508, 3 — RM509, 4 — RM5361, 5 — RM347, 6 — RM154, 7 — RM240, 8 — RM162, 9 — RM574, 10 — RM5707, 11 — RM245 [47].

Analysis of the revealed polymorphism showed that some of the markers variable in amplification products can separate groups of varieties with different efficiency of photosynthesis at $p \leq 0.05$. Three of them (RM154, RM600, RM5508) linked to loci that determine the carotenoid level, two markers are linked to loci controlling specific surface density (RM347, RM240) and chlorophyll a content (RM154, RM509). Figure 2 shows intragenic markers with the maximum number of alleles which are associated with photosynthetic efficiency and divide Russian rice varieties into contrasting groups according to the trait [47].

These markers can be involved in marker-assisted selection (MAS) for the traits that determine the efficiency of photosynthesis in Russian rice varieties. The importance of the genetic system which determines the photosynthetic potential of the variety, has been shown by many researchers [48-51]. It is noted that varieties for OFS, as a rule, use light more efficiently. They have a long period of photosynthesis and a high chlorophyll content, especially in the upper leaves. The photosynthetic function of plant organs is ensured by the optimal architectonics of crops. In particular, key traits are plant height, resistance to lodging, and the presence of long and wide erectoid leaves. Note, the plant height for OFS significantly exceeds that in varieties for intensive farming technologies, in some works, up to 119 cm, that is, 20-30 cm higher compared to plant height in traditional

agriculture) [44, 46].

Mineral nutrition response markers and chromosome regions. The potential for using genotypic differences in ability to assimilate minerals is enormous, since there is a 20-fold differences between the extreme manifestations of the trait [52-54]. The problem is that high-yielding varieties, as a rule, are not adapted to the lack of mineral nutrients. The mechanism of adaptation of rice plants to such a deficiency differs in different genotypes and includes an increase in root size, an intensification of absorption, and an increase in the internal efficiency of fertilizer use. However, there are reports that in most of the studied genotypes the latter indicator varies slightly [55-57].

Nutrition response of Russian rice varieties is poorly studied. Our studies have shown high yield variability in rice varieties at different levels of nitrogen fertilizers. Rapan variety shows the greatest variability of the trait (Cv = 33.4%), whereas Vodopad variety is the most stable (Cv = 15.7%). Without mineral fertilizers, the varieties reduced the yield by 49.05% on average with 55.93% reduction (variation from 53.33 to 58.71%) in varieties Rapan, Yubileyny 85, and Nautilus of intensive type. The grain yield of Vodopad variety decreased by 32%. In other words, the specialized "organic" varieties can additionally provide for more than 20 c/ha of rice grain and a 1.5-fold increase in OFS profitability [16].

Investigation of dose-dependent response to nitrogen fertilization revealed Vodopad variety to be in the lead without fertilization, 61 c/ha vs. an average yield of 48.2 c/ha. The yield of Nautilus (46.2 c/ha), Yubileiny 85 (43.1 c ha) and Rapan (40.8 c/ha) was lower than the average value of 48.2 c/ha. The change in the yield upon application of fertilizers was determined by the coefficient of linear regression of its relationship with the doses of applied nitrogen fertilizers [16].

On average, a 1 kg/ha nitrogen fertilizer provided 23 kg/ha increase in rice yield. The gain decreased with an increase in the dose of the fertilizer, from 32 kg/ha for N_0-N_{91} to 21 kg/ha for N_{92}-N_{137} and 15 kg/ha for N_{138}-N_{184} (Fig. 3). The revealed differences of Russian varieties allow breeding for increased nitrogen efficiency under different cultivation technologies [58].

Our previous works showed that the development of root system largely determines adaptability of rice plants to a deficient mineral nutrition, however, the question of which molecular markers can reliably group domestic samples contrasting in the manifestation of this trait during different phases of vegetation has not been studied [56].

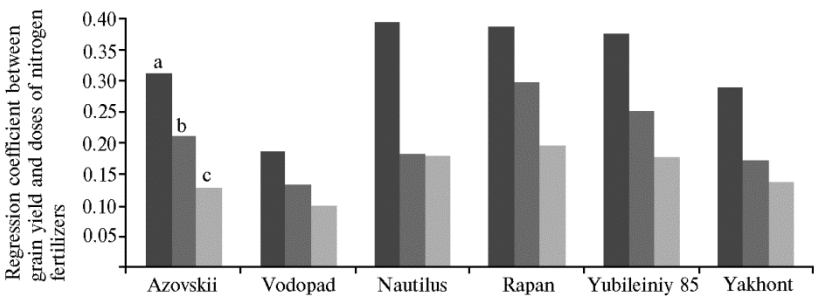

Fig. 3. Grain yields of Russian rice varieties depending on N-fertilizer doses (per active ingredient): a — 0-91 kg/ha, b — 92-137 kg/ha, c — 138-184 kg/ha (test plot of FSC of the Federal Rice Research Center rice, Krasnodar, 2017-2018) [16].

In a study of Russian rice varieties with microstellite markers, it was shown that the size of the root system is determined by genes in 14 loci [30], and these genes are not the same at different stages of plant development. Seven SSR

markers, RM261, RM6314, RM126, RM463, RM405, RM509, and RM242 differentiated the samples by the efficiency of mineral nutrition during germination. None of the markers reliably divided the samples by the efficiency of mineral nutrition at the tillering stage. The markers RM245, RM284, RM574, RM258, RM227, RM509, RM3428, RM440, and RM154 showed a high probability of association with the trait. Ten SSR markers, RM284, RM335, RM245, RM600, RM53, RM542, RM261, RM5361, RM5371, and RM6314 discriminated domestic rice varieties contrasting in adaptability to a lack of mineral nutrition during ripening (Table 1).

1. SSR markers discriminating Russian rice varieties with contrast adaptability to deficient mineral nutrition

Gene	Traits	SSR marker	Chromosome	References
TRN7-1	Root number, 65 days after planting	RM542	7	[59]
LFSNS	Leaf structure, the chlorophyll content in the second leaf at tasseling vs. day 30 of growth	RM 245	9	[60]
qTRN1-2	Root number, 65 days after planting	RM600	1	[59]
OSAD-JCAP	Osmotic regulation	RM284	8	[61]
rdgf4 LFSNS	Leaf senescence, the chlorophyll content in the second leaf at tasseling vs. day 30 of growth	RM335	4	[62]
AQEI046-LFSNS	Leaf senescence, the chlorophyll content in the second leaf at tasseling vs. day 30 of growth	RM53	2	[62]
qTRN4-1	Root number 85 days after planting	RM261	4	[59]
NCN	Efficiency of mineral nutrient utilization	RM5371	6	[63]
NCN	Efficiency of mineral nutrient utilization	RM5361	5	[63]
NCN	Efficiency of mineral nutrient utilization	RM6314	4	[30, 64]
OSADJCAP	Osmotic regulation	RM126	8	[61]
qRTT9-1	Root thickness 65 days after planting	RM242	9	[59]
qPHT12-1	Plant height	RM463	12	[59]
qFRP-12	Grain number per panicle			
NCN	Efficiency of mineral nutrient utilization	RM509	5	[63, 65]
qRTV5-1	Root volume, 65 days after planting	RM289	5	[59]
qPL-5	Panicle length	RM405	5	[66]
qYI-5	Spikelets per panicle			
NCN	Efficiency of mineral nutrient utilization	RM3155	8	[30, 64]

N o t e. NCN means that no common name of the gene is accepted.

Previously, both specific and nonspecific genes were mapped that enhance the responsiveness of rice plants to mineral nutrition; SSR markers were also located in the regions of localization of these genes [67-69]. The loci for the effectiveness of mineral nutrition in Russian rice varieties are defined in the scholar publications as being responsible for the number, volume and thickness of roots 65 days after planting, for the capability of osmotic regulation, and for leaf senescence assessed by the ratio of chlorophyll content in the second leaf at tasseling and after 30-day growth. Since there is a close correlation between the sizes of the aboveground and underground parts of plants and the pleiotropic influence of many genes is known, the loci that determine plant height, panicle length, and the grain number per panicle were also attributed to those that increase the efficiency of mineral nutrient utilization [70-72].

SSR markers of salt tolerance of Russian rice varieties. Table 2 shows SSR markers reliably grouping Russian rice varieties by different salt tolerance during flowering. Analysis of genes previously mapped in the regions of localization of these markers [72] showed that only two markers, RM25 and RM240 are associated with specific genes for salinity resistance enabling osmotic regulation. Other markers are linked to nonspecific loci that increase the viability and resistance to a number of stressors [72-75].

Markers enabling reliable discrimination of domestic rice varieties with contrast adaptability to salinity as assessed by changes in the length of the embryonic root have not been established. The obtained result is anticipated, since the

adaptability to salinity is determined by polygenes or gene clusters. The studied samples carry sets of genes that, acting together, hide the effect of one or another locus. However, this did not prevent the identification of loci most likely associated with the trait in question in seedlings. Identification was allowed due to an increase in the sensitivity threshold of the method by applying a significance level of $p \leq 0.09$ [76]. At the accepted level of significance, a numerous of loci were identified that determine the change in the root length during salinization in the early growing season. In most cases, the varieties are also separated due to nonspecific genes that increase resistance to salinity (76).

2. SSR markers associated with salt tolerance of Russian rice varieties [72]

SSR marker	Chromosome	Amplicon, bp (primer melting temperature, °C)	Traits
		Flowering	
RM574	5	155 (55)	Root system features
RM245	9	150 (55)	Photosynthesis efficiency during growth and maturation
RM240	2	132 (55)	Osmotic regulation, length of growing period
RM53	2	182 (55)	Leaf senescence, length of growing period
RM25	8	146 (55)	Length of growing period, osmotic regulation, leaf senescence,
RM590	10	137 (55)	Grain fracture
RM24	1	192(55)	Ratio of root number to stem number, length of growing period, total leaf parea
RM5361	5	138 (55)	No data available
		Seedlings (as per root length)	
RM574	5	155 (55)	Root volume, root thickness
RM245	9	150 (55)	Photosynthetic potential, length of growing period
RM542	7	113 (55)	Root volume, the angle of rice stem inclination, plant height
RM463	12	192 (55)	Plant height
RM242	9	225 (55)	Root length and thickness, cold tolerance, in vitro culturing, germination vigor, cell-membrane stability, plant height
RM3276	4	163 (50)	Salt tolerance
RM5508	7	177 (50)	Salt tolerance
		Seedlings (as per stem length)	
RM574*	5	155(55)	Root system features
RM154*	2	183 (61)	Photosynthetic potential
RM141	6	136 (55)	Photosynthetic potential
RM82	7	186 (55)	Photosynthetic potential, resistance to low temperatures
RM286	11	110 (55)	Length of growing period, photosynthetic potential
RM227	3	106 (55)	Leaf features, germination vigor, length of growing period, root dry weight
RM24	1	192 (55)	Ratio of root number to stem number, length of growing period, leaf features
RM542	7	113 (55)	Root weight, plant height
RM126	8	171 (55)	Osmotic regulation, length of growing period, Leaf senescence

* Markers reliably discriminating samples at $p \leq 0.05$.

Markers RM463, RM245, RM242, RM3276, RM5508, RM574, RM542 are linked to nonspecific loci that determine adaptability of seedlings to salinity. Markers RM463, RM242 are associated with genes encoding the photosynthetic potential. The RM242 is mapped in the chromosomal region where genes are localized that determine adaptability to stress due to higher cell-membrane tolerance and the growth rate. Markers RM574, RM542, and RM242 flank loci that determine root length, thickness, volume, and efficiency, thus allowing better rice plant responsiveness to mineral nutrition [77-79].

Microsatellite markers associated with growth rate of seedlings. According to our data [76], most loci which determine growth rate in Russian rice gene pool, are monomorphic, since for a long time, a high level of water was used to control weeds. The rapid appearance of plant above the water level

provides even sprouts and further high productivity. Nevertheless, statistical analysis revealed polymorphic regions determining the growth rate of domestic varieties. The data of http://www.gramene.org shows that genes associated with root system formation and germination vigor had already been found in the identified loci (Table 3).

3. SSR markers for clustering Russian rice varieties based on growth rate parameters [76]

SSR marker	Chromosome	Amplicons, bp	Traits
		Seeding stem length	
RM289	5	108	Stem and leaf size, growth vigor
		Root length	
RM242	9	225	Tolerance to low temperatures, root size and activity, ratio of root length to size and number of tillers and leaves, differentiation of explants, tolerance to membrane stressors, germination rate
RM126	4	125	Adaptiveness to deficit irrigation, tolerance to low temperatures, root size, the ratio of the root length to tiller length
		Seedling weight	
RM405	5	110	Stem length, panicle length, leaf size
RM261	4	125	Drought tolerance, tolerance to low temperatures, stem length, root length, panicle length, leaf size
RM242	9	225	In vitro culturing, root size, root activity and length compared to stem and panicle activity and length, tolerance to low temperatures, germination rate, tolerance to membrane stressors
RM463	12	192	Size of the aboveground organs
RM6314	4	169	No data available

The markers identified by us are mapped in the chromosomal regions bearing genes that determine the germination vigor, drought tolerance, tolerance to low temperatures, the root morphotype and size, the weight proportion of the aboveground to the underground parts of the plant, and cell-membrane stability under stress conditions [30, 56, 76]. Stem formation, according to our data, was determined by loci linked to the RM289 marker. In RM289 is mapped in the region that contains genes affecting the germination rate, plant height, differentiation of explants, the relative weight of roots, and the rate of seedling appearance [39, 40]. Differences in clustering varieties contrast in the growth rates of seedlings were significant ($p \leq 0.05$) for markers RM261, RM405, RM463, RM242, and RM6314 located on chromosomes 4, 5, 9, and 12, respectively (see Table 3).

We found a relationship between the growth rate of seedlings and several loci that determine adaptability to abiotic stresses [76]. Intensive growth, high photosynthetic activity and the efficiency of mineral nutrient utilization increase plant viability and allow the plant to pass through stress-sensitive phases as quickly as possible, which reduces the likelihood of damage by extreme temperatures or other factors reducing vitality, including those in organic farming [80-83]. Selection for an increase in nonspecific adaptability is the most promising in creating varieties with sustainable productivity for organic agriculture [84, 85].

Thus, rice varieties for organic farming should have a high adaptability to biotic and abiotic stresses, genotype competitiveness, efficiency of mineral nutrient utilization and photosynthesis. The high competitiveness of the genotype is a complex trait that includes high growth rate, effective tillering, a morphotype that provides minimal shading in a dense crop, and the root system with a high absorbing capacity. To achieve sustainable productivity in organic farming, varieties with nonspecific adaptability are the most promising. The non-specific adaptability is ensured by several genetic systems that control photosynthesis, the efficiency of mineral nutrition, the high rates of growth and development, and the resistance to salinity. In Russian rice varieties, microsatellite markers RM154, RM600, RM550, RM347, RM240, RM154, and RM509 are associated with loci for the efficiency of photosynthesis. Markers RM261, RM6314, RM126, RM463, RM405, RM509, and

RM242 differentiate rice samples by the effectiveness of mineral nutrition during germination, RM463, RM245, RM242, RM3276, RM5508, RM574, RM542 reliably discriminate by salt tolerance. Polymorphism by markers RM261, RM405, RM463, RM242 and RM6314 are associated with seedling growth rate. The listed genetic markers can be involved in breeding rice varieties for organic farming.

REFERENCES

1. Lal R. Global potential of soil carbon sequestration to mitigate greenhouse effect. *Critical Reviews in Plant Sciences*, 2003, 22(2): 151-184 (doi: 10.1080/713610854).
2. Fad'kin G.N., Vinogradov D.V., Shchur A.V., Gogmachadze G.D. *AgroEkoInfo*, 2015, 4: 1-12 (in Russ.).
3. Shchur A.V., Val'ko D.V., Vinogradov V.P. *Problemy regional'noi ekologii*, 2016, 3: 36-40 (in Russ.).
4. Pimentel D., Hepperly P., Hanson J., Douds D., Seidel R. Environmental, energetic, and economic comparisons of organic and conventional farming systems. *Bioscience*, 2005, 55(7): 573-582 (doi: 10.1641/0006-3568(2005)055[0573:EEAECO]2.0.CO;2).
5. Greene C. U.S. organic farming emerges in the 1990s: adoption of certified systems. *Agriculture Information Bulletin*, 2001, No. 770. Available: https://www.ers.usda.gov/webdocs/publications/42396/31544_aib770_002.pdf?v=2234.6. No date.
6. Uphoff N., Altieri M. *Alternatives to conventional modern agriculture for meeting world needs in the next century (Report of a conference on sustainable agriculture, evaluation of new paradigms and old practices, April 26-30, 1999, Bellagio, Italy)*. Cornell International Institute for Food, Agriculture and Development, Ithaca, NY, 1999.
7. Hokazono S., Hayashi K. Variability in environmental impacts during conversion from conventional to organ4htenberg E., Peters S. Organic versus conventional grain production in the mid-Atlantic. An economic overview and farming system overview. *American Journal of Alternative Agriculture*, 1997, 12(1): 2-9 (doi: 10.1017/S0889189300007104).
8. Kovalev N.G., Baranovskii I.N. *Organicheskie udobreniya v KHKHI veke (Biokonversiya organicheskogo syr'ya)*. Tver', 2006.
9. Uvarov R.A. *Tekhnologii i tekhnicheskie sredstva mekhanizirovannogo proizvodstva produktsii rastenievodstva i zhivotnovodstva*, 2015, 86: 139-147 (in Russ.).
10. Dorais M. Organic production of vegetables: state of the art and challenges. *Canadian Journal of Plant Science*, 2008: 1055-1066 (doi: 10.4141/CJPS07160).
11. Hepperly P., Seidel R., Pimentel D., Hanson J., Douds D. Jr. Organic farming enhances soil carbon and its benefits. In: *Soil carbon management*. J.M. Kimble, C.W. Rice, D. Reed, S. Mooney, R.F. Follett, R. Lal (eds.). CRC Press, Boca Raton, FL, USA, 2019: 129-153.
12. Teasdale J., Coffman C., Mangum R. Potential long-term benefits of no-tillage and organic cropping systems for grain production and soil improvement. *Agronomy Journal*, 2000, 99(5): 1297-1305 (doi: 10.2134/agronj2006.0362).
13. Moyer J., Nichols K., Bhosekar V. Fifteen year review summarizing effects of conventional and organic farming Systems on soils, nutrition, environment, economics and yields (1981-1995). *Asian Journal of Science and Technology*, 2017, 8(4): 4628-4634 (doi: 10.19080/ARTOAJ.2017.06.555678).
14. Fedulova E.A., Medvedev A.V., Kosinskiy P.D., Kononova S.A., Pobedash N. Modeling of the agribusiness enterprise activity on the basis of the balanced scorecard. *Foods and Raw Materials*, 2016, 4(1): 154-162 (doi: 10.21179/2308-4057-2016-1-154-162).
15. Goncharova J.K., Kharitonov E.M. Rice tolerance to the impact of high temperatures. In: *Agricultural research updates, V. 9*. Nova Science Publishers, Inc., NY, 2015: 97-125.
16. Chavas J., Posner J., Hedtcke J. Organic and conventional production systems in the Wisconsin Integrated Cropping Systems Trial: II. Economic and risk analysis 1993-2006. *Agronomy Journal*, 2009, 101(2): 288-295 (doi: 10.2134/agronj2008.0055x).
17. Voronkova N.A., Khamova O.F. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2009, 5: 24-29 (in Russ.).
18. Dobrokhotov S.A., Anisimov A.I. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Razvitie zemledeliya v Nechernozem'e: problemy i ikh reshenie»* [Proc. Int. Conf. «The development of agriculture in the Non-Black Earth region: problems and solutions»]. St. Petersburg, 2016: 119-124 (in Russ.).
19. Goncharova J.K., Kharitonov E.M. Genetic control of traits determining phosphorus uptake by rice varieties (*Oryza sativa* L.). *Vavilov Journal of Genetics and Breeding*, 2015, 19(2): 197-204.
20. Lammerts van Bueren E.T. Challenging new concepts and strategies for organic plant breeding and propagation. *Proceedings of the EUCARPIA Meeting on Leafy Vegetables Genetics and Breeding, Noordwijkerhout, The Netherlands*. Th.J.L. van Hintum, A. Lebeda, D. Pink, J.W. Schut (eds.). Centre for Genetic Resources, The Netherlands (CGN), Wageningen, The Netherlands, 2003: 17-22.
21. Lammerts van Bueren E.T., Struik P.C., Jacobsen E. Ecological concepts in organic farming and

their consequences for an organic crop ideotype. *NJAS — Wageningen Journal of Life Sciences*, 2002, 50(1): 1-26 (doi: 10.1016/S1573-5214(02)80001-X).
22. Morris M.L., Bellon M.R. Participatory plant breeding research: Opportunities and challenges for the international crop improvement system. *Euphytica*, 2004, 136: 21-35 (doi: 10.1023/b:euph.0000019509.37769.b1).
23. Mader P., Fliessbach A., Dubois D., Gunst L., Fried P., Niggli U. Soil fertility and biodiversity in organic farming. *Science*, 2004, 296(5573): 1694-1697 (doi: 10.1126/science.1071148).
24. Seufert V., Ramankutty N., Foley J.A. Comparing the yields of organic and conventional agriculture. *Nature*, 2012, 485: 229-232 (doi: 10.1038/nature11069).
25. Swer H., Dkhar M.S., Kayang H. Fungal population and diversity in organically amended agricultural soils of Meghalaya, India. *Journal of Organic Systems*, 2011, 6(2): 3-12.
26. Van Bruggen A.H. Plant disease severity in high-input compared to reduced-input and organic farming systems. *Plant Disease*, 1995, 79: 976-984 (doi: 10.1094/PD-79-0976).
27. Heyden B., Lammerts van Bueren E.T. *Bio-diversity of vegetables and cereals — chances for developments in organic agriculture*. Naturschutzbund (NABU), Bonn, 2000.
28. Leu A. Ameliorating the effects of climate change with organic systems. *Journal of Organic Systems*, 2009, 4(1): 4-7.
29. Vanaja T., Mammootty K.P. 'Kuthiru' and 'Orkayama' — newly identified genetic resources from Kerala, India for salinity tolerance in Indica rice. *Nature Proceedings*, 2010 (doi: 10.1038/npre.2010.4561.1).
30. Longping Y. Breeding of super hybrid rice. *Proc. Int. rice research conference «Rice Research for food security and poverty alleviation»*. S. Peng, B. Hardy (eds.). International Rice Research Institute, Los Bacos, Laguna (Philippines), 2001: 143-149.
31. Zhang O. Strategies for developing green super rice. *Proceedings of the National Academy of Sciences*, 2007, 104(42): 16402-16409 (doi: 10.1073/pnas.0708013104).
32. Vanaja T., Mammootty K.P., Govindan M. Development of organic indica rice cultivar (*Oryza sativa* L.) for the wetlands of Kerala. India through new concepts and strategies of crop improvement. *Journal of Organic Systems*, 2013, 8(2): 18-28.
33. Tammis W.L.M., van den Brink W.J. Conventional, integrated and organic winter wheat production in the Netherlands in the period 1993-1997. *Agriculture, Ecosystems and Environment*, 1999, 76(1): 47-59.
34. Lammerts van Bueren E.T., Jones S.S., Tamm L., Murphy K.M., Myers J.R., Leifert C., Messmer M.M. The need to breed crop varieties suitable for organic farming, using wheat, tomato and broccoli as examples: a review. *NJAS — Wageningen Journal of Life Sciences*, 2011, 58(3-4): 193-205 (doi: 10.1016/j.njas.2010.04.001).
35. Kharitonov E.M., Goncharova Yu.K., Ochkas N.A., Sheleg V.A., Bolyanova S.V. Application of multidimensional methods to separate varieties on their response to environment factors. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(1): 152-160 (doi: 10.15389/agrobiology.2017.1.152eng).
36. Goncharova J.K., Gontcharov S.V., Chicharova E.E. Localization of chromosome regions controlling high photosynthetic potential in Russian rice cultivars. *Russian Journal of Genetics*, 2018, 54(7): 796-804 (doi: 10.1134/S1022795418070037).
37. Akhtar N., Nazir M.F., Rabnawaz A., Mahmood T., Safdar M.E., Asif M., Rehman A. Estimation of heritability, correlation and path coefficient analysis in fine grain rice. *The Journal of Animal & Plant Sciences*, 2011, 21(4): 660-664.
38. Kumar C. Correlation and path coefficient analysis of yield components in aerobic rice (*Oryza sativa* L.). *The Bioscan*, 2014, 9(Supplement on Genetics and Plant Breeding): 907-913.
39. Nagaraju C., Sekhar R.M., Reddy H.K., Sudhakar P. Correlation between traits and path analysis coefficient for grain yield and other components in rice (*Oryza sativa* L.) genotypes. *International Journal of Applied Biology and Pharmaceutical Technology*, 2013, 4(3): 137-142.
40. Ramakrishnan S.H., Anandakumar C.R, Saravanan S., Malini N. Association analysis of some yield traits in rice (*Oryza sativa* L.). *Journal of Applied Sciences Research*, 2006, 2(7): 402-404.
41. Deshpande H.H., Devasenapathy P. Effect of green manuring and organic manures on yield, quality and economics of rice (*Oryza sativa* L.) under lowland condition. *Karnataka Journal of Agricultural Sciences*, 2010, 23(2): 235-238.
42. Dhurai S.Y., Reddy D.M., Bhati B.K. Correlation and path coefficient analysis for yield and quality traits under organic fertilizer management in rice (*Oryza sativa* L.). *Electronic Journal of Plant Breeding*, 2014, 5(3): 581-587.
43. Zahid M.A., Akhtar M., Sabir M., Manzoor Z., Awan T.H. Correlation and path analysis studies of yield and economic traits in Basmati rice (*Oryza sativa* L.). *Asian Journal of Plant Sciences*, 2006, 5(4): 643-645 (doi: 10.3923/ajps.2006.643.645).
44. Ali J., Jewel Z.A., Mahender A., Anandan A., Hernandez J., Li Z. Molecular genetics and breeding for nutrient use efficiency in rice. Rice breeding platform. *International Journal of Molecular Sciences Review*, 2018, 19(6): 1762 (doi: 10.3390/ijms19061762).
45. Goncharova Yu.K., Kharitonov E.M., Sheleg V.A., Bolyanova S.V. *Rossiiskaya*

sel'skokhozyaistvennaya nauka, 2016, 6: 3-8 (in Russ.).
46. Zhang Y.J., Dong Y.J., Zhang J.Z., Xiao K., Xu J.L., Terao H. Mapping QTLs for deficiency phosphorus response to root-growth of rice seedling. *Rice Genetics Newsletter*, 2006, 25: 36-37.
47. Manjunatha G.A., Saravana Kumar M., Jayashree M. Character association and path analysis in rice (*Oryza sativa* L.) genotypes evaluated under organic management. *Journal of Pharmacognosy and Phytochemistry*, 2017, 6(6): 1053-1058.
48. Hemamalini G.S., Shashidhar H.E., Hittalmani S. Molecular marker assisted tagging of morphological and physiological traits under two contrasting moisture regimes at peak vegetative stage in rice (*Oryza sativa* L.). *Euphytica*, 2000, 112: 69-78 (doi: 10.1023/A:1003854224905).
49. Yue B., Xue W.Y., Xiong L.Z., Yu X.Q., Luo L.J., Cui K.H., Jin D.M., Xing Y.Z., Zhang Q.F. Genetic basis of drought resistance at reproductive stage in rice: separation of drought tolerance from drought avoidance. *Genetics,* 2006, 172: 1213-1228 (doi: 10.1534/genetics.105.045062).
50. Robin S., Pathan M.S., Courtois B., Lafitte R., Carandang C., Lanceras S., Amante M., Nguyen H.T., Li Z. Mapping osmotic adjustment in an advanced back-cross inbred population of rice. *Theoretical and Applied Genetics*, 2003, 107: 1288-1296 (doi: 10.1007/s00122-003-1360-7).
51. Jiang G.H., He Y.Q., Xu C.G., Li X.H., Zhang Q. The genetic basis of stay-green in rice analyzed in a population of doubled haploid lines derived from an indica by japonica cross. *Theoretical and Applied Genetics*, 2004, 108: 688-698 (doi: 10.1007/s00122-003-1465-z).
52. Kharitonov E.M., Goncharova Y.K., Maliuchenko E.A. Genetics of the traits determining adaptability to abiotic stress in rice (*Oryza sativa* L.). *Russian Journal of Genetics: Applied Research*, 2017, 7(6): 684-697 (doi: 10.1134/S2079059717060089).
53. McCouch S.R., Teytelman L., Xu Y., Lobos K.B., Clare K., Walton M., Fu B., Maghirang R., Li Z., Xing Y., Zhang Q., Kono I., Yano M., Fjellstrom R., DeClerck G., Schneider D., Cartinhour S., Ware D., Stein L. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.) (supplement). *DNA Research*, 2002, 9(6): 257-279 (doi: 10.1093/dnares/9.6.257).
54. Temnykh S., DeClerck G., Lukashova A., Lipovich L., Cartinhour S., McCouch S. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. *Genome Research*, 2001, 11: 1441-1452 (doi: 10.1101/gr.184001).
55. Choi Y.H., Lee S.J., Yoon D.B., Moon H.P., Ahn S.N. Mapping of quantitative trait loci for cold tolerance in weedy rice. *Breeding Science*, 2004, 54: 373-380 (doi: 10.1270/jsbbs.54.373).
56. Mahender A., Anandan A., Pradhan S.K., Singh O.N. Traits-related QTLs and genes and their potential applications in rice improvement under low phosphorus condition. *Archives of Agronomy and Soil Science*, 2017, 64(4): 449-464 (doi: 10.1080/03650340.2017.1373764).
57. Berry P.M., Sylvester-Bradley R., Philipps L., Hatch D.H., Cuttle S.P., Rayns F.W., Gosling P. Is the productivity of organic farms restricted by the supply of available nitrogen? *Soil Use and Management*, 2002, 18(s1): 248-255 (doi: 10.1111/j.1475-2743.2002.tb00266.x).
58. Bhadoria P.B.S., Prakash Y.S., Kar S., Rakshit A. Relative efficacy of organic manures on rice production in lateritic soil. *Soil Use and Management*, 2003, 19(1): 80-82 (doi: 10.1111/j.1475-2743.2003.tb00283.x).
59. Bhattacharyya P., Chakraborty A., Bhattacharya B., Chakrabarti K. Evaluation of MSW compost as a component of integrated nutrient management in wetland rice. *Compost Science & Utilization*, 2003, 11(4): 343-350 (doi: 10.1080/1065657X.2003.10702144).
60. Bi L., Zhang B., Liu G., Li Z., Liu Y., Ye C., Yu X., Lai T., Zhang J., Yin J., Liang Y. Long-term effects of organic amendments on the rice yields for double rice cropping systems in subtropical China. *Agriculture, Ecosystems & Environment*, 2009, 129(4): 534-541 (doi: 10.1016/j.agee.2008.11.007).
61. Goncharova Yu.K., Kharitonov E.M. On genetic and physiological mechanisms of salt resistance in rice *Oryza sativa* l. (review). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2013, 3: 3-11 (doi: 10.15389/agrobiology.2013.3.3eng) (in Russ.).
62. Champagne E.T., Bett-Garber K.L., Grimm C.C., McClung A.M. Effects of organic fertility management on physicochemical properties and sensory quality of diverse rice cultivars. *Cereal Chemistry*, 2007, 84(4): 320-327 (doi: 10.1094/cchem-84-4-0320).
63. Champagne E.T., Bett-Garber K.L., Thomson J.L., Fitzgerald M.A. Unravelling the impact of nitrogen nutrition on cooked rice flavor and texture. *Cereal Chemistry*, 2009, 86(3): 274-280 (doi: 1010.1094/CCHEM-86-3-0274).
64. Hoad S., Topp C., Davies K. Selection of cereals for weed suppression in organic agriculture: a method based on cultivar sensitivity to weed growth. *Euphytica*, 2008, 163(3): 355-366 (doi: 10.1007/s10681-008-9710-9).
65. Kharitonov E.M., Goncharova Yu.K., Goncharov S.V., Bruyako V.N. Molecular markers associated with high early growth rate of Russian rice (*Oryza sativa* L.) varieties. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(5): 892-904 (doi: 10.15389/agrobiology.2019.5.892eng).
66. Baldani J.I., Baldani V., Seldin L., Döbereiner J. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *International Journal of Systematic and Evolutionary Microbiology*, 1986, 36(1): 86-93 (doi: 10.1099/00207713-36-1-86).
67. Chi F., Shen S.-H., Cheng H.-P., Jing Y.-X., Yanni Y.G., Dazzo F.B. Ascending migration

of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Applied and Environmental Microbiology*, 2005, 71(11): 7271-7278 (doi: 10.1128/AEM.71.11.7271-7278.2005).
68. Feng B., Chen K., Cui Y., Wu Z., Zheng T., Zhu Y., Ali J., Wang B., Xu J., Zhang W., Li Z. Genetic dissection and simultaneous improvement of drought and low nitrogen tolerances by designed QTL pyramiding in rice. *Frontiers in Plant Science*, 2018, 9(9): 306 (doi: 10.3389/fpls.2018.00306).
69. Huang L., Jun Y.U., Jie Y.A., Zhang R., Yanchao B.A., Chengming S.U., Zhuang H. Relationships between yield, quality and nitrogen uptake and utilization of organically grown rice varieties. *Pedosphere*, 2016, 26(1): 85-97 (doi: 10.1016/S1002-0160(15)60025-X).
70. Jeyabal A., Kuppuswamy G. Recycling of organic wastes for the production of vermicompost and its response in rice—legume cropping system and soil fertility. *European Journal of Agronomy*, 2001, 15(3): 153-170 (doi: 10.1016/S1161-0301(00)00100-3).
71. Yorobe J.M. Jr., Ali J., Pede V., Rejesus R.M., Velarde O.P., Wang W. Yield and income effects of rice varieties with tolerance of multiple abiotic stresses: the case of green super rice (GSR) and flooding in the Philippines. *Agricultural Economics*, 2016, 47(3): 261-271 (doi: 10.1111/agec.12227).
72. Van Quyen N., Sharma S.N. Relative effect of organic and conventional farming on growth, yield and grain quality of scented rice and soil fertility. *Archives of Agronomy and Soil Science*, 2003, 49(6): 623-629 (doi: 10.1080/03650340310001612979).
73. Xu M.-G., Li D.-C., Li J.-M., Qin D.-Z., Kazuyuki Y., Hosen Y. Effects of organic manure application with chemical fertilizers on nutrient absorption and yield of rice in Hunan of Southern China. *Agricultural Sciences in China*, 2008, 7(10): 1245-1252 (doi: 10.1016/S1671-2927(08)60171-6).
74. Zheng J., Zhang X., Li L., Zhang P., Pan G. Effect of longterm fertilization on C mineralization and production of CH_4 and CO_2 under anaerobic incubation from bulk samples and particle size fractions of a typical paddy soil. *Agriculture, Ecosystems & Environment*, 2007, 120(2-4): 129-138 (doi: 10.1016/j.agee.2006.07.008).

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SELECTION AND SEED PRODUCTION OF VEGETABLE CROPS IN RUSSIA (the 100th anniversary of the Federal Scientific Vegetable Center)

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FEDERAL SCIENTIFIC VEGETABLE CENTER — 100-YEAR HISTORY AS A BASIS FOR FUTURE DEVELOPMENTS
(review)

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Abstract

The review presents the history of the Gribovskaya Vegetable Breeding Experimental Station, the first in Russia and the USSR for vegetable breeding and seed production, on the basis of which the All-Russian Research Institute of Vegetable Breeding and Seed Production was established, farther reorganized into the Federal Scientific Vegetable Center. The center's activity dates back to 1920, when, under the leadership of Sergei I. Zhegalov, a theoretical and practical basis for the development of domestic breeding was laid. The century-old anniversary of the selection of vegetable crops allows us to trace the way of its formation in Russia, successes and future development. Since 1920, scientists paid much attention to the development and improvement of breeding methods that increase the efficiency of selection, as well as to accelerate the selection process to create targeted varieties and hybrids. With regard to the main vegetable crops, methods have been developed for interspecific hybridization (N.I. Timin et al., 2013; A.F. Agafonov et al., 2018), molecular labeling (T.P. Suprunova et al., 2011; E.A. Domblides et al., 2015), clonal micropropagation and production of doubled haploids successfully used in breeding (M.S. Bunin et al., 2004). Basic protocols have been proposed for in vitro culture of microspore for most cabbage crops (E.A. Domblides et al., 2016) and non-pollinated ovules for *Cucurbitaceae* (N.A. Shmykova et al., 2015). A technology has been developed for the production of doubled haploids in carrots in in vitro cultures of anthers, non-pollinated ovules and microspores (T.S. Vjurtts et al., 2016). The economic benefit of modern biotechnological in vitro methods when creating hybrids has been proven: the time for creating hybrids is reduced from 12 to 6 years, financial costs are reduced 2 times (A. Mineykina et al., 2019; T. Vjurtts et al., 2019). The aggravated situation with plant diseases and the expansion of the areas of new harmful pathogens on vegetable crops are discussed. Based on immunological, molecular and morphophysiological tests at artificial, provocative and natural infections, the sources of resistance to economically significant diseases are identified, in cabbage to *Plasmodiophora brassicae*, in table beet to *Cercospora beticola*, in vegetable beans to viral diseases, in onions to *Peronospora destructor* (I.A. Engalycheva et al., 2019). Physiological and biochemical methods are widely used when creating varieties with a high content of biologically active substances and antioxidants. Technologies have been developed for obtaining functional food products, including new types of teas with a therapeutic and prophylactic effect, soft drinks, food dyes, and confectionery (M.S. Gins et al., 2017). Recipes for gluten-free bakery products have been created using introduced yacon, amaranth and daikon cultures. Technologies for selenium enrichment of vegetable crops for fresh consumption and as raw materials for functional products have been developed (N.A. Golubkina et al., 2018). The intellectual potential accumulated over a hundred-year history is inextricably linked with the traditions laid down at the experimental station. Nowadays the Federal Scientific Vegetable Center coordinates scientific research on the selection, production and processing of vegetable and melon crops in Russia within the framework of state programs for the development of the industry and ensuring food security.

Keywords: history, anniversary, research, varieties, vegetables, breeding, biotechnology, immunity, molecular marking, biochemistry, functional products

The century-old history of the Federal Scientific Vegetable Center began with the Gribovskaya Vegetable Experimental Station, organized on March 1, 1920 at the initiative of the People's Commissariat of Agriculture for the state production of garden seeds and eliminating the deficit resulting from the introduction of sanctions from a number of foreign countries. By 1920, the Russian catalog of zoned crops included 70 varieties of vegetable, of which 50 were registered for foreign companies, which prompted the solution to the problem and the creation of a nursery for varieties of garden plants. By 1970, scientists from the Gribovskaya Vegetable Experimental Station created 240 varieties of vegetables and melons used in practice. In Erfurt (Germany) in 1961 and 1969, varieties of Gribovskaya Vegetable Experimental Station received 18 gold, 13 silver and two bronze medals, 11 varieties of white cabbage (by E.M. Popova) were awarded the Grand Prix. By the decision of the State Committee of the Council of Ministers of the USSR for Science and Technology dated October 28, 1970 and by the order of the USSR Ministry of Agriculture dated November 23, 1970 No. 377, the Gribovskaya Vegetable Experimental Station was turned into the All-Union Research Institute of Vegetable Breeding and Seed Production (VNIISSOK), which became a methodological center of fundamental and applied research in this area, with special attention paid to the organization of seed production and raising a high-quality elite. In 2017, after joining VNIISSOK eight branches, the Federal Scientific Vegetable Center was created as the coordinator of consolidated research on breeding, production and processing of vegetables and melons in Russia within the framework of state programs for the development of the industry and ensuring food security.

The stages of development of domestic selection and seed production of vegetable crops can be traced through the history of the Gribovskaya station, methods of work, the creation of unique varieties.

To start with, foreign varieties were multiplied in order to provide the country with seed material, however, due to the lack of initial samples or their inconsistency with varietal requirements, it became necessary to create new domestic varieties of vegetables. In this, agronomic and morphological features, varietal characteristics and their correlations were studied. A method to reveal early ripening variants by determining the growth rate of cabbage head was developed based on the correlations revealed in white cabbage. Correlations between early maturity and the proximity of female flowers to cotyledon leaves allowed accelerated selection of pumpkin crops. Analysis of trait dominance in vegetable peas provided identification of appropriate parents for crossing and selection of hybrid forms with desired characteristics given trait dominance, expected segregation, and the economic value of the parents. The used selection methods were continuous mass selection, individual selection using the method of halves for pumpkin, family selection with assessment by offspring, pure-linear selection for legumes, group selection for solanaceous, negative selection for flower varieties, clonal selection in vegetatively propagated crops, e.g. tarragon, rhubarb, selection from the population. Refractometers were used in breeding for increased dry matter content in cell sap, calipers were used to measure fruit diameter, and a weight method was applied for determining plant productivity. As worldwide, induced mutagenesis and distant hybridization were conducted in order to enrich the gene pool of cultivated plants [1].

The first interspecific onion hybrids were obtained in the USA in the 1930s [2], and in Russia A.A. Krivenko made the first crosses in 1936 [1]. Crossing *Allium cepa* L. with perennial onion *species A. altaicum* Pall., *A. fistulosum* L., *A. vavilovii* Popov & Vved. generated original interspecific hybrids with high resistance to peronosporosis. For the first time in breeding and genetic investigation of interspecific hybridization in the genus *Allium* L., the fertile hybrids between di- and tetraploid species were created, *A. cepa* L. (×2) × *A. nutans* L. (×4) and *A. cepa* L. (×2) × *A. schoenoprasum* L. (×4) [3]. Interspecific onion hybrids were used to create varieties Sigma, Zolotye Kupola, and Tseparius with low damage caused by downy mildew and a high yield [4]. At present, studies of interspecific onion hybrids continue both in Russia and abroad [5, 6]. The world practice of onion breeding shows that the use of *A. roylei* Stearn, *A. galanthum* Kar. & Kir., *A. vavilovii* Popov & Vved. is advisable to obtain new forms resistant to downy mildew, neck rot [7] and *Fusarium oxysporum* f. sp. *cepae* [8].

The creation and characterization of interspecific hybrids involves cytogenetic studies, which were started in 1931 to obtain cabbage and tomato polyploids [1]. Currently, fluorescent genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) are used to determine the degree of proximity of samples and predict a successful distant hybridization [9, 10].

In addition to distant hybridization, intervarietal crosses were used. Selection of parents from geographically and ecologically remote regions was widely used for breeding pumpkin crops, e.g., the cucumber variety Izyashchnyi, melon variety Gruntovaya Gribovskaya 149, watermelon variety Gribovskiy dlinnopetistyi, pumpkin variety Gribovskaya zimnyaya [1]. In addition to common methods of selection and pair-crosses, complex stepped backcrosses are currently used with an emphasis on the female type of flowering and selection under infectious load. Cucumber (*Cucumis sativus* L.) varieties and hybrids with group resistance to four or five diseases and adaptability to abiotic environmental factors, e.g. the Aquarius, Electron 2, Unity, F_1 Debyut, F_1 Krepysh, F_1 Bryunet, F_1 Krasotka, bush variety Korotyshka, etc., are widely grown in field conditions [11]. Unique varieties and hybrids of large-fruited pumpkin (*Cucurbita maxima* Duchesne) have been created, e.g. ultra-early maturing variety Vesnushka, early maturing varieties Ulybka, Konfetka, Olga with splendid taste quality; mid-season fruitful variety Rossiyanka; late ripening varieties Premiera, Gribovskaya zimnyaya and Moskvichka with a high content of dry matter and sugars. The last three varieties create a continuous consumption conveyor, and even in the conditions of the Moscow region can be grown by sowing seeds in open ground at the end of May [12].

New complex crossbreeding schemes involving varieties of local breeding and geographically distant foreign origin have been successfully used for legumes. In 1949, from the hybrid population of vegetable peas (*Pisum sativum* L.), a form with all leaflets transformed into tendrils was selected for the first time, and cultivar Usatyi 5 was created, which was involved in creation of the initial material and varieties resistant to lodging. Based on the developed methods for the selection of parents, modern pea varieties with an optimal combination of productive parameters were obtained, e.g., with canned green seeds, characterized by high yields, top-positioned beans and a slow transition of sugar into starch (varieties Sovinter 1, Fragment, Izumrud, Darunok, Viking, Barin, Korsar), and sugar peas without a parchment layer in the bean shells for fresh consumption (Neistoshchimyi 195, Sakhaenyi 2, Gigant) [13]. In addition, high-quality varieties of *Phaseolus vulgaris* L. Zolushka, Pagoda, Lika, Mriya, Antoshka, Svetlyachok have been created, as well as *Vicia faba* L. var. *major* Harz variety Russkie belye with light seeds, high protein content, resistance to diseases, and suitable for

mechanized cultivation technologies have been created [15].

Back in the 1930s, breeders were tasked with creating annual onion varieties throw sowing seeds in the ground. This task remains relevant nowadays. For growing from seeds, high-yielding varieties have been created that can form a harvest of marketable bulbs not only in the southern regions, but also in the Non-Black Earth Zone of Russia. These varieties are Chernyi Prints, Globus, Zolotnichok, Zolotye Kupola, Kolobok, Patryda, and Vermeles [16]. Intensive breeding is underway to create heterotic hybrids based on cytoplasmic male sterility (CMS). Using sterile lines, a number of heterotic hybrids have been created, including F_1 Vizit possessing high marketability, maturity, bulb yield, and disease resistance [17].

Hybridization method were also used to create cosmopolitan cabbage varieties Iyun'skaya 3200, Podarok 2500, Zimnyaya Gribovskaya 2176, Slava Gribovskaya 231, Slava 1305, Nomer pervyi Gribovskiy 147, Stakhanovka 1315, Amager 611 [1]. Almost the entire assortment of *Brassica oleracea* L. convar. *capitata* (L.) Alef. var. *alba* DC from the Gribovskaya station and the All-Russian Research Institute of vegetable breeding and seed production (VNIISSOK), zoned more than half a century ago, comprises a unique gene pool for creation of new heterotic hybrids and varieties. Thus, heterotic hybrids F_1 Aurora, F_1 Snezhinka, F_1 Zarnitsa, F_1 Mechta, F_1 Severyanka were obtained, which make a conveyor of fresh products for consumers [18].

At the dawn of the selection of table root crops, free pollination was the main method of hybridization. Using polycross-pollination of a group of samples, varieties of table beet (*Beta vulgaris* L. ssp. *vulgaris* var. *conditiva* Alef.) Bordo 237, carrots (*Daucus carota* L.) Nantes 4, radish (*Raphanus sativus* L. var. *sativus*) Teplichnyi Gribovsky and Soffit were produced [1]. By the method of paired crosses, i.e. a kind of free pollination of two parents, direct and reverse, the carrot variety Moscovskaya zimnyaya A-515 was obtained. Intra-family crossing and crossbreeding within groups were used to obtain monogerm forms of table beet (Bordo odnosemyannaya, Lyubava, Gaspadynya). In recent years, inbreeding, backcrossing and crossbreeding have become the main methods for creating lines. The sib cross method is used to overcome inbred depression [19].

Over time, the requirements for the varieties have changed. The need arose to obtain varieties and hybrids with modified biological properties. The scope of the study was biochemical features and chemical composition of plants, e.g. concentration of carbohydrates in green peas, protein in vegetable beans, ascorbic acid in large-leaved sorrel *Rumex acetosa* L., watercress *Lepidium sativum* L., in the leaves of Brussel cabbage *Brassica oleracea* L. var. *gemmifera* Zenker and Savoy cabbage *Brassica oleracea* L. convar. *capitata* (L.) Alef. var. *sabauda* L. [20]. Adverse conditions due to technogenic factors create a special need for these products, since vegetables are considered as a necessary product for normal human life. In recent years, functional food products containing components that have a positive effect on the physiological functions of a person have become widespread in Japan and the EU countries [21-23].

The world market of functional products is intensively developing, annually increasing by 15-20%, which reflects the modern trend to healthy and balanced nutrition. Japan remains the leader in the functional food market with about 40% of the global product, the second place is occupied by the United States with slightly more than 30%, and the share of European countries is less than 30% [24, 25]. The creation of functional food products is focused on obtaining varieties with an increased content of carotenoids, flavonoids and other bioactive components [26-28].

In Russia, breeding for a high content of biologically active compounds,

including antioxidants and micronutrients, is also being successfully carried out. The required product quality is achieved through regular assessment of biochemical parameters during breeding [29]. These studies result in functional products aimed to boost immunity and to increase human life expectancy. Technologies have been developed for the production of new types of tea with therapeutic and prophylactic properties, soft drinks, food dyes and confectionery products, including for diabetics. Formulations of gluten-free bakery products have been created using yacon, amaranth and daikon varieties [30]. Technologies for enriching vegetable crops with selenium for fresh consumption and use as raw materials for functional products have been developed [31-34].

Research on immunity and plant protection has always been a priority. The study began with the most harmful pathogens and the methodology of the assessment of plant resistance [1]. Nowadays, the plant immunity and protection are acquiring special relevance. In the world, millions of tons of vegetable products are annually lost due to various epiphytoties [35]. Monitoring of the pathogenic complex on agricultural crops over the past 10-15 years indicates an expansion of the areas of new harmful pathogens, a change in abundance populations of plant pathogens, the nature and size of the ecological niches they occupy, a change in dominant species in communities, an increase in virulence. and aggressiveness of previously low-pathogenic organisms [36, 37].

The population changes are largely associated with environmental factors that affect relationships in the pathogen—plant system [38, 39]. Fungal pathogens *Sclerotinia nivalis*, *Gleocladium roseum*, *Trichotecium roseum*, *Chaetomium* spp., *Typhula ishikariensis* were for the first time discovered on the roots of canteen carrots during storage. An increase in the prevalence and aggressiveness of *Pectobacterium carotovora* was detected. In recent years, the species composition of micromycetes of the genus *Fusarium* has expanded on vegetable crops in the Moscow region. Many of these causative agents of fusarium rot and wilting are thermophilic species and have not previously been found in this region. *F. oxysporum*, *F. avenacium*, *F. nivale*, *F. chlamidosporum*, *F. solani*, *F. culmorum*, and *F. semitectum* have been identified on winter garlic; *F. chlamidosporum*, *F. equiseti*, *F. proliferatum* were found on dining carrots [40]. *Aspergillus niger*, a new pathogen for the Central Russia identified on onions causes black mold by the end of the growing season and during storage [41, 42]. *Typhula ishikariensis* was isolated from beet roots, and *Drechslera* Bondartseva from radish seeds [43].

Recently, the epiphytoties of phytoviruses are mainly due to more aggressive new strains, the cultivation of varieties with poor resistance, uncontrolled trade in planting and seed material, the emergence of new vectors and insufficient combating them [44, 45]. In recent years, the harmfulness of phytoviruses for vegetable crops has been increasing, which leads to a decrease in the productivity and quality of vegetable crops. Therefore, the identification and study of viruses and viral diseases on vegetable crops remain relevant in immunological research.

On lettuce plants (*Lactuca sativa* L.), harmful infections of *Lettuce mosaic virus* (LMV, *Potyvirus*, *Potyviridae*) and *Tomato aspermy virus* (AsTV, *Cucumovirus*, *Bromoviridae*) was identified [46]. In the Moscow region, *Bean common mosaic virus* (BCMV, *Potyvirus*, *Potyviridae*), *Bean yellow mosaic virus* (BYMV, *Potyvirus*, *Potyviridae*), and *Pea mosaic virus* (PMV, *Potyvirus*, *Potyviridae*) cause great damage to *Fabaceae* crops (beans, sweet peas) [47]. Immunodiagnostic methods detected the most harmful and economically important *Tobacco mosaic virus* (TMV, *Tobamovirus*, *Virgaviridae*), *Tomato spotted wilt virus* (TSWV, *Tospovirus*, *Bunyaviridae*), *Cucumber mosaic virus* (CMV, *Cucumovirus*, *Bromoviridae*), *Potato virus X* (PVX, *Potexvirus*, *Alphaflexiviridae*), *Potato virus Y* (PVY,

Potyvirus, *Potyviridae*), and *Alfalfa mosaic virus* (AMV, *Alfamovirus*, *Bromoviridae*) infecting *Solanaceae* plants (sweet pepper and tomato) [48, 49]. The methods used to isolate and select disease-resistant forms are constantly being modified depending on the biological characteristics of host plants and phytopathogens within the framework of targeted breeding. Particular attention is paid to the development of express methods of detection at early stages of plant development, in seeds and seedlings. The use of etiolated and photosynthetic seedlings allows screening of a wide range of genetically diverse samples [40, 50, 51]. A methodical approach is suggested to assessing the resistance of white cabbage to *Xanthomonas campestris* pv. *campestris* using etiolated and photosynthetic seedlings. The influence of this pathogen on plant growth were studied depending on the race composition of the pathogen and the cultivar resistance [52].

Immunological, molecular, and morphophysiological assessments of the collection and breeding material upon artificial infection, provocative conditions and natural infection revealed the sources of resistance to economically significant diseases, e.g., in cabbage to keel [53], in beetroot to cercosporosis [54], in vegetable beans to viral diseases [47], and onions to downy mildew [4].

Wide experimental network in various geographic zones (Russia, Ukraine, Turkmenistan, Uzbekistan, Azerbaijan) provide conditions to reproduce high-quality elite seeds in various soil and climatic conditions. Since the 1970s, ecological studies have been aimed at increasing the role of the variety in the genotype-environment system, which has become the main method for obtaining plastic and high adaptive varieties [55]. Mechanized technologies for seed production, small-scale mechanization, methods of economic evaluation in seed production and plant breeding were developed. The range of crops expanded to involve green, spicy-flavoring and less widespread crops (110 items in total).

Nowadays, the expansion of the spectrum of genetic resources and the enhancement of the morphogenesis are the most important to obtain a fundamentally new source material. At the Federal Scientific Vegetable Center (FSVC), a rich indicative collection has been created, numbering more than 16 thousand accessions of 120 cultures. In 2017, the collection was registered as USI (unique scientific installation) Genetic collection of plant resources VNIISSOK. In the collection, there are varieties of vegetables, melons and flowers, breeding forms used as genetic sources and donors of selectively valuable traits, as well as folk varieties.

To speed up the breeding, the development and application of innovative methods is of no small importance. Since the late 1980s, in vitro tissue and cell culture technologies has been actively using by the FRCVG researchers. First studies were aimed at obtaining a virus-free planting material for garlic in a meristem culture. A.V. Polyakov and colleagues found that the use of air bulbs isolated from unopened inflorescences up to 25 mm in diameter to produce winter garlic in vitro culture makes it possible to obtain plants free from internal infection [56]. The technology of clonal micropropagation of white cabbage was developed to unlimitedly obtain plants with male sterility [57]. The developed technology of clonal micropropagation of eggplant (*Solanum melongena* L.) and pepper (*Capsicum annuum* L.) [58] formed the basis of embryoculture for the rescue of embryos during interspecific hybridization [59].

Since the discovery of the first haploids in 1922 [60], many geneticists and plant breeders have become interested in using such haploids to obtain homozygous lines. To date, almost 300 varieties of agricultural crops have been created in world practice via haploid biotechnology. The list of species in which haploids and doubled haploids (DH) are obtained is constantly lengthening, new review publications on haploidy appear, genetic and fundamental studies are car-

ried out on DH lines, new varieties and hybrids are created based on lines of doubled haploids, including vegetable crops [61-65]. At VNIISSOK, the methods of haploidy began to develop in the 1990s on carrots and white-headed cabbage. As a result, doubled haploids of carrot varieties of various origins were obtained, e.g. NIIOH 336, Vitaminnaya, Moskovskaya Zimnyaya A-515, Losinoostrovskaya 13, Leandr, Shantane 2461, Nape, Rondo, hybrids F_1 Karatan, F_1 Calisto [66].

The developed innovative biotechnologies make it possible to significantly accelerate breeding for most vegetable crops. The basic protocol for doubled haploid technology based on the in vitro culture of isolated microspores was developed in the early 1980s [67] for rapeseed and adapted for genus *Brassica* [68, 69]. A success of the protocol is reported by scientists from India [70], Canada [71], Czech Republic [72]. A basic protocol for in vitro microspore culture for cabbage crops has been developed at the FRCVG [73], and doubled haploids have been obtained, including white cabbage [74, 75], broccoli cabbage *Brassica oleracea* L. convar. *botrytis* (L.) Alef. var. *cymosa* Duch. [76, 77], turnip [78], purple cabbage *Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *purpuraria* (L.H. Bailey) Hanelt [79], Sarepta mustard *Brassica juncea* (L.) Czern., Indau *Eruca sativa* Mil. [80], and even the most unresponsive culture in this family, the European radish [81].

Research on generating carrot doubled haploids has been going on for a long time, but the first successes have been achieved recently. Back in 1995, the formation of multinucleated structures in the culture of isolated carrot microspores was reported for the first time, but no plants were obtained [82]. There are publications on the use of this approach by Polish [83] and Chinese [84] researchers. At the FRCVG, a technology was developed for the production of doubled haploids of table carrots in in vitro cultures of anther, non-pollinated ovules and microspores, resulting in doubled haploids for eight varieties [85]. Calculation of the cost for pure lines of white cabbage [86] and table carrots [87] proved the economic benefit of the method of isolated microspores in vitro when creating hybrids. The time to obtain hybrids is reduced from 12 to 6 years, and financial costs are halved.

Experiments are underway to optimize production of doubled haploids in in vitro culture of non-pollinated ovules for pumpkin crops. DH plants of large-fruited pumpkin have been obtained [88]. Considerable progress has been achieved for homozygous zucchini [89] and cucumber [90] lines. The basic protocol for production of doubled haploids of pumpkin crops via in vitro culture of non-pollinated ovules was developed in the second half of the 20th century but patented only in 2017 [91]. Over time, it was adapted for various members of *Cucurbitaceae* family [92, 93], in particular for squash (*Cucurbita pepo* L.), large-fruited and hard pumpkin [94], and cucumber [95]. It was shown that the in vitro non-pollinated ovule-based doubled haploid technologies for pumpkin crops developed at the FRCVG are more effective compared to foreign analogues, since the number of regenerants per ovary was greater than indicated in foreign publications. For the first time, the formation of ugly abnormal flowers was discovered in the progeny of DH lines derived from in vitro culture of non-pollinated ovules [96], which is of interest for genetic studies of sex determinants in *Cucurbita pepo* L. Cytological analysis of R_0 regenerant plants showed that 7% were haploids, about 20% were mixoploids, and the rest were doubled haploids ($2n = 2\times = 40$). For the first time, micrographs were obtained of the chromosomes of the squash *C. pepo* subsp. *brevicaulis* var. *giraumons* Duch, its distant

hybrid with *C. pepo* subsp. *pepo* var. *pepo*, and their doubled haploids [97].

At the FRCVG, the molecular marker technologies have been actively developing since the 1990s [98]. At present, molecular markers are the main method to produce CMS-based hybrids of vegetable crops. A system has been developed for DNA identification of all types of cytoplasm in cabbage crops with a new allelic variant of the *orf138* locus responsible for sterile cytoplasm of the Ogura type in white cabbage [99].

The mitochondrial gene*s coxII* and a*tp6* responsible for the CMS, have been identified in sweet pepper and interspecific hybrids of *Capsicum frutescens* and *C. chinense*, which makes it possible to identify samples with sterile and fertile cytoplasm [100]. Samples of onions with mitochondrial genes *orfA501* and *cob* were identified, and the type of sterile cytoplasm (S- or T-plasmotype) was determined [101]. Additional markers were used for detection of cytoplasmic gene *orf725* and nuclear genes to more fully assess the initial material of onion, which revealed the samples suitable for hybridization [102].

Using modern breeding methods, heterosis hybrids of pepper F_1 Natali, F_1 Gusar [100], medium late cabbage F_1 Natali [74], large-fruited pumpkin F_1 Vega [103], and broccoli cabbage F_1 Sparta [104] were created.

Thus, the breeding of vegetable crops in Russia went through several stages: introduction, the use of various methods of selection among local and foreign populations, the production of new varieties by crossing species and genera, the use of biotechnology and molecular markers to quickly achieve the final result. A century after its founding, the Federal Scientific Vegetable Center remains the leader in the Russian Federation in creating vegetable varieties and hybrids for open ground and greenhouses, hydroponic and aeroponic installations in combination with growing technologies, fertilization and plant protection protocols in line with the world trends. Innovative solutions are being developed for obtaining functional food products. The Federal Scientific Vegetable Center coordinates research on the breeding, production and processing of vegetable and melon crops in Russia within the framework of state programs for the development of the industry and ensuring food security.

REFERENCES

1. *Selektsiya i semenovodstvo ovoshchnykh kul'tur na Gribovskoi opytnoi stantsii za 50 let* /Pod redaktsiei Z.V. Kuptsovoi [Selection and seed production of vegetable crops at the Gribovskaya experimental station for 50 years. Z.V. Kuptsova (ed.)]. Moscow, 1970 (in Russ.).
2. Emsweller S.L., Jones H.A. An interspecific hybrid in *Allium* L. *Hitgardis*, 1935, 5(9): 265-273.
3. Timin N.I., Pyshnaya O.N., Agafonov A.F., Mamedov M.I., Titova I.V., Kan L.Yu., Logunova V.V., Romanov V.S., Shmykova N.A., Timina L.T., Gurkina L.K., Dzhos E.A., Suprunova T.P., Krivosheev S.M., Engalycheva I.A. *Mezhvidovaya gibridizatsiya ovoshchnykh rastenii (Allium L. — luk, Daucus L. — morkov', Capsicum L. — perets)* /Pod redaktsiei V.F. Pivovarova [Interspecific hybridization of vegetable plants (*Allium* L. — onion, *Daucus* L. — carrot, *Capsicum* L. — pepper). V.F. Pivovarov (ed.)]. Moscow, 2013 (in Russ.).
4. Agafonov A.F., Logunova V.V., Gurkina L.K. *Vegetable crops of Russia*, 2018, 4(42): 3-5 (doi: 10.18619/2072-9146-2018-4-3-5) (in Russ.).
5. Umehara M., Sueyoshi T., Shimomura K., Iwai M., Shigyo M., Hirashima K., Nakahara T. Interspecific hybrids between *Allium fistulosum* and *Allium schoenoprasum* reveal carotene-rich phenotype. *Euphytica*, 2006, 148(3): 295-301 (doi: 10.1007/s10681-005-9029-8).
6. Hirschegger P., Jakše J., Trontelj P., Bohanec B. Origins of *Allium ampeloprasum* horticultural groups and a molecular phylogeny of the section *Allium (Allium: Alliaceae*). *Molecular Phylogenetics and Evolution*, 2010, 54(2): 488-497 (doi: 10.1016/j.ympev.2009.08.030).
7. Scholten O.E., van Kaauwen M.P.W., Shahin A., Hendrickx P.M., Keizer L.C.P., Burger K., van Heusden A.W., van der Linden C.G., Vosman B. SNP-markers in *Allium* species to facilitate introgression breeding in onion. *BMC Plant Biology*, 2016, 16(1): 187-196 (doi: 10.1186/s12870-016-0879-0).

8. Ariyanti N.A., Hoa V.Q., Khrustaleva L.I., Hirata S., Abdelrahman M., Ito S., Yamauchi N., Shigyo M. Production and characterization of alien chromosome addition lines in *Allium fistulosum* carrying extra chromosomes of *Allium roylei* using molecular and cytogenetic analyses. *Euphytica*, 2015, 206(2): 343-355 (doi: 10.1007/s10681-015-1476-2).
9. Kan L.Yu. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii, posvyashchennoi 131-oi godovshchine so dnya rozhdeniya akademika N.I. Vavilova «Vavilovskie chteniya — 2018»* [Proc. Int. Conf. «Vavilov Readings-2018»]. Moscow, 2018: 59-63 (in Russ.).
10. Suzuki G., Ogaki Y., Hokimoto N., Xiao L., Kikuchi-Taura A., Harada C., Okayama R., Tsuru A., Onishi M., Saito N., Do G.S., Lee S.H., Ito T., Kanno A., Yamamoto M., Mukai Y. Random BAC FISH of monocot plants reveals differential distribution of repetitive DNA elements in small and large chromosome species. *Plant Cell Rep.*, 2012, 31(4): 621-628 (doi: 10.1007/s00299-011-1178-8).
11. Korottseva I.B. *Vegetable crops of Russia,* 2015, 3(28): 54-57 (doi: 10.18619/2072-9146-2015-3-4-54-57) (in Russ.).
12. Khimich G.A., Korottseva I.B., Gins M.S., Gins V.K., Baikov A.A. *Novye i netraditsionnye rasteniya i perspektivy ikh ispol'zovaniya*, 2016, 12: 271-273 (in Russ.).
13. Kotlyar I.P., Ushakov V.A., Kaigorodova I.M., Pronina E.P. *Vegetable crops of Russia*, 2019, 2(46): 34-38 (doi: 10.18619/2072-9146-2019-2-34-38) (in Russ.).
14. Pivovarov V.F., Pronina E.P. *Vegetable crops of Russia,* 2013, 1(18): 4-11 (doi: 10.18619/2072-9146-2013-1-4-11) (in Russ.).
15. Pronina E.P., Ushakov V.A., Kotlyar I.P., Soldatenko A.V. *Vegetable crops of Russia*, 2019, 6: 50-52 (doi: 10.18619/2072-9146-2019-6-50-52) (in Russ.).
16. SHimanskii L.P., Kopylovich V.L., Sikorskii A.V., Sirota S.M., Agafonov A.F., Pronina E.P. *Vegetable crops of Russia*, 2014, 4(25): 23-27 (doi: 10.18619/2072-9146-2014-4-23-27) (in Russ.).
17. Logunova V.V., Krivenkov L.V., Gurkina L.K., Grashchenkova N.N. *Izvestiya FNTSO*, 2019, 2: 45-49 (doi: 10.18619/2658-4832-2019-2-45-49) (in Russ.).
18. Bondareva L.L. *Vegetable crops of Russia*, 2017, 1(34): 22-23 (doi: 10.18619/2072-9146-2017-1-22-23) (in Russ.).
19. Fedorova M.I., Stepanov V.A. *Vegetable crops of Russia*, 2017, 4(37): 16-22 (doi: 10.18619/2072-9146-2017-4-16-22) (in Russ.).
20. Gins M.S., Gins V.K. *Fiziologo-biokhimicheskie osnovy introduktsii i selektsii ovoshchnykh kul'tur* [Physiological and biochemical bases of the introduction and selection of vegetable crops]. Moscow, 2011 (in Russ.).
21. Hasler C.M., Bloch A.S., Thomson C.A., Enrione E., Manning C. Position of the American dietetic association: functional foods. *Journal of the American Dietetic Association*, 2004, 104(5): 814-826 (doi: 10.1016/j.jada.2004.03.015).
22. Martirosyan D.M. *Functional foods and chronic diseases: science and practice (volume 8)*. Oxford, 2011.
23. Lewandowska U., Szewczyk K., Hrabec E., Janecka A., Gorlach S. Overview of metabolism and bioavailability enhancement of polyphenols. *J. Agric. Food Chem.*, 2013, 61(50): 12183-12199 (doi: 10.1021/jf404439b).
24. European Commission. *Functional foods.* Brussels, 2010 (doi: 10.2777/82512).
25. Mudry J. Functional foods, marketing of. In: *Encyclopedia of food and agricultural ethics.* P.B. Thompson, D.M. Kaplan (eds.). Springer, Dordrecht, 2014 (doi: 10.1007/978-94-007-0929-4_417).
26. Woo K.S., Hwang I.G., Kim T.M., Kim D.J., Hong J.T., Jeon H.S. Changes in the antioxidant activity of onion (*Allium cepa*) extracts with heat treatment. *Food Sci. Biotechnol.*, 2007, 16(5): 828-831.
27. Ficco D.B.M., de Simone V., Colecchia S.A., Pecorella I., Platani C., Nigro F., Finocchiaro F., Papa R., de Vita P. Genetic variability in anthocyanin composition and nutritional properties of blue, purple, and red bread (*Triticum aestivum* L.) and durum (*Triticum turgidum* L. ssp. *turgidum* convar. *durum*) wheats. *J. Agric. Food Chem.*, 2014, 62(34): 8686-8695 (doi: 10.1021/jf5003683).
28. Zhang Z., Lei M., Liu R., Gao Y., Xu M., Zhang M. Evaluation of alliin, saccharide contents and antioxidant activities of black garlic during thermal processing. *Journal of Food Biochemistry*, 2015, 39(1): 39-47 (doi: 10.1111/jfbc.12102).
29. Adzhieva V.F., Babak O.G., Shoeva O.Y., Kilchevsky A.V., Khlestkina E.K. Molecular genetic mechanisms of the development of fruit and seed coloration in plants. *Russian Journal of Genetics: Applied Research*, 2016, 6(5): 537-552 (doi: 10.1134/S2079059716050026).
30. Gins M.S., Gins V.K., Kononkov P.F. *Vestnik Rossiiskoi sel'skokhozyaistvennoi nauki*, 2016, 2: 55-58 (in Russ.).
31. Gins M.S., Romanova E.V., Plyushchikov V.G., Gins V.K., Pivovarov V.F. *Funktsional'nye produkty pitaniya iz rastitel'nogo syr'ya* [Functional plant-based foods]. Moscow, 2017 (in Russ.).

32. Kryachko T.I., Malkina V.D., Martirosyan V.V., Smirnova S.A., Golubkina N.A., Bondareva L.L. *Izvestiya vysshikh uchebnykh zavedenii. Pishchevaya tekhnologiya*, 2019, 1(367): 22-26 (in Russ.).
33. Malkina V.D., Kryachko T.I., Martirosyan V.V., Golubkina N.A., Seredin T.M., Pavlov L.V. *Konditerskoe i khlebopekarnoe proizvodstvo*, 2019, 3-4(180): 31-35 (in Russ.).
34. Golubkina N.A., Seredin T.M., Koshevarov A.A., Shilo L.M., Baranova E.V., Pavlov L.V. *Mikroelementy v meditsine*, 2018, 19(1): 43-50 (doi: 10.19112/2413-6174-2018-19-1-43-50) (in Russ.).
35. Pethybridge S.J., Kikker J.R., Hanson L.E., Nelson S.C. Challenges and prospects for building resilient disease management strategies and tactics for the New York table beet industry. *Agronomy*, 2018, 8(7): 112 (doi: 10.3390/agronomy8070112).
36. West J.S., Townsend J.A., Stevens M., Fitt B.D.L. Comparative biology of different plant pathogens to estimate effects of climate change on crop diseases in Europe. *European Journal of Plant Pathology*, 2012, 133(1): 315-331 (doi: 10.1007/s10658-011-9932-x).
37. Sanin S.S. V sbornike: *Plodovodstvo i yagodovodstvo Rossii* [In: Fruit and berry growing in Russia]. Moscow, 2015, Iss. 43: 178-183 (in Russ.).
38. Velasquez A.C., Castroverde C.D.M., He S.Y. Plant—pathogen warfare under changing climate conditions. *Current Biology*, 2018, 28(10): R619-R634 (doi: 10.1016/j.cub.2018.03.054).
39. Ryabushkina N.A. *Biotekhnologiya. Teoriya i praktika*, 2005, 5: 5-15 (in Russ.).
40. Chen J., Shang Y.-T., Wang W.-H. Chen X.-Y., He E.-M., Zheng H.-L., Shangguan Z. Hydrogen sulfide-mediated polyamines and sugar changes are involved in hydrogen sulfide-induced drought tolerance in *Spinacia oleracea* seedlings. *Frontiers in Plant Science*, 2016, 7: 1173 (doi: 10.3389/fpls.2016.01173).
41. Timina L.T., Engalycheva I.A. *Selektsiya i semenovodstvo ovoshchnykh kul'tur*, 2014, 45: 530-539 (in Russ.).
42. Agafonov A.F., Timina L.T., Shestakova K.S. *Vegetable crops of Russia*, 2012, 3(16): 48-51 (in Russ.).
43. Nayuolu M.V. *Plant viruses.* India, 2007.
44. Makkouk K.M., Kumari S.G. Epidemiology and integrated management of persistently transmitted aphid-borne viruses of legume and cereal crops in West Asia and North Africa. *Virus Research*, 2009, 141(2): 209-218 (doi: 10.1016/j.virusres.2008.12.007).
45. Hampton R.O., Jensen A., Hagel G.T. Attributes of bean yellow mosaic potyvirus transmission from clover to snap beans by four species of aphids (*Homoptera*: *Aphididae*). *Journal of Economic Entomology*, 2005, 98(6): 1816-1823 (doi: 10.1603/0022-0493-98.6.1816).
46. Engalycheva I.A., Pavlova O.V. *Vestnik zashchity rastenii*, 2016, 3(89): 68-70 (in Russ.).
47. Engalycheva I.A., Kozar' E.G. *Agrarnaya nauka*, 2019, S3: 79-85 (doi: 10.32634/0869-8155-2019-326-3-79-85) (in Russ.).
48. Almási A., Csilléry G., Csömör Z., Nemes K., Palkovics L., Salánki K., Tóbiás I. Phylogenetic analysis of Tomato spotted wilt virus (TSWV) NSs protein demonstrates the isolated emergence of resistance-breaking strains in pepper. *Virus Genes,* 2015, 50(1): 71-78 (doi: 10.1007/s11262-014-1131-3).
49. Scholthof K.B.G., Adkins S., Czosnek H., Palukaitis P., Jacquot E., Hohn T., Hohn B., Saunders K., Candresse T., Ahlquist P., Hemenway C., Foster G.D. Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology*, 2011, 12(9): 938-954 (doi: 10.1111/j.1364-3703.2011.00752.x).
50. Yu L., Zhang C., Shang H., Wang X., Wei M., Yang F., Shi Q. Exogenous hydrogen sulfide enhanced antioxidant capacity, amylase activities and salt tolerance of cucumber hypocotyls and radicles. *Journal of Integrative Agriculture*, 2013, 12(3): 445-456 (doi: 10.1016/S2095-3119(13)60245-2).
51. Janicka M., Reda M., Czyzewska K., Kabała K. Involvement of signalling molecules NO, H_2O_2 and H_2S in modification of plasma membrane proton pump in cucumber roots subjected to salt or low temperature stress. *Functional Plant Biology*, 2018, 45(4): 428-439 (doi: 10.1071/FP17095).
52. Ushakov A.A., Kozar' E.G., Engalycheva I.A. *Vegetable crops of Russia*, 2019, 6: 133-140 (doi: 10.18619/2072-9146-2019-6-133-140) (in Russ.).
53. Ushakov A.A., Bondareva L.L., Engalycheva I.A. *Vegetable crops of Russia*, 2018, 6(44): 97-100 (doi: 10.18619/2072-9146-2018-6-97-100) (in Russ.).
54. Kozar' E.G., Vetrova S.A., Engalycheva I.A., Fedorova M.I. *Vegetable crops of Russia*, 2019, 6: 124-132 (doi: 10.18619/2072-9146-2019-6-124-132) (in Russ.).
55. Pivovarov V.F., Dobrutskaya E.G. *Ekologicheskie osnovy selektsii i semenovodstva ovoshchnykh kul'tur* [Ecological bases of selection and seed production of vegetable crops]. Moscow, 2000 (in Russ.).
56. Polyakov A.V., Azopkova M.A., Lebedeva N.N., Murav'eva I.V. *Vestnik Moskovskogo gosudarstvennogo oblastnogo universiteta. Seriya: estestvennye nauki*, 2018, 4: 115-124 (doi: 10.18384/2310-7189-2018-4-115-124) (in Russ.).

57. Bunin M.S., Shmykova N.A. *Ispol'zovanie biotekhnologicheskikh metodov dlya polucheniya iskhodnogo selektsionnogo materiala kapusty* [The use of biotechnological methods to obtain the initial breeding material of cabbage]. Moscow, 2004 (in Russ.).
58. Verba V.M., Mamedov M.I., Pyshnaya O.N., Shmykova N.A. *Vestnik RASKHN*, 2010, 6: 57-59 (in Russ.).
59. Verba V.M., Mamedov M.I., Pyshnaya O.N., Suprunova T.P., Shmykova N.A. Isolation of eggplant interspecific hybrids by the method of embryo culture. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2010, 45(5): 66-71 (in Engl.).
60. Blakeslee A.F., Belling J., Farnham M.E., Bergner A.D. A haploid mutant in the jimson weed, «Datura stramonium». *Science*, 1922, 55(1433): 646-647 (doi: 10.1126/science.55.1433.646).
61. Maluszynski M., Kasha K.J., Szarejko I. Published doubled haploid protocols in plant species. In: *Doubled haploid production in crop plants.* M. Maluszynski, K.J. Kasha, B.P. Forster, I. Szarejko (eds.). Springer, Dordrecht, 2003: 309-335 (doi: 10.1007/978-94-017-1293-4_46).
62. Forster B.P., Heberle-Bors E., Kasha K.J., Touraev A. The resurgence of haploids in higher plants. *Trends in Plant Science*, 2007, 12(8): 368-375 (doi: 10.1016/j.tplants.2007.06.007).
63. *Advances in haploid production in higher plants.* A. Touraev, B.P. Forster, S.M. Jain (eds.). Netherlands, 2009 (doi: 10.1007/978-1-4020-8854-4).
64. Dunwell J.M. Haploids in flowering plants: origins and exploitation. *Plant Biotechnology Journal*, 2010, 8(4): 377-424 (doi: 10.1111/j.1467-7652.2009.00498.x).
65. Germanà M.A. Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Reports,* 2011, 30(5): 839-857 (doi: 10.1007/s00299-011-1061-7).
66. Tyukavin G.B. *Osnovy biotekhnologii morkovi* [Carrot biotechnology basics]. Moscow, 2007 (in Russ.).
67. Lichter R. Induction of haploid plants from isolated pollen of *Brassica napus. Zeitschrift fur Pflanzenphysiologie*, 1982, 105(5): 427-434 (doi: 10.1016/S0044-328X(82)80040-8).
68. Pechan P.M., Keller W.A. Identification of potentially embryogenic microspores in Brassica napus. *Physiologia Plantarum*, 2006, 74(2): 377-384 (doi: 10.1111/j.1399-3054.1988.tb00646.x).
69. Seo M., Sohn S., Park B., Ko H., Jin M. Efficiency of microspore embryogenesis in *Brassica rapa* using different genotypes and culture conditions. *Journal of Plant Biotechnology*, 2014, 41(3): 116-122 (doi: 10.5010/JPB.2014.41.3.116).
70. Reeta B., Dey S.S., Parkash C., Sharma K., Sood S., Kumar R. Modification of important factors for efficient microspore embryogenesis and doubled haploid production in field grown white cabbage (*Brassica oleracea* var. *capitata* L.) genotypes in India. *Scientia Horticulturae*, 2018, 233: 178-187 (doi: 10.1016/j.scienta.2018.01.017).
71. Ferrie A.M.R. Microspore culture of Brassica species. In: *Doubled haploid production in crop plants.* M. Maluszynski, K.J. Kasha, B.P. Forster, I. Szarejko (eds.). Springer, Dordrecht, 2003: 205-215 (doi: 10.1007/978-94-017-1293-4_31).
72. Smýkalová I., Větrovcová M., Klíma M., Macháčková M., Griga M. Efficiency of microspore culture for doubled haploid production in the breeding project «Czech Winter Rape». *Czech Journal of Genetics and Plant Breeding,* 2006, 42(2): 58-71 (doi: 10.17221/3655-CJGPB).
73. Domblides E.A., Shmykova N.A., Shumilina D.V., Zayachkovskaya T.V., Mineykina A.I., Kozar' E.V., Akhramenko V.A., Shevchenko L.L., Kan L.Yu., Bondareva L.L., Domblides A.S. *Tekhnologiya polucheniya udvoennykh gaploidov v kul'ture mikrospor semeistva kapustnye: metodicheskie rekomendatsii* [The technology for obtaining doubled haploids of *Brassicaceae* family in the culture of microspores: guidelines]. Moscow, 2016 (in Russ.).
74. Pivovarov V.F., Bondareva L.L., Shmykova N.A., Shumilina D.V., Mineykina A.I. New generation hybrids of white cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba* DC) based on doubled haploids. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 1(52): 143-151 (doi: 10.15389/agrobiology.2017.1.143eng).
75. Korottseva K.S., Domblides E.A., Domblides A.S. *Materialy konferentsii «Biotekhnologiya v rastenievodstve, zhivotnovodstve i sel'skokhozyaistvennoi mikrobiologii»* [Proc. Conf. "Biotechnology in crop production, animal husbandry and agricultural microbiology"]. Moscow, 2019, 17-19 (in Russ.).
76. Domblides E.A., Kozar' E.V., Shumilina D.V., Zayachkovskaya T.V., Akhramenko V.A., Soldatenko A.V. *Vegetable crops of Russia*, 2018, 1(39): 3-7 (doi: 10.18619/2072-9146-2018-1-3-7) (in Russ.).
77. Zablotskaya E.A., Bondareva L.L., Sirota S.M. *Vegetable crops of Russia*, 2018, 1(39): 8-11 (doi: 10.18619/2072-9146-2018-1-8-11) (in Russ.).
78. Shumilina D., Kornyukhin D., Domblides E., Soldatenko A., Artemyeva A. Impact of genotype and culture conditions on microspore embryogenesis and plant regeneration in *Brassica rapa* L. ssp. *rapa*. *Plants*, 2020, 9(2): 278 (doi: 10.3390/plants9020278).
79. Kozar' E.V., Korottseva K.S., Romanova O.V., Chichvarina O.A., Kan L.Yu., Akhramenko V.A., Domblides E.A. *Vegetable crops of Russia*, 2019, (6): 3-7 (doi: 10.18619/2072-9146-2019-6-10-18) (in Russ.).
80. Domblides E.A., Chichvarina O.A., Mineykina A.I., Kurbakov E.L., Kharchenko V.A.,

Domblides A.S., Soldatenko A.V. *Vegetable crops of Russia*, 2019, 4: 8-12 (doi: 10.18619/2072-9146-2019-4-8-12) (in Russ.).
81. Kozar' E.V., Domblides E.A., Soldatenko A.V. *Vavilovskii zhurnal genetiki i selektsii*, 2020, 24(1): 31-39 (doi: 10.18699/VJ20.592) (in Russ.).
82. Matsubara S., Dohya N., Murakami K. Callus formation and regeneration of adventitious embryos from carrot, fennel and mitsuba microspores by anther and isolated microspore cultures. *Acta Horticulturae*, 1995, 392: 129-138 (doi: 10.17660/ActaHortic.1995.392.15).
83. Kiszczak W., Kowalska U., Kapuścińska A., Burian M., Górecka K. Comparison of methods for obtaining doubled haploids of carrot. *Acta Societatis Botanicorum Poloniae*, 2017, 86(2): 3547 (doi: 10.5586/asbp.3547).
84. Li J.-R., Zhuang F.-Y., Ou C.-G., Hu H., Zhao Z.-W., Mao J.-H. Microspore embryogenesis and production of haploid and doubled haploid plants in carrot (*Daucus carota* L.). *Plant Cell Tiss. Organ Cult.*, 2013, 112: 275-287 (doi: 10.1007/s11240-012-0235-5).
85. Vyurtts T.S., Shmykova N.A., Fedorova M.I., Zayachkovskaya T.V., Domblides E.A. *Vestnik zashchity rastenii,* 2016, 3(89): 43-44 (in Russ.).
86. Mineikina A., Bondareva L., Domblides E. The economic benefits of the production of double haploid for selection of white cabbage. *IOP Conference Series: Earth and Environmental Science*, 2019, 395: 012081 (doi: 10.1088/1755-1315/395/1/012081).
87. Vjurtts T., Domblides E., Soldatenko A. Economic efficiency of obtaining carrot lines using classical and biotechnological methods. *IOP Conference Series: Earth and Environmental Science*, 2019, 395: 012084 (doi: 10.1088/1755-1315/395/1/012084).
88. Shmykova N.A., Khimich G.A., Korottseva I.B., Domblides E.A. *Vegetable crops of Russia*, 2015, 3(28): 28-31 (doi: 10.18619/2072-9146-2015-3-4-28-31) (in Russ.).
89. Domblides E.A., Shmykova N.A., Zayachkovskaya T.V., Khimich G.A., Korottseva I.B., Kan L.Yu., Domblides A.S. V sbornike: *Biotekhnologiya kak instrument sokhraneniya bioraznoobraziya rastitel'nogo mira (fiziologo-biokhimicheskie, embriologicheskie, geneticheskie i pravovye aspekty)* [Biotechnology as a tool for preserving the biodiversity of the plant world (physiological, biochemical, embryological, genetic and legal aspects)]. Simferopol', 2016: 28-29 (in Russ.).
90. Domblides EA., Shmykova N.A., Belov S.N., Korottseva I.B., Soldatenko A.V. *Vegetable crops of Russia*, 2019, 6: 3-9 (doi: 10.18619/2072-9146-2019-6-3-9) (in Russ.).
91. Chen J., Vanek E., Pieper M. *Method for producing haploid, dihaploid and doubled haploid plants by isolated microspore culture. A01H 1/00 (2006.01)*. Application filed by Vilmorin and Cie (FR). № PCT/EP20 16/067825. Priority data 26.07.2016. Publication 02.02.2017 WO 2017/017108A1.
92. Lofti M., Alan A.R., Henning M.J., Jahn M.M., Earle E.D. Production of haploid and double haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. *Plant Cell Rep.*, 2003, 21(11): 1121-1128 (doi: 10.1007/s00299-003-0636-3).
93. Baktemur G., Taşkın H., Buyukalaca S. Comparison of different methods for separation of haploid embryo induced through irradiated pollen and their economic analysis in melon (*Cucumis melo* var. *inodorus*). *The Scientific World Journal*, 2013, 10: 529502 (doi: 10.1155/2013/529502).
94. Galązka J., Niemirowicz-Szczytt K. Review of research on haploid production in cucumber and other cucurbits. *Folia Horticulturae*, 2013, 25(1): 67-78 (doi: 10.2478/fhort-2013-0008).
95. Galazka J., Slomnicka R., Goral-Radziszewska K., Niemirowicz-Szczytt K. From pollination to DH-lines — verification and optimization of protocol for production of double haploids in cucumber. *Acta Scientiarum Polonorum Hortorum Cultus*, 2015, 14(3): 81-92.
96. Domblides E.A., Shmykova N.A., Khimich G.A., Korottseva I.B., Domblides A.S. *Vegetable crops of Russia*, 2018, 5(43): 13-17 (doi: 10.18619/2072-9146-2018-5-13-17) (in Russ.).
97. Domblides E.A., Kan L.Yu., Khimich G.A., Korottseva I.B., Domblides A.S. *Vegetable crops of Russia*, 2018, 6(44): 3-7 (doi: 10.18619/2072-9146-2018-6-3-7) (in Russ.).
98. Kochieva E.Z., Suprunova T.P. Identification of inter- and intraspecific polymorphism in tomato. *Genetika,* 1999, 35(10): 1386-1389.
99. Domblides E.A., Domblides A.S., Zayachkovskaya T.V., Bondareva L.L. *Vavilovskii zhurnal genetiki i selektsii*, 2015, 19(5): 529-537 (doi: 10.18699/VJ15.069) (in Russ.).
100. Pyshnaya O.N., Mamedov M.I., Shmykova N.A., Shumilina D.V., Suprunova T.P., Dzhos E.A., Matyukina A.A. *Trudy Kubanskogo gosudarstvennogo agrarnogo universiteta*, 2015, 55: 213-216 (in Russ.).
101. Suprunova T.P., Logunov A.N., Logunova V.V., Agafonov A.F. *Vegetable crops of Russia*, 2011, 4(13): 20-21 (in Russ.).
102. Domblides A.S. *Vegetable crops of Russia*, 2019, 5: 15-19 (doi: 10.18619/2072-9146-2019-5-15-19) (in Russ.).
103. Khimich G.A. *Vegetable crops of Russia*, 2016, 1(30): 48-49 (doi: 10.18619/2072-9146-2016-1-48-49) (in Russ.).
104. Pivovarov V.F., Shmykova N.A., Bondareva L.L., Zablotskaya E.A. *Vestnik Rossiiskoi sel'skokhozyaistvennoi nauki*, 2015, 5: 33-35 (in Russ.).

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LARGE FRUIT OF TOMATO *Solanum lycopersicum* L.: GENETIC DETERMINANTS, ORGANOGENESIS AND FRUIT DEVELOPMENT
(review)

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Abstract

Large fruit in *Solanum lycopersicum* L. is the result of domestication. We were interested in the appearance of large fruits in tomato in connection with the practice task to get new tomato forms with large fruits for multi-tiered hydroponic and aeroponic installations for vertical fruit production in greenhouses. Using the technology of target tomato breeding we obtained the first special dwarf tomato varieties Natasha and Timosha with small fruits for multi-tiered hydroponic installations. Obtaining of large fruit in tomato is connected with genetic and epigenetic control of the trait (An. Frary et al., 2000; B. Cong et al., 2006; Z. Huang et al., 2011; S. Wang, et al., 2011; A.J. Monforte et al., 2014; L. Azzi et al., 2015). The goal of this review is to summarize data on genetic determinants the trait of "size/weight of the fruit", analysis processes of organogenesis, hormone and metabolic regulation of fruit development. Analysis of papers dedicated to fruit weight increasing during domestication shows the availability of 37 loci involved in regulation of cell division and enlargement at four different stages of fruit development, starting from the phases of ovary development and fruit set to the phases of cell development and enlargement of cells which form the mature fruit. Some of these loci are connected with processes of hormonal plant development at the phase of anthesis, fertilization, formation of fruits and seeds, and so, they are involved in auxin (*SlPIN4*, *SlTIR1*, *SlARF7*, *SlARF8*, *SlIAA9*) and gibberellin (*SlGA20ox1*, *SlDELLA1*) signaling pathways. Others control cell enlargement during fruit development and maturing, and so, they are involved in regulation of primary (*HXK1*, *SuSY*, *LIN5*, *TIV1*, *mMDH*, *cpFBP*, *SPA*) and secondary (*NOTABILIS/NCED1*, *FLACCA*, *Gal-LDH*, *GME*) metabolism. Individual group of loci controls cell cycle at the period of ovary development (*TAGL1*, *FAS*, *LC*, *SlWUS*, *SlIMA*) and fruit growth (*SlCDKA1*, *SlCDCB1*, *SlCDKB2* and *SlCCS52A*, *SlWEE1*, *SlKRP1*) (L. Azzi et al., 2015). The *fw2.2* is the first locus which has been described in detail (An. Frary et al., 2000). Locus *fw2.2* controls the small fruit size in *S. lycopersicum* and is semidominant to allele *FW2.2* of large fruit size. With transgenic lines, it had been established, that locus *fw2.2* is carried by cos50. Sequence analysis of the cos50 had identified two open reading frames. One of them contain a single recombinant event, which delimited "the rightmost" end of the *fw2.2* (XO33). Because genetic mutation(s) causing change in fruit size must be to the left of XO33, cDNA44 cannot be involved and open reading frame is the likely cause of the small-fruit phenotype. Next studies indicated that *fw2.2* acts as a negative regulator of cell division during the very early stages of fruit development following pollination. Thus, *fw2.2* is one of regulatory QTLs, such as *achaete-scute*, *scabrous and Delta* QTLs in fruit flies, *teosinte-branched 1*(*tb1*) in maize and *Hox* genes in animals (cited by B. Cong et al., 2006). Possible, locus *FW2.*2 is positive regulator of cell division, which is involved in interaction with cytoplasmic membranes mediated by the regulatory (beta)-subunits of CKII kinase, that is well known in yeast and animals where it forms part of cell cycle related with signaling pathway (B. Cong et al., 2006).

Keywords: *Solanum lycopersicum* L., tomato, breeding, heritability, large fruits, average fruit weight, dwarfism, regulatory QTLs, fruit development

The main modern trend in greenhouse vegetable growing is multi-tier narrow-shelving hydroponic and aeroponic installations (vertical vegetable growing)

which produce greenery yield 530 times as much as in field conditions [1, 2]. To fill the capacious [1] and fast-growing [2] vertical vegetable growing market with the main crops (tomato, cucumber, sweet pepper), new plant breeding technologies are required. Knowing the peculiarities of the phenotypic manifestation of genes that control the key trait (dwarfism) allowed us to developed a target technology to select forms of vegetable crops for vertical vegetable growing [3, 4] and to produce the world's first small-fruited tomato varieties Natasha and Timosha for multi-tiered narrow-shelled hydroponics [5]. Application of genetic analysis [6] and targeted hybridization with large-fruited maternal forms almost doubled the average weight of fruits in the F_3 generation [7]. But the tomato fruit size is a complex quantitative trait. Consequently, to effectively produce large-fruited varieties, it is necessary to know not only the genetic determinants of the trait, but also the mechanisms that modulate phenotypic manifestation of these genes. In our review, we focused on the analysis of data on genes involved in the control of fruit weight in tomato, and the possibilities to regulate their expression, which, in our opinion, are of primary interest for breeding.

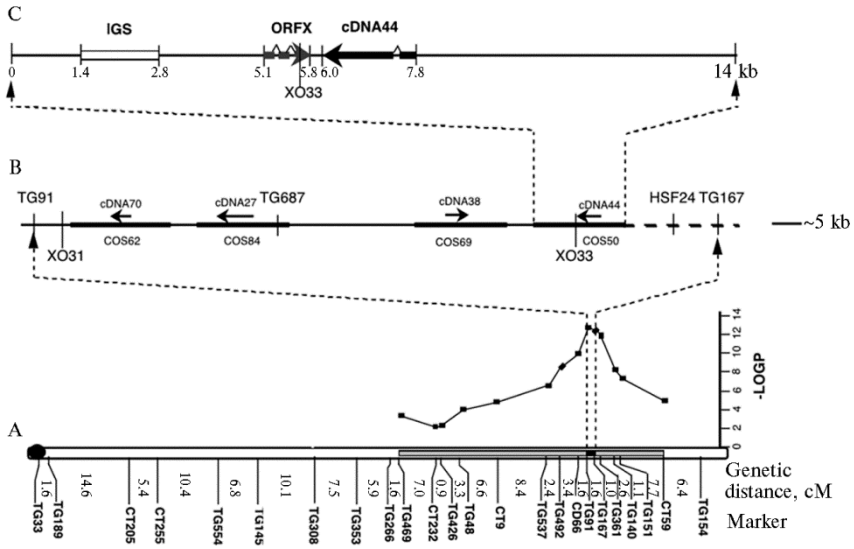
This review aims to summarize information on the genetic determinants of fruit weight in tomato and their relationship with organogenesis, hormonal and metabolic regulation of fruit development.

The tomato (*Solanum lycopersicum* Mill.) fruit is a multilocular berry widely used as a model of a juicy fruit in both agronomic and basic research [8-10]. The ancestral form of the domestic tomato had fruits less than 1 cm in diameter and weighing several grams. Changes in tomato fruit size are associated with domestication. We recorded a tomato fruit weight of 780 g (2018) [2], but in modern tomatoes it can reach 1000 g with a diameter of more than 15 cm [11].

Loci controlling fruit size. In tomato, fruit size is a polygenic trait. Most of the 37 loci involved in the evolution and domestication of tomato from small-fruited forms to larger-fruited ones are genetically mapped [12, 13].

The first mapped locus for tomato fruit size was *fw2.2*. Determining small fruit size, this locus behaves as semi-dominant vs. the semi-recessive large-fruit allele *FW2.2* [11]. All studied wild species of *S. lycopersicum* carry small-fruit alleles *fw2.2*, while modern varieties carry large-fruit alleles *FW2.2*. An international group of researchers cloned and sequenced a 19 kb segment containing *fw2.2* locus, and also identified the genes responsible for the effect of this locus [11]. The same authors constructed a high-resolution genetic map for the *fw2.2* locus using four unique transcripts identified in 3472 plants of the F_2 generation derived from crossing of two near-isogenic lines (NILs) different in *fw2.2* alleles (Fig.) [11]. Four cDNAs corresponding to these transcripts were used to screen a library of cosmids carrying fragments of *S. pennellii* genomic DNA. As a result, four positive non-overlapping cosmids were identified, the cos50, cos62, cos69 and cos84, each corresponding to one of the unique transcripts. Using transgenic lines, *fw2.2* was detected in cos50. Sequencing of this cosmid revealed two open reading frames (see Fig.). The first ORF corresponded to cDNA44 (one of the four unique cDNAs by which cos50 was identified), for the second ORF (663 nt), corresponding transcripts were not initially found in the library. The insertion contained a highly repetitive AT-rich (80%) 1.4 kbp region (see Fig., C). Previous mapping of *fw2.2* revealed a single recombination event [XO33] that delimited the "rightmost" end of the *fw2.2*. Comparison of the genomic DNA sequence in this recombinant plant with that in the two parental lines showed the localization of XO33 between the 43rd and 80th nucleotides from the 5′-end of the open reading frame X (ORFX) (see Fig., A). Since the genetic mutation(s) causing fruit size change can only be to the left of XO33, cDNA44 cannot be involved in the fruit size increase, and the ORFX or an upstream region is the likely cause of a standard small-fruited

fw2.2 phenotype.



High-resolution genetic mapping of *fw2.2* locus [11].

A. Location of *fw2.2* on chromosome 2 in crosses between *Solanum lycopersicum* and an isogenic line containing small introgression from *S. pennellii.*

B. A contig form the candidate region *fw2.2* delimited by recombination events at XO31 and XO33. Arrows mark four original candidate cDNA (cDNA70, cDNA27, cDNA38, and cDNA44), bold horizontal lines indicate four cosmids (cos62, cos84, cos69, cos50) isolated with these cDNAs as probes.

C. The sequence of cos50 spanning XO33 recombination event.

The same authors [11] found an open reading frame (ORFX) in flower organs (petals, carpels, sepals, and stamens) before flowering. Since ORFX is transcribed at a level too low to be detected using standard Northern hybridization protocols, the authors used reverse transcription polymerase chain reaction (RT-PCR) and revealed the highest level of ORFX expression in carpels. The study of the relative expression levels of ORFX transcripts in carpels in different isogenic lines showed a significantly higher level of expression of ORFX transcripts in carpels in small-fruited isogenic lines as compared to large-fruited ones. The study of ORFX transcription in carpels before flowering confirms that *fw2.2* is enhanced in the early stages of plant development. In order to test this hypothesis, the authors compared the masses of flower organs in small-fruited and large-fruited isogenic lines [11). Carpels which later develop into fruits, pistils and sepals before flowering were always heavier in large-fruited isogenic lines than in small-fruited lines. The cell size before flowering was similar in both types of isogenic lines, which means that carpels of large-fruited genotypes contain more cells. Analysis of allelic differences between *fw2.2* and *FW2.2* by comparing the 830 bp fragment containing ORFX in *S. pennellii* and *S. lycopersicum* led to the conclusion that, in the case of *fw2.2*, the phenotype is due to one or more upstream changes in the ORFX promoter region. The reduction in cell division in carpels of small-fruited isogenic lines correlates with the general increase in levels of the ORFX transcripts, confirming that ORFX can be a negative regulator of cell division [11]. Convincing evidence of this was obtained in later works carried out in the same laboratory [14]. It was found that *fw2.2* acts as a negative regulator of cell division in the earliest stages of fruit development, i.e., after pollination. Thus, the *fw2.2* is one of the regulatory quantitate trait loci (QTL) for an increase in fruit size, similar, for example, to the *achaete-scute*, *scabrous*, and *Delta* loci in fruity-cultures,

teosinte-branched 1 (*tb1*) in maize and *Hox* genes of animals, in which morphological changes reflect variations in gene regulation rather than modification of protein functions [cited from 14]. As for the *FW2.2* locus, it most likely plays the role of a positive regulator, direct or indirect, of cell division. Interaction occurs between the *FW2.2* locus and cytoplasmic membranes via the regulatory β-subunit of CKII kinase. CKII kinases are well studied in yeast and animals, in which theses kinases are involved in the cell cycle and are associated with signaling pathways. Thus, although *FW2.2* is a specific plant protein [11] and regulates cell division in a specialized organ (fruit), it appears to be involved in the cellular control of signal transduction [14].

The locus *FW2.2* belongs to the multigene family which in the tomato plants is comprised of 17 homologues. The family is usually referred to as the *FW2.2-like* or *FWL* genes. FW2.2 and FWL proteins contain an uncharacterized Placenta-specific 8 (PLAC8) motif which was originally found in the mammalian placental proteins [15]. The PLAC8 motif contains two cysteine-rich conservative domains separating the variable region that precedes the transmembrane segments. In tomato, the original FW2.2 proteins possess two transmembrane domains which fix the protein on the plasmalemma [14]. The earlier reports have shown that cysteine-rich domains may be involved in the transmembrane transfer of heavy metals such as cadmium and zinc. This was first established in proteins of cadmium-resistant *Arabidopsis* plants. This type of proteins can multimerize into a homopentamer to form a transmembrane pore, which makes it possible to transport metal cations [16].

FW3.2 is the second major QTL for tomato fruit size/weight that has been mapped and cloned [17]. The genes of this locus encode the P450 enzyme from the CYP78A5 subfamily, previously identified as KLUH [18]. The effect of *SlKLUH* is to increase the fruit volume through an increase in the cell number in the pericarp and septum tissues. *SlKLUH* function suppression using RNA interference strategy led to a decrease in fruit and seed sizes [17].

FW11.3 is another important QTL responsible for tomato fruit weight [19]. Genetic mapping revealed an overlap of *FW11.3* with the *fasciated* (*fas*) locus which determines the fruit shape and is located on chromosome 11, but *FW11.3* and *fas* are not alleles [20]. The large-fruit allele *FW11.3*, in contrast to *FW2.2* and *fas*, is partially dominant [19].

The complex family of loci that control the tomato fruit size/weight often overlaps with the loci responsible for the fruit shape, which, according to the opinion of some authors, was the result of domestication and is closely related to the regulatory functions of these loci [21]. Therefore, we will consider QTLs for fruit shape in *S. lycopersicum*. In contrast to the ancestral round shape, modern tomatoes are round, flat, ellipsoidal, pear-shaped, heart-shaped, oval and elongated in shape. But all the diversity of these forms is controlled by only four mutant genes, the *OVATE, SUN*, *FASCIATED* (*FAS*), and *LOCULE NUMDER* (*LC*) [22].

OVATE is the first gene for fruit shape identified by positional cloning [23]. *OVATE* is a platform for the ovate family of proteins, the functions of which are not fully understood [23, 24]. The *ovate* mutation is expressed in the appearance of elongated, pear-shaped, and ellipsoidal fruits, depending on the genotypic background of the plant carrying the *ovate* mutation [25]. This diversity confirms that *OVATE* is not the only gene responsible for the observed phenotype, but interacts epistatically with other genes [23]. It is assumed that the *OVATE* mutation is associated with the lost function of a negative plant growth regulator, the role of which remains to be clarified. For example, in *Arabidopsis*, proteins of the

OVATE family act as transcriptional repressors of the expression of AtGA20ox1, a key factor in the biosynthesis of gibberellic acid, which reduces cell elongation and, therefore, can affect fruit size [24, 26, 27].

Elongated fruit shape is associated with the *SUN* gene. The retrotransposon, which places this gene under the control of the *DEFL1* defensin gene promoter, provides duplication of this gene, which leads to its high expression in tomato fruits [28, 29]. Overexpression of the *SUN* gene increases the cell number in the direction of fruit elongation, which ultimately forms a phenotype with an elongated fruit [30].

The number of locules (gene *LOCULE NUMDER*, LC) is determined by the number of carpels within the flower. Wild tomato species have fruits with 2-4 locules, while modern varieties and hybrids can have more than 15 locules per fruit. As a result, not only the shape, but also the size/weight of the fruit changes, sometimes by more than 50% [9].

QTL *FASCIATED* (*FAS*) has been identified as a locus that regulates tomato fruit size via an increased number of locules, from 2 to 7 or more, while the *lc* mutation has a weaker effect [31, 32]. *FAS* encodes a YABBY-like transcription factor [33], and *LC* is located in a non-coding region between two potential candidate genes, the *WUSCHEL* which is a member of a plant-specific transcription factor gene family *WUS* (*WOX*), and a gene encoding a protein carrying a WD40 repeat [34]. The functions of most *WOX* genes have been known for a very long time [35]. More specific *WUS* genes are involved in maintaining stem thickness and meristem size, and therefore *WUSCHEL* can influence the number of locules. *FAS* and *LC* are able to epistatically interact and produce fruits with a very large number of locules [36]. Both of these loci control the size of the floral meristem; therefore, the development of a large number of carpels (locules) is possible, leading to the appearance of enormous fruits [33, 34].

Functional analysis using *TOMATO AGAMOUS-LIKE1* (*TAGL1*), an ortholog of the duplicated *SHATTERPROOF* (*SHP*) *MADS* box gene of *Arabidopsis thaliana*, showed the involvement of this transcription factor in the regulation of fruit development [37]. Tomato plants in which *TAGL1* expression was suppressed produced small fruits with a thin pericarp consisting of several layers of cells, and the pigmentation of the fruits also changed during ripening, which indicated the participation of *TAGL1* in the regulation of these processes.

Organogenesis: development of the tomato fruit. Fruits usually develop from anterior organs, for example, from carpels inside a flower. In tomato, carpels are formed during 17-20 cycles of cell division which occurs before flowering inside the L3 layer of the floral meristem not involved in cell expansion [38, 39]. Obviously, the number of cells formed before flowering is critical for the final size of the fruit, and such a positive correlation is often observed [9]. From the beginning of flowering to double fertilization that occurs in the ovules [8, 40], the morphogenesis and growth of carpel and ovules require the synthesis of auxins, cytokinins, and gibberellins, which act as a complex organized spatially and temporally. In order to protect the ovule and keep it dormant for a certain time, the abscisic acid and ethylene inside the ovary inhibit the growth of the ovule for a short period before flowering until it is ripe [41]. Only after successful pollination and fertilization of the ovules the process is completed with the involvement of fruit set triggers, the auxins and gibberellins synthesized by the ovary [42].

An increase in the tomato fruit size is the first and longest phase of a fruit development, it takes 5-8 weeks, depending on the genotype. Growth is due to the first period of intense mitotic activity in accordance with the spatial and temporal organization of cell division. Active cell division within the pericarp is usually limited to an initial period of 1-2 weeks after fruit setting. Remarkably, cell

division begins within discrete cell layers according to a certain scenario: two subepidermal layers of the pericarp undergo several cycles of periclinal division, thus leading to an increase in the number of periclinal cell layers, while two epidermal cell layers in response undergo anticlinal divisions, which leads to an increase in fruit volume [43]. These different types of cell division are regulated differentially, because cell division promotes the formation of cell layers that arise only within 5-8 days after flowering, while cell division less pronounced orientation occurs within 10-18 days after flowering [43]. Cell division in growing fruits covers about 80-97% of newly formed cells that arise after flowering and successful pollination.

During the second phase of growth, cell expansion occurs independently, but concomitant with cell division [8]. In fact, cell expansion begins a few days after fruit set [43] and continues during the entire period of fruit growth. At the end of the cell expansion phase, individual cells in the fleshy part of the fruit (mesocarp tissue) increase in volume by more than 30,000 times, which leads to an increase in the cell diameter by more than 0.5 mm [43). The increase in cell volume occurs mainly due to a significant increase in the volume of the vacuolar compartment and the vacuolar index of the cell. This expected cell hypertrophy is due to an increase in the amount of nuclear DNA as a result of endopolyploidization. Endopolyploidy means the appearance of different ploidy levels within the organism. In plants, it occurs as a result of endoreduplication, which is observed in 90% of angiosperms according to various estimates [44, 45]. Endoreduplication leads to the emergence of chromosomes with 2*n* chromatids or occurs without any changes in the number of chromosomes. Then hypertrophied nuclei arise from the successive cycles of DNA replication without separation of sister chromatids, which ultimately leads to the formation of polytene chromosomes [46]. The physiological relevance of endoreduplication is still a matter of debate. However, it is often noted that cell size and ploidy correlate highly and positively with each other in many plant species, in different organs, and in different cell types [47]. At each stage of organogenesis, certain groups of regulatory polygenic loci, associated with a change in the tomato fruit size in one way or another, are active [13]. The loci *TAGL1*, *FAS*, *LC*, *SlWUS*, and *SlIMA* are involved in the development of the ovule cell. During the flowering period, the genes *SlPIN4*, *SlTIR1*, *SlARF7*, *SlARF8*, and *SlIAA9* are involved in auxin signaling, and the *SlGA20ox1* and *SlDELLA1* participate in gibberellin signaling. During fruit growth, *SlCDKA1*, *SlCDCB1*, *SlCDKB2* (cell cycle control), *FW2.2*, *SlKLUH/FW3.2*, *FW11.3*, *OVATE*, *SUN*, *SlIMA* (cell division control), and *SlPIN4* (auxin signaling) are active. *SlCCS52A*, *SlWEE1*, *SlKRP1* (cell cycle control), *HXK1*, *SuSY*, *LIN5*, *TIV1*, *mMDH*, *cpFBP* (primary metabolism), *SlIAA17* (auxin signaling), *SPA* (regulation of primary metabolism), *NOTABILIS/NCED1*, *FLACCA* (biosynthesis of abscisic acid), *Gal-LDH*, *GME* (biosynthesis of ascorbates) are involved in increasing fruit volume (cell expansion) [13].

Hormonal regulation of fruit growth and development. After successful pollination of a flower and fertilization of an ovule and setting of fruits and seeds, the stage of ovary formation begins with the subsequent development of fruits and seeds, which occurs synchronously in accordance with a precise, genetically controlled process mediated by phytohormones [8]. Auxin and gibberellic acid seem to precede the phytohormones necessary for fruit set in response to pollination, since the exogenous use of these phytohormones leads to the formation of the ovary and the development of parthenocarpy [48]. The role of cytokinin, ethylene and abscisic acid was demonstrated later, but not well documented [49]. Early fruit development processes, controlled by auxins distributed in tissues and cells, initiate signal transduction pathways. Temporal and spatial distribution of *PIN* and *AUX/LAX* expression suggests that their coordinated action

regulates auxin transport during fruit development in tomato [50]. Silencing of the *SlPIN4* gene, which is first expressed in flower buds and young developing fruits, leads to the parthenocarpy of small fruits, which indicates the premature development of these fruits [51]. The auxin signaling pathway involves an auxin receptor, the transport inhibitor response protein (TIR1). In the presence of auxin, TIR1 involves the auxin-indolyl-3-butyric acid (Aux/IAA) transcription repressors in the process and induces their degradation by the 26S proteasomes. Degradation of the Aux/IAA protein repressor leads to the emergence of Aux/IAA-related auxin response factors (ARFs). The erroneous expression of the *TIR1* gene for the auxin receptor in tomato, as well as the erroneous expression of specific members of this gene family, the *Aux/IAA* and *ARF*, disrupts the flowering and formation of the ovary, as a result, normal pollination and fertilization does not occur, which increases the number of parthenocarpic fruits on a tomato plant [48, 52, 53]. In tomato, fruit setting is partly due to gibberellic acids in the complex of hormonal information exchange with auxin [54]. Auxin synthesized in the oocyte and apical shoots prevents the appearance of non-fertilized oocytes by reducing the transcription of genes encoding the biosynthesis of gibberellic acid enzymes, in particular, GA-20 oxidases [55]. Thus, phytohormones play the role of mediators in the signaling pathways of transport proteins and transcription factors during fruit setting and development in tomato. Phytohormones are involved in fruit size regulation with the participation of a number of genes organized into complex systems.

Metabolic control of fruit development. The early stages of fruit development are critical for the formation of economically valuable characteristics, for example, organoleptic composition, which ultimately determines fruit quality. Water, organic acids (primarily citrate and malate), and minerals accumulate inside the vacuoles of expanding cells [38], while starch is rapidly converted to simple sugars [56]. Fruit softness, color and taste are formed during ripening [57, 58]. The development and weight of the fruit is closely related to the content of primary and secondary metabolites [59, 60]. Consequently, modification of the expression of genes associated with metabolism can affect the organoleptic properties and weight of the tomato fruit. The development of the fruit as a succulent organ is more dependent on the accumulation of photoassimilates: a change in accumulation of assimilates significantly affects the development and size of the fruit through modulation of the number and size of cells [61, 62]. When a tomato plant is kept in the dark, fruit growth is significantly slowed down as a result of strong suppression of cell cycle genes in the fruit tissues [63]. On the contrary, an increase in the photoassimilation capacity of a fruit with a decrease in the number of fruits per plant led to an increase in the rate of flower formation and fruit growth. This is evidenced by an increase in the number of cells inside the carpel due to an increase in mitotic activity [64]. Thus, modification of carbohydrate and photoassimilate metabolism, driven partially by key enzymes involved in primary carbohydrate metabolism and photosynthesis, may affect fruit growth.

QTL *Lin5* has been identified as the main QTL controlling fruit weight and sugar content [65]. It was found that genes associated with it encode cell wall invertase [66]. When *Lin5* was silenced, fruit yield, fruit and seed size, and seed number were significantly reduced [67]. In transgenic plants, the changes affected the sugar metabolism, therefore, the sucrose content increased while the glucose and fructose content decreased at the full-ripening stage. Silencing of the vacuolar invertase gene (*TIR1*) in tomato led to generally similar results. The formation of small fruits was caused by a high rate of sucrose accumulation and a decrease in the amount of hexose at the final stage of fruit development [68]. Interestingly, changes in the concentration of osmotically active soluble sugars occurred during the expansion phase of the cell and affected the size of the fruit. This supports the

idea that the concentration of soluble sugars is associated with an increase in water volume, which is an important determinant of an increase in fruit size.

When searching for genomic QTL regions associated with yield traits, Bermudez et al. [69)] identified 9 candidate genes located on chromosome 4. In particular, a gene encoding a protein similar to the DnaJ chaperone was identified, and an assumption was made about its connection with the primary metabolism in tomato during fruit development. Functional analysis of this gene, later named *SPA* (*sugar partitioning-affecting*) in planta using the silencing method, showed that the weight of the ripe fruit, the number of fruits per plant, and the harvesting index are significantly higher in transgenic plants than in wild plants [70]. A detailed analysis of metabolic and enzymatic activity showed that during silencing, intermediate metabolites (sugar phosphates) accumulated in the photosynthetic organs of plants, while the activity of phosphoglucomutase, sugar kinases, and invertases decreased. The SPA protein of tomatoes interacts with the thylakoid membranes of chloroplasts, plays an important role in metabolism, affects the redistribution of carbohydrates and, as a result, changes the harvest index [70].

In recent studies of QTLs that determine the size and shape of the tomato fruit, emphasis is placed on the complex nature of the alleles. Chu et al. [71] quite definitely state that the number of locules and fruit size in tomato are controlled by natural alleles *lc* and *fas*. *LC* encodes the WUSCHEL tomato ortholog (WUS), while the *FAS* encodes the CLAVATA3 tomato ortholog (CLV3). The leading role of the WUS-CLV3 in the organization of the meristem was demonstrated in several plant species. The authors of this work showed that mutation of both loci in tomato leads to an increase in the *SlWUS* expression level in flower buds 2-3 days after initiation. Single and double mutant alleles *lc* and *fas* retain a high level of *SlWUS* expression during the development of carpel in a flower bud [71]. Other authors, combining the sequence mapping technique and the CRISPR-Cas9 genome editing method, identified the AP2/ERF transcription factor locus which regulates the activity of the flower meristem [72]. They named this locus *EXCESSIVE NUMBER OF FLORAL ORGANS* (*ENO*) [72]. Mutation of the *ENO* gene leads to an increase in the number of multilocular fruits per plant as a result of the proliferation of the flower meristem. Genetic analysis revealed a synergistic effect of *LOCULE NUMDER* (*SlWUS* locus) and *FASCIATED* (*SlCLV3* locus) mutations, the two key mutations in the evolution of tomato fruit size upon domestication [72]. As a result of extensive research carried out by traditional (Tomato Analyzer) and modern (EcoTILLING) methods, a group of Indian scientists found that a population of one tomato variety with a low level of polymorphism detected by EcoTILLING, nevertheless, showed a wide phenotypic diversity. The authors explain the obtained results by the fact that phenotypic diversity is the result of interaction between the genome, transcriptome, proteome, and metabolome [73]. In the context of the studied topic, this means that not so much single genes control the size and weight of the tomato fruit, but regulatory QTLs which was mentioned above [71, 72]. Of particular interest are works devoted to the influence of regulatory QTLs involved in the metabolic pathways of auxin and gibberellin on the setting and regulation of fruit size in tomato [74, 75], but we believe that these aspects should be the subject of special review.

So, summarizing data on the genetic determinants of the fruit size in tomato, led us to the following conclusions. The fruit size in *Solanum lycopersicum* L. is controlled by a group of loci that regulate the processes of cell division and expansion during four stages of fruit development, from the development of the ovule and the ovary formation after fertilization to cell division and expansion of cells that form a mature fruit. To date, 37 such loci are known. These loci can

overlap with loci that control the fruit shape in *S. lycopersicum* and are likely involved in phytohormone signaling pathways and processes of primary and secondary metabolism. Genetic determinants of cell division and expansion are involved in the signaling pathways of auxin and gibberellin, and therefore changing fruit size through these phytohormones is quite likely. The development and weight of the tomato fruit is closely related to the amount of primary and secondary metabolites. Modification of the expression of genes associated with primary and secondary metabolism can change the organoleptic composition and weight of tomato fruits by adjusting the harvest index and distribution of carbohydrates, which will ultimately improve the biochemical composition of tomato fruits.

REFERENCES

1. *Global Industry Report*, *2014-2025*, April, 2017, Report ID: IVR 1-68038-797-1.
2. Balashova I.T., Sirota S.M., Pinchuk E.V. Vertical vegetable growing: creating tomato varieties for multi-tiered hydroponic installations. *International Conference on Sustainable Development of Cross-Border Regions. IOP Conference Series: Earth and Environmental Science*. Barnaul, 2019, 395 (012079): 1-8 (doi: 10.1088/1755-1315/395/1/012079).
3. Sirota S.M., Balashova I.T., Kozar' E.G., Pinchuk E.V. *Ovoshchi Rossii*, 2016, 4(33): 3-9 (in Russ.).
4. Balashova I.T., Sirota S.M., Kozar E.G., Pivovarov V.F. Target tomato breeding for special hydroponic technology. *Abstracts of 20th EUCARPIA Congress*. Switzerland, Zurich, 2016: 343.
5. Balashova I.T., Sirota S.M., Kozar' E.G., Pinchuk E.V. *Vestnik Orlovskogo gosudarstvennogo agrarnogo universiteta*, 2017, 3(66): 71-74 (in Russ.).
6. Pivovarov V.F., Balashova I.T., Sirota S.M., Kozar' E.G., Pinchuk E.V. Improvement of sporophyte selection for the purpose of acceleration of tomato breeding for narrow shelf hydroponics technology. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2013, 1: 95-101 (doi: 10.15389/agrobiology.2013.1.95eng).
7. Pivovarov V.F., Balashova I.T., Sirota S.M., Kozar' E.G., Pinchuk E.V. Analysis of hybridization effect by the appearance of target tomato traits in F_2, F_3 progenies in breeding for multi circle hydroponics. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(5): 1049-1055 (doi: 10.15389/agrobiology.2017.5.1049eng).
8. Gillaspy G., Ben-David H., Gruissem W. Fruits: a developmental perspective. *The Plant Cell*, 1993, 5(10): 1439-1451 (doi: 10.1105/tpc.5.10.1439).
9. Tanksley S.D. The genetic, developmental and molecular bases of fruit size and shape variation in tomato. *The Plant Cell Online*, 2004, 16(suppl_1): 181-189 (doi: 10.1105/tpc.018119).
10. Klee H.J., Giovannoni J.J. Genetics and control of tomato fruit ripening and quality attributes. *Annual Review of Genetics*, 2011, 45(1): 41-59 (doi: 10.1146/annurev-genet-110410-132507.
11. Frary An., Nesbitt T.C., Frary Am., Grandillo S., Van der Knaap E., Cong B., Liu J.P., Meller J., Elber R., Alpert K.B., Tanksley S.D. *fw2.2*: A quantitative trait locus key to the evolution of tomato fruit size. *Science, New Series*, 2000, 289(5476): 85-88 (doi: 10.1126/science.289.5476.85).
12. Grandillo S., Ku H.M., Tanksley S.D. Identifying loci responsible for natural variation in fruit size and shape in tomato. *Theoretical and Applied Genetics*, 1999, 99(6): 978-987 (doi: 10.1007/s001220051405).
13. Azzi L., Deluche C., Gévudant F., Frangne N., Delmas F., Hernould M., Chevalier C. Fruit growth-related genes in tomato. *Journal of Experimental Botany*, 2015, 66(4): 1-12 (doi: 10.1093/jxb/eru527).
14. Cong B., Tanksley S.D. FW2.2 and cell cycle control in the developing tomato fruit: a possible example of gene co-option in the evolution of a novel organ. *Plant Molecular Biology*, 2006, 62(6): 867-880 (doi: 10.1007/s11103-006-9062-6).
15. Guo M., Rupe M.A., Dieter J.A., Zou J., Spielbauer D., Duncan K.E., Howard R.J., Hou Z., Simmons S.R. Cell Number Regulator 1 affects plant and organ size in maize: implications for crop yield enhancement and heterosis. *The Plant Cell*, 2010, 22(4): 1057-1073 (doi: 10.1105/tpc.109.073676).
16. Song W.Y., Choi K.S., Kim D.Y. Geisler M., Park J., Vincenzetti V., Schellenberg M., Kim S.H., Lim Y.P., Noh E.W., Lee Y., Martinoia E. *Arabidopsis* PCR2 is a zinc exporter involved in both zinc extrusion and long-distance zinc transport. *The Plant Cell*, 2010, 22(7): 2237-2252 (doi: 10.1105/tpc.109.070185).
17. Chakrabarti M., Zhang N., Sauvage C., Mucios S., Blanca J., Cacizares J., Diez M.J., Schneider R., Mazourek M., McClead J., Causse M., Van der Knaap E., A cytochrome P450 regulates a domestication trait in cultivated tomato. *Proceedings of the National Academy of Sciences*, 2013, 110(42): 17125-17130 (doi: 10.1073/pnas.1307313110).

18. Anastasiou E., Kenz S., Gerstung M., MacLean D., Timmer J., Fleck C., Lenhard M. Control of plant organ size by KLUH/CYP78A5-depended intercellular signaling. *Developmental Cell*, 2007, 13(6): 843-856 (doi: 10.1016/j.devcel.2007.10.001).
19. van der Knaap E., Tanksley S.D. The marking of a bell-pepper shaped tomato fruit: identification of loci controlling fruit morphology in Yellow Stuffer tomato. *Theoretical and Applied Genetics*, 2003, 107(1): 139-147 (doi: 10.1007/s00122-003-1224-1).
20. Huang Z., van der Knaap E. Tomato fruit weight 11.3 maps close to fascinated on the bottom on chromosome 11. *Theoretical and Applied Genetics*, 2011, 123(3): 465-474 (doi: 10.1007/s00122-011-1599-3).
21. Monforte A.J., Diaz A., Caco-Delgrado A., van der Knapp E. The genetic basis of fruit morphology in the horticultural crops: lessons from tomato to melon. *Journal of Experimental Botany*, 2014, 65(16): 4525-4537 (doi: 10.1093/jxb/eru017).
22. Rodriguez G.R., Mucos S., Anderson C., Sim S.C., Michel A., Causse M., McSpadden Gardener B.B., Francis D., van der Knapp E. Distribution of *SAN*, *OVATE*, *LC* and *FAS* in the tomato germplasm and the relationship to fruit shape diversity. *Plant Physiology*, 2011, 156(1): 275-285 (doi: 10.1104/pp.110.167577).
23. Liu J., Van Eck J., Cong B., Tanksley S.D. A new class of regulatory genes underlying the cause of pear-shaped tomato fruit. *Proceedings of the National Academy of Sciences*, 2002, 99(20): 13302-13306 (doi: 10.1073/pnas.162485999).
24. Wang S., Chang Y., Guo J., Zeng Q., Ellis B.E., Chen J.G. Arabidopsis Ovate family proteins, a novel transcriptional repressor family, control multiply aspects of plant growth and development. *PLoS ONE*, 2011, 6(8): 23896 (doi: 10.1371/journal.pone.0023896).
25. Gonsalo M.J., van der Knapp E. A comparative analysis into the genetic basis of morphology in tomato varieties exhibiting elongated fruit shape. *Theoretical and Applied Genetics*, 2008, 116(5): 647-656 (doi: 10.1007/s00122-007-0698-7).
26. Hackbusch J., Richter K., Mьller J., Salamini F., Uhrig J.F. A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of a 3-aa loop extension homeodomain proteins. *Proceedings of the National Academy of Sciences*, 2005, 102(13): 4908-4912 (doi: 10.1073/pnas.0501181102).
27. Wang S., Chang Y., Guo J., Chen J.-G. Arabidopsis Ovate Family Protein1 is a transcriptional repressor, that suppresses cell elongation. *The Plant Journal*, 2007, 50(5): 858-872 (doi: 10.1111/j.1365-313X.2007.03096.x).
28. Xiao H., Jiang N., Schaffner E., Stockinger E.J., van der Knapp E. A retrotransposon-mediated gene duplication underlines morphological variation of tomato fruit. *Science*, 2008, 319(5869): 1527-1530 (doi: 10.1126/science.1153040).
29. Jiang N., Gao D., Xiao H., van der Knapp E. Genome organization of the tomato *sun* locus and characterization of unusual retrotransposon reader. *The Plant Journal*, 2009, 60(1): 181-193 (doi: 10.1111/j.1365-313X.2009.03946.x).
30. Wu S., Xiao H., Cabrera A., Meulia T., van der Knapp E. *SUN* regulate vegetative and reproductive organ shape by changing cell division patterns. *Plant Physiology*, 2011, 157(3): 1175-1186 (doi: 10.1104/pp.111.181065).
31. Lippman Z., Tanksley S.D. Dissecting the genetic pathway to extreme fruit size in tomato using a cross between the small-fruited wild species *Lycopersicum pimpinellifolium* and *L. esculentum* var. Giant Heirloom. *Genetics*, 2001, 158(1): 413-422.
32. Barrero L.S., Cong B., Wu F., Tanksley S.D. Developmental characterization of the *fascinated* locus and mapping of the *Arabidopsis* candidate genes involved in the control of floral meristem size and carpel number in tomato. *Genome*, 2006, 49(8): 991-1006 (doi: 10.1139/g.06-059).
33. Cong B., Barrero L.S., Tanksley S.D. Regulatory change in YABBY-like transcriptional factor led to evolution of extreme fruit size during tomato domestication. *Nature Genetics*, 2008, 40(6): 800-804 (doi: 10.1038/ng.144).
34. Mucos S., Ranc N., Botton E. Bйrard A., Rolland S., Duffé P., Carretero Y., Le Paslier M.-C., Delalande C., Bouzayen M., Brunel D., Causse M. Increase in tomato locule number is controlled by two single-nucleotide polymorphisms located near *WUSCHEL*. *Plant Physiology*, 2011, 156(4): 2244-2254 (doi: 10.1104/pp.111.173997).
35. van der Graaff E., Laux T., Rensing S.A. The WUS homeobox-cotaining (WOX) protein family. *Genome Biology*, 2009, 10(12): 248 (doi: 10.1186/gb-2009-10-12-248).
36. Barrero L.S., Tanksley S.D. Evaluating the genetic basis of multiple-locule fruit in a broad cross section of tomato cultivars. *Theoretical and Applied Genetics*, 2004, 109(3): 669-679 (doi: 10.1007/s00122-004-1676-y).
37. Vrebalov J., Pan I.L., Arroyo A.J.M., McQuinn R., Chung M.Y., Poole M., Rose J., Seymour G., Grandillo S., Giovannoni J., Irish V.F. Fleshy fruit expansion and ripening are regulated by the tomato *SHATTERPROOF* gene *TAGL1*. *The Plant Cell*, 2009, 21(10): 3041-3062 (doi: 10.1105/tpc.109.066936).
38. Coombe B. *The development of fleshy fruits. Annual review of plant physiology*. Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, South Australia, 1976: 507-528.
39. Ho L.C. Fruit growth and sink strength. In: *Fruit and seed production: aspect of development,*

environmental physiology and ecology. C. Marshall, J. Grace (eds.). University Press, Cambridge, 1992: 101-124.
40. Brukhin V., Hernould M., Gonzalez N., Chevaleir C., Mouras A. Flower development schedule in tomato, *Lycopersicum esculentum* cv. Sweet Cherry. *Sexual Pant Reproduction*, 2003, 15: 311-320 (doi: 10.1007/s00497-003-0167-7).
41. Vriezen W.H., Feron R., Maretto F., Keijman J., Mariani C. Changes in tomato ovary transcriptome demonstrate complex hormonal regulation of fruit set. *New Phytologist*, 2008, 177(1): 60-76 (doi: 10.1111/j.1469-8137.2007.02254.x).
42. Ruan Y.-L., Patrick J.W., Bouzayen M., Osorio S., Fernie A.R. Molecular regulation of seed and fruit set. *Trends in Plant Science*, 2012, 17(11): 656-665 (doi: 10.1016/j.tplants.2012.06.005).
43. Cheniclet C., Rong W.Y., Causse M., Frangne N., Bolling L., Carde J.-P., Ranaudin J.-P. Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly tomato fruit growth. *Plant Physiology*, 2005, 139(4): 1984-1994 (doi: 10.1104/pp.105.068767).
44. Nagl W. DNA endoreduplication and polyteny understood as evolutionary strategies. *Nature*, 1976, 261(5561): 614-615 (doi: 10.1038/261614a0).
45. D'Amato F. Role of polyploidy in reproductive organs and tissues. In: *Embryology of angiosperms*. Springer, Berlin, Heidelberg, 1984: 519-566 (doi: 10.1007/978-3-642-69302-1_11).
46. Bourdon M., Pirello J., Cheniclet C., Coriton O., Bourge M., Brown S., Moïse A., Peypelut M., Rouyère V., Renaudin J.-P., Chevalier C., Frangne N. Evidence for karyoplasmic homeostasis during endoreduplication and a ploidy-dependent increase in gene transcription during tomato fruit growth. *Development*, 2012, 139(20): 3817-3826 (doi: 10.1242/dev.084053).
47. Chevalier C., Nafati M., Mathieu-Rivet E., Bourdon M., Frangne N., Cheniclet C., Ranaudin J.P., Gévaudant F., Hernould M. Elucidation the functional role of endoreduplication in tomato fruit development. *Annals of Botany*, 2011, 107(7): 1159-1169 (doi: 10.1093/aob/mcq257).
48. De Jong M., Wolters-Arts M., Feron R., Mariani C., Vriezen W.H. The *Solanum lycopersicum* auxin response factor 7 (SlARF7) regulates auxin signaling during tomato fruit set and development. *The Plant Journal*, 2008, 57(1): 160-170 (doi: 10.1111/j.1365-313X.2008.03671.x).
49. Kumar R., Khurana A., Sharma A. Role of plant hormones and their interplay in development and ripening of fleshy fruits. *Journal of Experimental Botany*, 2014, 65(16): 4561-4575 (doi: 10.1093/jxb/eru277).
50. Pattison R.J., Catalá C. Evaluating of auxin distribution in tomato (*Solanum lycopersicum*) through an analysis of the *PIN* and *AUX/LAX* gene families. *The Plant Journal*, 2012, 70(4): 585-598 (doi: 10.1111/j.1365-313x.2011.04895.x).
51. Mounet F., Moing A., Kowalczyk M., Rohrmann. J., Petit J., Garcia V., Maucourt M., Yano K., Deborde C., Aoki K., Bergès H., Granell A., Fernie A.R., Bellini C., Rothan C., Lemaire-Chamley M. Down-regulation of a single auxin efflux transport protein in tomato induces precocious fruit development. *Journal of Experimental Botany*, 2012, 63(13): 4901-4917 (doi: 10.1093/jxb/ers167).
52. Wang H., Jones B., Li Z., Frasse P., Delalande C., Regard F., Chaabouni S., Latchй A., Pech J.C., Bouzayen M. The tomato *Aux/IAA* transcription factor *IAA9* is involved in fruit development and leaf morphogenesis. *The Plant Cell*, 2005, 17(10): 2676-2692 (doi: 10.1105/tpc.105.033415).
53. Ren Z., Li Z., Miao Q., Yang Y., Deng W., Hao Y. The auxin receptor homolog in *Solanum lycopersicum* stimulates tomato fruit set and leaf morphogenesis. *Journal of Experimental Botany*, 2011, 62(8): 2815-2826 (doi: 10.1093/jxb/erq455).
54. Serrani J.C., Ruiz-Rivero O., Fos M., García - Martínez J.L. Auxin-induced fruit set in tomato is mediated in part by gibberellins. *The Plant Journal*, 2008, 56(6): 922-934 (doi: 10.1111/j.1365-313X.2008.03654.x).
55. Serrani J.C., Sanjuan R., Ruiz-Rivero O., Fos M., Garsia-Martinez J.L. Gibberellin regulation of fruit set and growth in tomato. *Plant Physiology*, 2007, 145(1): 246-257 (doi: 10.1104/pp.107.098335).
56. Wang F., Sanz A., Brenner M.L., Smith A. Sucrose synthase, starch accumulation, and tomato fruit sink strength. *Plant Physiology*, 1993, 101(1): 321-327 (doi: 10.1104/pp.101.1.321).
57. Giovannoni J.J. Genetic regulation of fruit development and ripening. *The Plant Cell Online*, 2004, 16(suppl_1): S170-S180 (doi: 10.1105/tpc.019158).
58. Gapper N.E., MacQuinn R.P., Giovannoni J.J. Molecular and genetic regulation of fruit ripening. *Plant Molecular Biology*, 2013, 82(6): 575-591 (doi: 10.1007/s11103-013-0050-3).
59. Carrari F., Fernie A.R. Metabolic regulation underlying tomato fruit development. *Journal of Experimental Botany*, 2006, 57(9): 1883-1897 (doi: 10.1093/jxb/erj020).
60. Tohge T., Alseekh S., Fernie A.R. On the regulation and function of secondary metabolism during fruit development and ripening. *Journal of Experimental Botany*, 2014, 65(16): 4599-4611 (doi: 10.1093/jxb/ert443).
61. Bohner J., Bangerth F. Cell number, cell size and hormone levels in semi-isogenic mutants of *Lycopersicum pimpinellifolium* differing in fruit size. *Physiologia Plantarum*, 2006, 72(2): 316-320 (doi: 10.1111/j.1399-3054.1988.tb05839.x).

62. Bertin N., Cuatier H., Roche C. Number of cells in tomato fruit depending on fruit position and source-sink balance during plant development. *Plant Growth Regulation*, 2002, 36(2): 105-112 (doi: 10.1023/A:1015075821976).
63. Baldet P., Devaux C., Chevalier C., Brouquisse R., Just D., Raymond P. Contrasted responses to carbohydrate limitation in tomato fruit at two stages of development. *Plant, Cell and Environment*, 2002, 25(12): 1639-1649 (doi: 10.1046/j.1365-3040.2002.00941.x).
64. Baldet P., Hernould M., Laporte F., Mounet F., Just D., Mouras A., Chevalier C., Rothan C. The expression of cell proliferation-related genes in early developing flower is affected by fruit load reduction in tomato plants. *Journal of Experimental Botany*, 2006, 57(4): 961-970 (doi: 10.1093/jxb/erj082).
65. Fridman E., Pleban T., Zamir D. A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. *Proceeding of the National Academy of Sciences*, 2000, 97(9): 4718-4723 (doi: 10.1073/pnas.97.9.4718).
66. Fridman E., Carrari F., Liu Y.S., Fernie A.R., Zamir D. Zooming in on a quantitative trait for tomato yield using interspecific introgressions. *Science*, 2004, 305(5691): 1786-1789 (doi: 10.1126/science.1101666).
67. Zanor M.I., Osorio S., Nunes-Nesi A., Carrari F., Lohse M., Usadel B., Kühn C., Bleiss W., Giavalisco P., Willmitzer L., Sulpice R., Zhou Y.-H., Fernie A.R. RNA interference of LIN5 in tomato confirms its role in controlling Brix content, uncovers the influence of sugar on the levels of fruit hormones and demonstrate the importance of sucrose cleavage for normal fruit development and fertility. *Plant Physiology*, 2009, 150(3): 1204-1218 (doi: 10.1104/pp.109.136598).
68. Gilbert L., Alhagdow M., Nunes-Nesi A., Quemener B., Guillon F., Bouchet B., Faurobert M., Gouble B., Page D., Garcia V., Petit J., Stevens R., Causse M., Fernie A.R., Lahaye M., Rothan C., Baldet P. GDP-d-mannose 3,5- epimerase (GME) plays a key role in the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. *The Plant Journal*, 2009, 60(3): 499-508 (doi: 10.1111/j.1365-313x.2009.03972.x).
69. Bermúdez L., Urias U., Mistein D., Kanenetzky L., Asis R., Fernie A.R., Van Sluys M.A., Carrari F., Rossi M. A candidate gene survey of quantitative trait loci, affecting chemical composition in tomato fruit. *Journal of Experimental Botany*, 2008, 59(10): 2875-2890 (doi: 10.1093/jxb/ern146).
70. Bermúdez L., de Godoy F., Baldet P., Demarco D., Osorio S., Quadrana L., Almeida J., Asis R., Gibon Y., Fernie A.R., Rossi M., Carrari F. Silencing of the tomato Sugar Partitioning Affecting protein (SPA), modifies sink strength through a shift a leaf sugar metabolism. *The Plant Journal*, 2014, 77(5): 676-687 (doi: 10.1111/tpj.12418).
71. Chu Yi-H., Jang J.-Ch., Huang Z., Van der Knapp E. Tomato locule number and fruit size controlled by natural alleles of *lc* and *fas*. *Plant Direct*, 2019, 3(7): e00142 (doi: 10.1002/pld3.142).
72. Yuste-Lisbona F.J., Fernández-Lozano A., Pineda B., Bretones S., Ortiz-Atienza A., Garcia-Sogo B., Müller N.A., Angosto T., Capel J., Moreno V., Jiménez-Gómez J.M., Lozano R. *ENO* regulates tomato fruit size through the floral meristem development network. *Proceeding of the National Academy of Sciences*, 2020, 117(14): 8187-8195 (doi: 10.1073/pnas.1973688117).
73. Mohan V., Gupta S., Thomas S., Mickey H., Charakana Ch., Chauhan V.S., Sharma K., Kumar R., Tyagi K., Sarma S., Gupta S.K., Kilambi H.V., Nongmaithem S., Kumari A., Gupta P., Sreelakshmi Ye., Sharma R. Tomato fruits show phenomic diversity but fruit developmental genes show low genomic diversity. *PLoS ONE*, 2016, 11(4): e0152907 (doi: 10.1371/journal.pone.0152907).
74. Quinet M., Angosto T., Yuste-Lisbona F.J., Blanchard-Gros R., Bigot S., Martinez J.-P., Lutts S. Tomato fruit development and metabolism. *Frontiers in Plant Science*, 2019, 10: 1554 (doi: 10.3389/fpls.2019.01554).
75. Liu S., Zhang Y., Feng Q., Qin L., Pan Ch., Lamin-Samu A.T., Gang L. Tomato AUXIN RESPONSE FACTOR 5 regulates fruit set and development via the mediation of auxin and gibberellin signaling. *Science Report*, 2018, 8: 2971 (doi: 10.1038/s41598-018-21315-y).

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ASSESSMENT OF GENETIC DIVERSITY AMONG HEADED CABBAGE (*Brassica oleracea* L.) ACCESSIONS BY USING SSR MARKERS

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Abstract

In the Russian Federation, headed cabbage *B. oleracea* L. convar. *capitata* (L.) (both traditional cultivars and hybrids) is the most common cole crop. Application of DNA marker technologies allow rapid identification of valuable genotypes and their genetic relationships in order to produce genetically diverse breeding forms. Microsatellite (SSR) markers are widely involved in genetic identification and genotyping of crops. Particularly, these markers effectively reveal the polymorphism among and within cultivars of *B. oleracea* L. In the present work we have estimated for the first time the genetic relationship among local accessions of headed cabbage on the basis of SSR-loci polymorphism. The goal of the work was to reveal genetic relationship between breeding accessions of *B. oleracea* L. convar. *capitata* (L.) Alef. var. *capitata* L. f. *alba*, var. *capitata* L. f. *rubra*, and var. *sabauda* L. based on DNA typing and genetic classification using SSR markers, and to compare DNA data of studied genotypes with defined cabbage varietal and maturity groups. Twenty-four breeding accessions of headed cabbage including red and Savoy varieties from collection of the Federal Scientific Vegetable Center (FSVC) and also developed at FSVC were involved. Genomic DNA was extracted from young plant leaves at 2-3 leaf stage with DNA extraction kit Sorb-GMO (Syntol, Russia). Final DNA purity and concentration were identified with the SmartSpec Plus spectrophotometer (Bio-Rad, USA). Twenty-one microsatellite loci with known primer sequences were chosen to perform SSR analysis. The amplification was run in C1000 Touch thermocycler (Bio-Rad, USA). PCR products were separated in a 6 % polyacrylamide sequencing gel with the use of Sequi-Gen GT electrophoresis system (Bio-Rad, USA). The fragments sizes were detected in comparison with molecular weight markers GeneRuler100 bp plus DNA ladder (Thermo Fisher Scientific, USA). The digital images of electrophoregrams were analyzed with Image Lab 3.0 software (Bio-Rad, USA). STRUCTURE 2.3.4 (https://web.stanford.edu/group/pritchardlab/home.html) software was used to study population structure. The genetic distances were calculated using GenAlEx 6.5 software for Microsoft Excel by Nei's method. To construct the UPGMA dendrogram the algorithm of MEGA 5.2 program was used. As a result of analysis 103 alleles were obtained with an average 4.9 alleles per locus. PCR product sizes were between 130 and 410 bp. The PIC value varied from 0.3 to 0.9. Population analysis revealed six clusters to distribute all breeding accessions. Calculated Nei's genetic distances varied from 0.060 to 0.186. The UPGMA deprogram constructed on distances matrix reflected the origin of cabbage accessions taken. Thus, cultivars Belorusskaya 455, Podarok 2500, Amager 611 and Zimovka 1474 originated from Northwestern Europe were joined into one cluster, there was also hybrid Severiynka F_1 developed with the use of these cultivars. Early-maturing varietal group Ditmarskaya Raniya represented by cultivars Ijunskaya 3200, Stakhanovka 1513, Nomer Perviy Gribovskiy 147 formed a separate cluster which also included an early-maturing hybrid Avrora F_1 being of a partial origin from Ijunskaya 3200. Two breeding lines obtained from Avrora F_1 were genetically distant and disposed in another subcluster. Cultivars Slava 1305 and Slava 231 belonging to the separate varietal group Slava formed a branch of the dendrogram. Cultivar Parus and hybrids Zarnitsa F_1, Mechta F_1 developed relatively recently were disposed distantly from other accessions. Moscovskaya pozdnyaya 15 a local cultivar formed its own branch of the dendrogram. Three cultivars of Savoy cabbage were grouped together with sufficient genetic distance between each other, where a new early-maturing cultivar Moskovskaya kruzhevnitsa was more distant from others. The group of red cabbage accessions situated distantly from other clusters with great difference inside the group. The obtained results based on SSR

marker variation were in accordance with data on the origin of headed cabbage accessions confirming that they belong to defined varietal and maturity groups. This provides information for nearest breeding program for new cabbage breeding forms.

Keywords: *Brassica oleracea* L., headed cabbage, SSR markers, genetic identification, cultivar genotype polymorphism, varietal group

China, India and the Russian Federation are the world's largest cabbage producers, and Russia retains the leadership in cabbage consumption [1]. Cabbage is rich in antioxidants, e.g. polyphenols, anthocyanins, gallic, vanillic and coumaric acids, and has anti-inflammatory properties due to glutamine and flavonoids. Cabbage is also a rich source of vitamin C and glucosinolates [2].

Of the species *Brassica oleracea* L. (CC, $2n = 18$), the white cabbage *B. oleracea* L. convar. *capitata* L. Alef. var. *capitata* (L.) f. *alba* DC. is the most common in Russia, while the red-headed cabbage *B. oleracea* L. convar. *capitata* (L.) Alef. var. *capitata* (L.) f. *rubra* (L.) Thell., Savoy cabbage *B. oleracea* L. convar. *capitata* (L.) Alef. var. *sabauda* L., cauliflower *B. oleracea* L. convar. *botrytis* (L.) Alef. var. *botrytis* L., broccoli *B. oleracea* L. convar. *botrytis* (L.) Alef. var. *italica* Plenck, Brussels sprouts *B. oleracea* L. convar. *gemmifera* (DC.) Gladis var. *gemmifera* DC., kohlrabi *B. oleracea* L. convar. *acephala* (DC.) Alef. var. *gongylodes* L., and leafy kale *B. oleracea* L. convar. *acephala* (DC.) Alef. var. *sabellica* L. are less popular. Numerous cultivars and high-yielding hybrids of *B. oleracea* have been produced due to breeding activities performed worldwide. The forms with the longest period of vernalization and frost resistance are a distinguishing feature of the convar. *capitata*. The initial biodiversity of cabbage originated in the Western Mediterranean Sea, Western and Northern Europe, and then spread to Eastern Europe, America, Asia (China, Japan), Australia and Africa. High yield and transportability were the properties of cabbage that contributed to widespread distributed of the crop. Cabbage varieties significantly differ in early maturity properties. The crop is not demanding for heat, positively responds to long daylight hours and cultivation technologies making the growing period longer [3].

Genetic characterization enables identification of parental forms with a high level of heterosis and a stable expression of economically valuable traits, which should be involved in crossing, thus allowing for a better use of plant resources. In addition, with the advent of a new breeding material, it is necessary to distinguish generated genotypes and identify the same or closely related ones. The use of SSR (simple sequence repeats) DNA markers facilitates the precise genetic typing of breeding samples. The reliability and detection of high genetic polymorphism are the advantages of microsatellite markers, which make them the most popular and universal in genetic studies of agricultural crops [4-6].

The database, which accumulates information on the variability of microsatellite loci in the genus *Brassica* L., including *B. oleracea*, contains 398 microsatellite markers [7]. Based on microsatellite loci, genetic relationships between species of the genus *Brassica* were established and confirmed [8-10]. Genetic analysis within the B. oleracea species revealed the relationships both between cabbage varieties, and varieties and breeding lines [11-14]. Phylogenetic studies of the *B. oleracea* were also based on the variability of microsatellites [15]. In a set of 91 commercial white cabbage genotypes, a total of 359 alleles grouped into six clusters were detected by 69 microsatellite markers. The first two clusters grouped the white cabbage genotypes, with broccoli, cauliflower, kohlrabi and kale varieties grouped in the remaining clusters [16]. In breeding samples of white cabbage, a high genetic variability was determined with accurate typing of each plant based on microsatellite polymorphism [17].

Thus, summarizing previous works, there is a scarcity of published literature on the genetic diversity of headed cabbage traditional Russian varieties and hybrids. Currently, the varieties used in breeding have been bred and zoned by E.M. Popova back in the 1940s at the Gribovskaya Experimental Station.

This work is the first to establish the genetic relationships between breeding samples of domestic head cabbage based on polymorphism of microsatellite loci. When comparing the three varieties, a close genetic relationship was found between the genotypes of Savoy cabbage and white cabbage.

The aim of our work was to identify genetic relationships between breeding samples of *Brassica oleracea* L. convar. *capitata* (L.) Alef. var. *capitata* L. f. *alba*, var. *capitata* L. f. *rubra*, and var. *sabauda* L. using SSR markers, and to compare the obtained data with assignment of the examined genotypes to cultivar types and maturity groups.

Materials and methods. Genomic DNA of 24 breeding samples of head cabbage (Genetic collection of the Federal Scientific Vegetable Center (FSVC) was extracted from leaves in the 2-3rd leaf phase by the CTAB buffer-based method using Sorb-GMO-B reagent kit (Syntol LLC, Russia) as per the manufacturer's protocol. Young leaves, from five plants per sample, were homogenized in an extraction buffer (a ball mill TissueLyser II, Qiagen, Germany; 26 Hz, 1560 vibrations per min, 1.7 min). The final DNA purity and concentration were determined spectrophotometrically (a SmartSpec Plus, Bio-Rad, USA). DNA solutions with $OD_{260/280}$ = 1.6-1.8 were used in PCR.

Twenty-one microsatellite loci with known primer sequences [11, 12] and polymorphic information content (PIC value) of 0.5 or above were involved in SSR analysis. For 25 μl PCR, 2.5 μl of 10× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM individual dNTPs, 0.3 μM of each primer, 1.5 U Taq DNA polymerase (Syntol LLC, Russia), and 3 μl of individual DNA template were mixed. The basal PCR protocol was as follows: 45 s at 92-95 °C (denaturation), 30 s at 52 to 58 °C, depending on primer pairs (annealing), and 30 s to 1 min at 72 °C (elongation). A 35-cycle amplification was performed (a C1000 Touch™ thermal cycler, Bio-Rad, USA).

Amplification products were separated by vertical electrophoresis (a Sequi-Gen GT system, Bio-Rad, USA) in a 6% polyacrylamide sequencing gel at 1600 V for 1.5-2 h. After electrophoresis, gels were stained (SYBR™ Safe DNA Gel Stain, Invitrogen, USA). The ChemiDoc XRS + system (Bio-Rad, USA) was used for gel documentation. The size of the amplified fragments was determined using GeneRuler100 bp plus DNA ladder (Thermo Fisher Scientific, USA). The digital photographs of electrophoregrams were analyzed using the Image Lab 3.0 software (Bio-Rad, USA).

For each SSR locus, the presence and absence of amplification product were designated as 1 and 0, respectively, to construct the final binary matrix. For each primer, the PIC value was calculated [18]. Population structures were analyzed using STRUCTURE 2.3.4 software (http://web.stanford.edu/group/pritchardlab/home.html) [19]; to determine the ΔK value, the STRUCTURE HARVESTER v0.6.1 program was applied (http://taylor0.biology.ucla.edu/structureHarvester/) [20]. The data analysis in STRUCTURE did not provide the number of clusters, so their probable number was set from 1 to 12, followed by a comparison of the K value for each option. Genetic distances were calculated in GenAlEx 6.5 (https://biology-assets.anu.edu.au/GenAlEx/Download.html) [21] by the Nei's method [22, 23]. The MEGA5.2 algorithm was used to construct the UPGMA dendrogram (https://mega.software.informer.com/5.2/) [24].

Results. Table 1 shows the samples of *B. oleracea* involved in the study.

1. A set of headed cabbage (*Brassica oleracea* L.) breeding samples involved in SSR analysis of genetic diversity (Genetic collection of the Federal Research Center for Vegetable Growing, FRCVG)

Name	Cultivar	Designation
Samples of *Brassica oleracea* convar. *capitata* (L.) Alef. var. *capitata* L. f. *alba* DC		
Amager 611	Amager	Ama
Avrora F_1	Ditmarskaya rannyaya	Avr
Avrora F_1 line 1	Ditmarskaya rannyaya	AvL1
Avrora F_1 line 2	Ditmarskaya rannyaya	AvL2
Belorusskaya 455	Belorusskaya	Bel
Zarnitsa F_1	Ditmarskaya rannyaya	Zar
Zimovka 1474	Langendeiskaya zimyaya	Zim
Iyunskaya 3200	Ditmarskaya rannyaya	Iju
Moskovskaya pozdnyaya 15/reproduction	Moskovskata posdnyaya	MoPo/MoPo1
Mechta F_1	Langendeiskaya zimyaya	Mech
Nomer pervyi gribovskii 147	Ditmarskaya rannyaya	N1
Parus/reproduction	Dutch group	Par/Par4
Podarok 2500	Belorusskaya	Pod
Severyanka F_1	Belorusskaya	Sev
Slava 1305	Slava	Sl13
Slava gribovskaya 231	Slava	Sl23
Stakhanovka 1513	Ditmarskaya rannyaya	Sta
Samples of *Brassica oleracea* var. *sabauda* L.		
Virtue 1340	Virtue	Ver
Moskovskaya kruzhevnitsa	Rosetochnaya	MosKr
Yubileinaya 2170	Ulmskaya	Ubi
Samples of *Brassica oleracea* var. *capitata* L. f. *rubra*		
Gako 741	Gako	Gak
Kamennaya golovka 447	Erfurtskaya	KamG
Rubin	Turnovskaya	Rub
Krasnokochannaya line I3	Erfurtskaya	RedL1

Note. Reproduction means that the sample is grown from the elite seeds.

Amplification of 21 microsatellite loci yielded 103 alleles, 4.9 per locus on average, of which 13 alleles were specific. The breeding samples Severyanka F_1, Belorusskaya 455, Podarok 2500, Amager 611, and Zimovka 1474 have the largest number of specific alleles (seven alleles). The amplification products vary in length from 130 to 410 bp. Sets of five alleles each were characteristic of the most informative SSR loci AF458409 and BZ523957 with the PIC values 0.90 and 0.86 for the primers, respectively (Fig. 1). However, less informative SSR loci with the PIC value 0.69 for the primers could contain a larger number of alleles, up to 13, like in CC969431, or 11, like in CC956699, CC969507, and AF113918. The lowest PIC value for the primers to AF180355 locus was 0.30, with only two alleles found.

2. Polymorphic microsatellite loci for marker-based estimation of the genetic relationships among headed cabbage (*Brassica oleracea* L.) breeding samples (Genetic collection of the Federal Scientific Vegetable Center)

Marker (GenBank NCBI)	Genes with polymorphic microsatellite sequences	Motif	Number of alleles	PIC
AF051772	Reproductive meristem gene 1 (REM1) mRNA	(gaa)5	3	0.81
AF051772(2)	B3 domain-containing protein REM1	(ct)6-1(ct)4-1(tcc)3	4	0.78
AF458409	Deoxycytidine deaminase (DCTD1), mRNA	(aga)6	5	0.90
AJ427337	mRNA for calmodulin 1 (cam1 gene)	(ga)5	3	0.61
BZ523957	Genomic clone BOKAH45	(ttg)6	5	0.86
CC956628	Brassica oleracea genomic clone BOIAA94, genomic survey sequence	(tc)5	2	0.50
CC956699	Brassica oleracea genomic clone BOIAB20, genomic survey sequence	(cac)9	11	0.69
CC969431	Genomic clone BOIAB19	(ga)6	13	0.61
CC969459	Genomic clone BOIAB94	(cgg)5	2	0.31
CC969497	Genomic clone BOIAA26	(tgc)5	2	0.83
CC969507	Genomic clone BOIAB15	(ct)5	11	0.74
X94979	mRNA for pollen coat protein	(atg)5	2	0.44

Continued Table 2

AF241115	Isolate HRI/CGN 5688 cauliflower gene	(at)5(ta)6	6	0.51
X92955	mRNA for pollen coat protein	(tttta)2(ata)7	3	0.57
AF180355	Isolate B265 ABI1 protein (ABI1) gene	(tc)16	2	0.30
AF113919	Phospholipase D2 (PLD2) gene	(at)6(gt)5	3	0.58
AF273844	Thioredoxin-h-like protein 1 (THL1) mRNA	(ctt)7	4	0.73
AF230693	Stearoyl-ACP desaturase (DELTA9-BO-1) gene	(ctt)3(ct)6(cttg)6	4	0.81
AF113918	Phospholipase D1 (PLD1) gene	(ct)7(at)7-1	11	0.60
U67451	Brassica oleracea homeotic protein boi1AP1 (Boi1AP1) mRNA	(at)9-1	6	0.81
AF241115(2)	Isolate HRI/CGN 5688 cauliflower gene	(ta)6-1	2	0.63

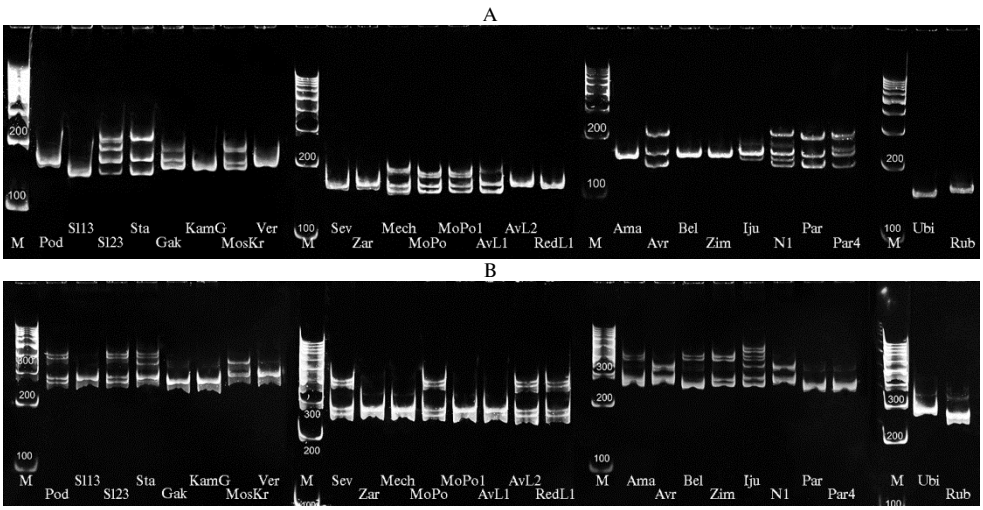

Fig. 1. Electophoregrams of microsatellite loci AF458409 (A) **and BZ523957** (B) **PCR amplification products in headed cabbage (*Brassica oleracea* L.) breeding samples** (Genetic collection of the Federal Research Center for Vegetable Growing, FRCVG). M — molecular weight marker (GeneRuler100 bp plus DNA ladder, Thermo Fisher Scientific, USA). For designations and description of samples, see Table 1.

In our study, the calculated PIC values were consistent with the values reported for the same markers by other researchers ($\chi^2 = 2.16$ at $p = 1.0$) [11, 12]. The markers for loci with a PIC value ≥ 0.5 turned out to be effective for genetic discrimination of related genotypes [25]. As a result, these markers allowed us to revealed 76% polymorphism among the studied breeding samples.

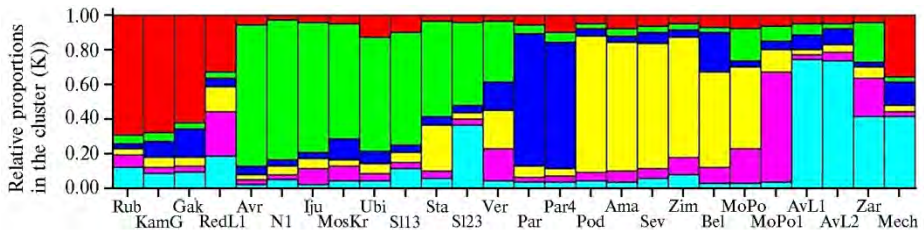

Fig. 2. Structure of the set of headed cabbage (*Brassica oleracea* L.) breeding samples (Genetic collection of the Federal Scientific Vegetable Center) **based on Bayesian analysis** (built with STRUCTURE software for six clusters, K = 6). For designations and description of samples, see Table 1.

It was shown that the tested breeding samples were best distributed among six clusters ($\Delta K = 2.05$) where they were grouped according to origin (Fig. 2). The calculated genetic distances varied from 0.060 to 0.186, with the greatest genetic distance between the Parus cultivar and the Mechta F_1 hybrid. Cabbage samples Severyanka F_1, Belorusskaya 455, Podarok 2500, Amager 611, and Zimovka 1474

of the Dutch group originating from North-Western Europe clustered together (Fig. 3). The high genetic similarity of cultivars Amager 611, Belorusskaya 455, and Podarok 2500 confirms that the latter was obtained through complex hybridization of the first two cultivars. The cultivars Belorusskaya 455, Zimovka 1474, and Podarok 2500 originated at the Gribovskaya Experimental Station were involved in production of a relatively new hybrid Severyanka F_1 advanced in quality and resistance to fusarium wilt, clubroot, and bacterioses. The cultivar Belorusskaya 455 obtained by selection of the earliest maturing forms in the Vitebsk region, has been zoned since 1943. The late-ripening cultivar Moskovskaya Pozdnaya 15, zoned in the same year and having a high productivity, formed a separate sub-cluster, closer to the samples of later ripeness, which explains local origin of the cultivar Moskovskaya Pozdnaya 15 from the Pyshkinskaya cultivar [26]. The genetic remoteness of this variety is also confirmed by its separate varietal type in the Central Russian group of varieties. The Parus cultivar with high quality parameters formed its own cluster, which proves the genetic remoteness of this cultivar from the general group of varieties and indicates a complex origin from various lines with economically valuable properties.

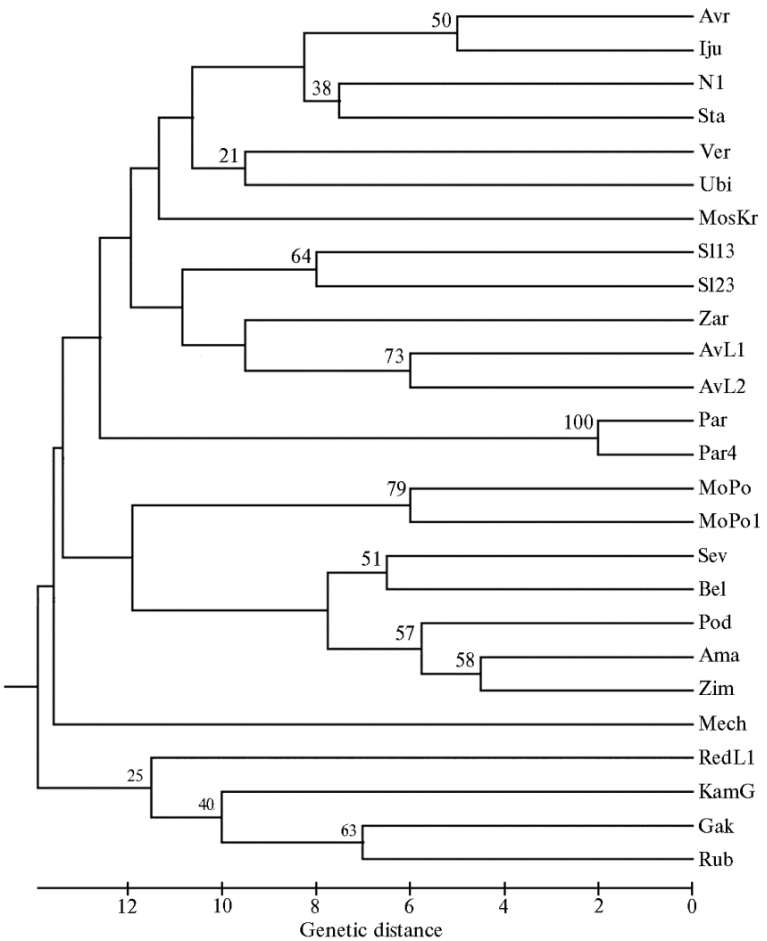

Fig. 3. UPGMA tree derived from data on microsatellite loci polymorphism of headed cabbage (*Brassica oleracea* L.) breeding samples (Genetic collection of the Federal Scientific Vegetable Center). Bootstrap replicates of 1000. For designations and description of samples, see Table 1.

Cultivars of the Ditmarskaya early type originating from Central Europe (Iyunskaya 3200, Stakhanovka 1513, Nomer pervyi gribovskii 147 and the early

ripening hybrid Avrora F_1 which has the Iyunskaya 3200 cultivar in the pedigree) formed a separate cluster. All these samples were early maturing. The cultivar Nomer pervyi gribovskii 147 zoned since 1943 was used to create cultivar Iyunskaya 3200, which is also consistent with its position in the dendrogram. The lines derived from the Avrora F_1 hybrid formed a separate subcluster. This confirms that the derived lines can be genetically quite distant from the hybrid. The traditional mid-season cultivars Slava Gribovskaya 231 and Slava 1305 of the Slava type, which were bred at the Gribovskaya Experimental Station and released in 1940, grouped in a separate subcluster.

The new mid-early hybrid Zarnitsa F_1 formed a separate branch of the dendrogram closer to the early ripening specimens, while the late-ripe hybrid Mechta F_1 formed a cluster at a 0.123 distance from the general group of cabbage specimens.

Savoy cabbage cultivars grouped into a separate subcluster within the white cabbage cluster. All of them belonged to different types of varieties, therefore, they were located genetically distantly from each other. The early-ripe cultivar Yubileynaya 2170 resulted from crossing the Savoy Viennese early cabbage and the white cabbage variety Nomer pervyi gribovskiy 147. The new mid-ripe cultivar Moskovskaya kruzhevnitsa with bubbly leaves turned out to be the most genetically distant from the other two cultivars of Savoy cabbage.

Traditional cultivars of red cabbage Kamennaya golovka 447 and Gako 741, bred at the Gribovskaya Experimental Station and released in 1943, belong to the Erfurt and Gako variety types, respectively, which is confirmed by their genetic remoteness. The Rubin variety and the I3 424-17 breeding line were grouped in the same cluster. Traditional varieties are well adapted to local growing conditions and can be donors of valuable genes conferring genetic variability and plasticity.

It should also be noted that the relatively recently bred cultivars Parus, Moskovskaya kruzhevnitsa, hybrids Zarnitsa F_1 and Mechta F_1 were quite distant from the main clusters consisting of traditional varieties. In addition, the breeding lines derived from promising F_1 hybrids significantly differed from the original form, which makes it possible to use DNA markers in inbreeding when obtaining hybrid-based lines.

In this study, a relatively small set of microsatellite markers successfully classified unique domestic head cabbages. El-Esawi et al. [27] used only twelve microsatellite loci to revealed a 75.7% polymorphism among twenty-five *Brassica oleracea* L. genotypes of which fifteen genotypes were cabbages, which is consistent with our findings.

Despite significant advances in the genetics of cabbage crops, the researchers mainly focus on the pathogen resistance [28-30], flowering time [31, head 32], morphology [33] and cracking [34, 35], with the lack of information on the genetic identification of cabbage varieties from different ripeness groups. Based on the genetic assessment of the studied breeding samples, we identified two main groups of white cabbage genotypes, with early maturation (the cluster of Avrora F_1, Iyunskaya 3200, Nomer pervyi gribovskiy 147, and Stakhanovka 1513) and late maturation (Moskovskaya pozdnyaya 15, Severyanka F_1, Belorusskaya 455, Amager 611, and Zimovka 1474). The Savoy cabbage genotypes in general were more precocious, which may explain their close genetic location to the group of more early maturing cultivars. Of these, the cultivar Virtue 1340 is the most late-ripening (the ripening period is about 130 days), while the white cabbage late-ripen genotypes need more than 140 days to reach ripeness.

Since environmental factors can influence varietal morphological traits and characteristics, cause phenotypic variability and hamper the assessment of varietal traits, DNA markers are the only tool for accurately identifying, maintaining and preserving genotypes for further selection [36]. DNA analysis with a set of known SSR markers allows reliable identification and selection of breeding samples from different ripeness groups and among valuable domestic cultivars, which are suitable for growing in all regions of Russia.

Thus, SSR markers were effective in detecting genetic variability among twenty-four genotypes of headed cabbage, including those similar in origin and belonging to the same cultivar. The results of DNA analysis confirm pedigrees of the traditional varieties and new hybrids. The genetic background of the genotypes can be decisive for design of crossing combinations in obtaining new breeding forms. The results of SSR analysis and genetic distance evaluation are "decision support information". This helps to select breeding material, control crossing combinations within groups of different ripeness, with preserving characters of the original cultivar types, and identify breeding lines in hybrid and/or varietal populations.

REFERENCES

1. Chepkemoi J. *The world leaders in cabbage production.* WorldAtlas, 2017. Available: worldatlas.com/articles/the-world-leaders-in-cabbage-production.html. Accessed: 15.03.2020.
2. Novotny C., Schulzova V., Krmela A., Hajslova J., Svobodova K., Koudela M. Ascorbic acid and glucosinolate levels in new czech cabbage cultivars: effect of production system and fungal infection. *Molecules*, 2018, 23(8): 1855 (doi: 10.3390/molecules23081855).
3. Lizgunova T.V. *Kul'turnaya flora SSSR. Tom 11. Kapusta* [Cultural flora of the USSR. V. 11. Cabbage]. Leningrad, 1984 (in Russ.).
4. Formisano G., Roig C., Esteras C., Ercolano M.R., Nuez F., Monforte A.J., Pico M.B. Genetic diversity of Spanish *Cucurbita pepo* landraces: an unexploited resource for summer squash breeding. *Genetic Resources and Crop Evolution*, 2012, 59(6): 1169-1184 (doi: 10.1007/s10722-011-9753-y).
5. Collard B.C.Y, Mackill D.J. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 2008, 363(1491): 557-572 (doi: 10.1098/rstb.2007.2170).
6. Guichoux E., Lagache L., Wagner S., Chaumeil P., Léger P, Lepais O., Lepoittevin C., Malausa T., Revardel E., Salin F., Petit R.J. Current trends in microsatellite genotyping. *Molecular Ecology Resources*, 2011, 11(4): 591-611 (doi: 10.1111/j.1755-0998.2011.03014.x).
7. Lowe A.J., Moule C., Trick M., Edwards K.J. Efficient large-scale development of microsatellites for marker and mapping application in *Brassica* crop species. *Theoretical and Applied Genetics*, 2004, 108(6): 1103-1112 (doi: 10.1007/s00122-003-1522-7).
8. Márquez-Lema A., Velasco L., Pérez-Vich B. Transferability, amplification quality, and genome specificity of microsatellites in *Brassica carinata* and related species. *Journal of Applied Genetics*, 2010, 51(2): 123-131 (doi: 10.1007/BF03195720).
9. Thakur A.K., Singh K.H., Singh L., Nanjundanet J., Khan Y.J., Singh D. SSR marker variations in *Brassica* species provide insight into the origin and evolution of *Brassica* amphidiploids. *Hereditas*, 2018, 155(6): 1-11 (doi: 10.1186/s41065-017-0041-5).
10. Raza A., Mehmood S.S., Ashraf F., Khan R.S.A. Genetic diversity analysis of *Brassica* species using PCR-based SSR markers. *Gesunde Pflanzen*, 2019, 71(1): 1-7 (doi: 10.1007/s10343-018-0435-y).
11. Tonguc M., Griffiths P.D. Genetic relationships of *Brassica* vegetables determined using database derived sequence repeats. *Euphytica*, 2004, 137(2): 193-201 (doi: 10.1023/B:EUPH.0000041577.84388.43).
12. Louarn S., Torp A.M., Holme I.B., Andersen S.B., Jensen B.D. Database derived microsatellite markers (SSRs) for cultivar differentiation in *Brassica oleracea*. *Genetic Resources and Crop Evolution*, 2007, 54(8): 1717-1725 (doi: 10.1007/s10722-006-9181-6).
13. Saxena B., Kaur R., Bhardwaj S.V. Assessment of genetic diversity in cabbage cultivars using RAPD and SSR markers. *Journal of Crop Science and Biotechnology*, 2011, 14(3): 191-196 (doi: 10.1007/s12892-011-0018-2).
14. Zhu S., Zhang X., Liu Q., Luo T., Tang Z., Zhou Y. The genetic diversity and relationships of cauliflower (*Brassica oleracea* var. *botrytis*) inbred lines assessed by using SSR markers. *PLoS ONE*, 2018, 13(12): e0208551 (doi: 10.1371/journal.pone.0208551).
15. Artem'eva A.M., Kloke E., Chesnokov Yu.V. *Informatsionnyi vestnik VOGiS*, 2009, 13(4): 759-771 (in Russ.).

16. Izzah N.K., Lee J., Perumal S., Park J.Y., Ahn K., Fu D., Kim G.-B., Nam Y.-W., Yang T.-J. Microsatellite-based analysis of genetic diversity in 91 commercial *Brassica oleracea* L. cultivars belonging to six varietal groups. *Genetic Resources and Crop Evolution*, 2013, 60(7): 1967-1986 (doi: 10.1007/s10722-013-9966-3).
17. Shapturenko M., Khotyleva L., Vakula S., Jakimovich A.V., Zabara Yu.M., Khotyleva L.V. Informative EST-SSR markers for genotyping and intraspecific differentiation of *Brassica oleracea* var. *capitata* L. *Vavilov Journal of Genetics and Breeding*, 2016, 20(1): 51-56.
18. Chesnokov Yu.V., Artem'eva A.M. Evaluation of the measure of polymorphism information of genetic diversity. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2015, 50(5): 571-578 (doi: 10.15389/agrobiology.2015.5.571eng).
19. Pritchard J.K., Stephens M., Donnelly P. Inference of population structure using multilocus genotype data. *Genetics*, 2000, 155(2): 945-959.
20. Earl D.A., vonHoldt B.M. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 2012, 4(2): 359-361 (doi: 10.1007/s12686-011-9548-7).
21. Peakall R., Smouse P.E. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 2012, 28(19): 2537-2539 (doi: 10.1093/bioinformatics/bts460).
22. Nei M. Genetic distance between populations. *American Naturalist*, 1972, 106(949): 283-392 (doi: 10.1086/282771).
23. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 1978, 89(3): 583-590.
24. Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. MEGA5: molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 2011, 28(10): 2731-2739 (doi: 10.1093/molbev/msr121).
25. DeWoody J.A., Honeycutt R.L., Skow L.C. Microsatellite markers in white-tailed deer. *Journal of Heredity*, 1995, 86(4): 317-319 (doi: 10.1093/oxfordjournals.jhered.a111593).
26. *Elena Mikhailovna Popova. Uchenyi, selektsioner. Osnovopolozhnik nauchnoi otechestvennoi selektsii po kapustnym kul'turam. Nauchnoe nasledie vospominaniya, razvitie nauchnykh idei* /Pod redaktsiei V.F. Pivovarova [Elena Mikhailovna Popova. Scientist, breeder. The founder of domestic scientific selection of cabbage crops. Scientific heritage — memories, the development of scientific ideas. V.F. Pivovarov (ed.]. Moscow, 2007 (in Russ.).
27. El-Esawi M.A., Germaine K., Bourke P., Malone R. Genetic diversity and population structure of *Brassica oleracea* germplasm in Ireland using SSR markers. *Comptes Rendus Biologies*, 2016, 339(3-4): 130-140 (doi: 10.1016/j.crvi.2016.02.002).
28. Peng L., Zhou L., Li Q., Wei D., Ren X., Song H., Mei J., Si J. Qian W. Identification of quantitative trait loci for clubroot resistance in *Brassica oleracea* with the use of *Brassica* SNP microarray. *Frontiers in Plant Science*, 2018, 9(822): 1-8 (doi: 10.3389/fpls.2018.00822).
29. Kifuji Y., Hanzawa H., Terasawa Y., Ashutosh, Nishio T. QTL analysis of black rot resistance in cabbage using newly developed EST-SNP markers. *Euphytica*, 2013, 190(2): 289-295 (doi: 10.1007/s10681-012-0847-1).
30. Mei J.Q., Ding Y.G., Lu K., Wei D.Y., Liu Y., Disi J.O., Li J., Liu L., Liu S., McKay J., Qian W. Identification of genomic regions involved in resistance against *Sclerotinia sclerotiorum* from wild *Brassica oleracea*. *Theoretical and Applied Genetics*, 2013, 126(2): 549-556 (doi: 10.1007/s00122-012-2000-x).
31. Okazaki K., Sakamoto K., Kikuchi R., Saito A., Togashi E., Kuginuki Y., Matsumoto S., Hirai M. Mapping and characterization of FLC homologs and QTL analysis of flowering time in *Brassica oleracea*. *Theoretical and Applied Genetics*, 2007, 114(4): 595-608 (doi: 10.1007/s00122-006-0460-6).
32. Uptmoor R., Schrag T., Stützel H., Esch E. Crop model based QTL analysis across environments and QTL based estimation of time to floral induction and flowering in *Brassica oleracea*. *Molecular Breeding*, 2008, 21(2): 205-216 (doi: 10.1007/s11032-007-9121-y).
33. Lv H., Wang Q., Zhang Y., Yang L., Fang Z., Wang X., Liu Y., Zhuang M., Lin Y., Yu H., Liu B. Linkage map construction using InDel and SSR markers and QTL analysis of heading traits in cabbage. *Molecular Breeding*, 2014, 34(1): 87-98 (doi: 10.1007/s11032-014-0019-1).
34. Pang W., Li X., Choi S.R., Nguyen V.D., Dhandapani V., Kim Y.Y., Ramchiary N., Kim J.G., Edwards D., Batley J., Na J., Kim H.R., Lim Y.P. Mapping QTLs of resistance to head splitting in cabbage (*Brassica oleracea* L. var. *capitata* L.). *Molecular Breeding*, 2015, 35(5): 1-12 (doi: 10.1007/s11032-015-0318-1).
35. Su Y., Liu Y., Li Z., Fang Z., Yang L., Zhuang M., Zhang Y. QTL analysis of head splitting resistance in cabbage (*Brassica oleracea* L. var. *capitata*) using SSR and InDel makers based on whole-genome re-sequencing. *PLoS ONE*, 2015, 10(9): e0138073 (doi: 10.1371/journal.pone.0138073).
36. Korir N.K., Han J., Shangguan L., Wang C., Kayesh E., Zhang Y., Fang J. Plant variety and cultivar identification: advances and prospects. *Critical Reviews in Biotechnology*, 2013, 33(2): 111-125 (doi: 10.3109/07388551.2012.675314).

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DEVELOPMENT PECULIARITIES OF BEAN COMMON MOSAIC VIRUS (*Potyvirus*, *Potyviridae*) IN MOSCOW REGION AND INITIAL MATERIAL FOR RESISTANCE BREEDING

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Abstract

Recent years have seen a significant expansion in the distribution area of bean common (*Phaseolus vulgaris* L.) mosaic virus (BCMV) that has become an economically significant disease agent for the non-chernozem part of Russia. As early as 2014, the epiphytotics were observed in the Moscow region, but no BCMV resistance screening in both Russia and foreign bean accessions has been performed yet in these agroclimatic conditions. Thus, the presented study is the first one that has described the features of BCMV development in the Moscow region and defines climatic factors affecting the disease progression. An assortment of bean accessions has been estimated on a level of resistance to BCMV using different techniques including molecular markers. The goal of the study was to find an initial breeding material as a source for development of new competitive BCMV-resistant local yardlong bean cultivars. The research was carried out in the Moscow Province in 2014-2019. The research methods included visual and serological diagnosis and phytopathological monitoring of disease progression of artificial and natural infection. Field testing of disease resistance in accessions of various genetic and geographical origins over time was performed using a four-point scale; the accessions were ranked into resistance groups based on the degree of the disease with regard to the stability of expression of the characteristic in various years. DNA analysis of the main resistance genes, i.e., dominant gene *I* and recessive genes *bc-1²* and *bc-3*, was performed using the respective markers SW13, SBD5, and ROC11, following the developed procedures. The result of the study was the identification of the biological features of the BCMV isolate from the Moscow region affecting *Phaseolus vulgaris* L. and *Pisum sativum* L. from the *Fabaceae* family in biotest. The expression of symptoms and intensity of the disease in indicator plants in a greenhouse and bean accessions in field trials significantly depended on temperature, and the spread of the virus — on the accumulated precipitation. In general, reduced precipitation in combination with elevated temperatures served as a deterrent preventing the pathogen from further spreading in the climatic conditions of the Moscow region. At the same time, this combination facilitated viral infection manifestations on the plant leaf apparatus, especially during the vegetation period. Out of 207 accessions studied, only 6 % demonstrated a persistently high BCMV resistance in the context of epiphytotics. Screening of 30 accessions with different resistance levels showed that recessive genes *bc-1²* and *bc-3* were present in the majority of the accessions and dominant gene *I* only in half of all accessions. Most accessions had genotypes *I*/*bc-1²*/*bc-3* (33 %) and *bc-1²*/*bc-3* (47 %), among which only 1/3 demonstrated a persistently high virus resistance. The plants lacking the genes *I* and *bc-1²* were severely damaged by the virus. The chi-square test (χ^2) revealed a more significant effect of the gene *bc-1²* on the field resistance of common bean accessions to BCMV. Based on the results obtained, as an initial breeding material for developing yardlong bean cultivars with high BCMV resistance we recommend 17 most promising accessions of different origin, including five cultivars (Khavskaya Universalnaya, Rant, Zolushka, Marlinka, Svetlyachok) and two perspective hybrids (SP-232, KP-

84) selected at Federal Research Center for Vegetable Growing that are distinguished by several agronomic characters.

Keywords: *Phaseolus vulgaris* L., green bean, bean common mosaic virus, BCMV, virus resistance, resistance genes, sustainable resistance, DNA-markers, initial material

Common bean (*Phaseolus vulgaris* L.) is the third most important and worldwide grown food legume crop following soybeans *Glycine max* (L.) Merr. and peanuts *Arachis hypogea* L. [1]. Recently, it has been used as a functional food product with a high content of proteins, vitamins, antioxidants and trace elements, and, moreover, with an excellent taste [1, 2]. According to the FAO (Food and Agriculture Organization) report for 2018, vegetable bean is the crop occupying the largest areas in the countries of Latin America, Eastern and Southern Africa [3]. In recent years, the industrial production of this culture has been actively expanding in Russia [4].

Viruses, especially aphid-transmitted, constitute a large group of pathogenic microorganisms infecting *Ph. vulgaris*. To date, about ten viruses are known, among which the *Bean common mosaic virus* (BCMV), *Bean yellow mosaic virus* (BYMV), *Bean common mosaic necrosis virus* (BCMNV) of *Potyviridae* family, and *Bean leafroll virus* (BLRV) of *Luteoviridae* family are the most harmful and wide-spread. Other species are classified as endemic, the appearance of which is due to certain conditions in different countries and regions [1, 5, 6].

BCMV was first discovered in 1917 in the USA [7]. At present, BCMV is the most harmful. In some countries, the epiphytoties caused by BCMV, according to various estimates, annually result in a 50-100% loss of legumes [8-13]. In the Russian Federation, BCMV was first described in the 1980s on meadow clover in the Far East, and a decade later on bean culture in other regions [14, 15].

BCMV is typically transmitted vertically by pollen and seeds. The virus is found in the seed coat, cotyledons, and embryos [6, 8, 9]. The seed infection is significantly influenced by the time of plant infection: the most vulnerable period is the phase of differentiation of flower organs, while during infection after flowering, the probability of seed transmission of BCMV is significantly reduced [16]. Eleven species of aphids are actively involved in the vector transmission of the virus in a non-persistent way form the source of infection. Most of them effectively transmit the virus as winged migrants in a matter of minutes, but they also quickly lose this ability [6, 9, 17]. The range of natural reserves of BCMV is mainly limited to cultivated and wild species of the *Fabaceae* family, the genera *Phaseolus*, *Pisum*, *Trifolium*, and *Vicia* [11, 18-20].

In fact, BCMV is a complex of strains subdivided into eight pathogroups (PG), five for BCMV (PG-1, PG-2, PG-4, PG-5, PG-7), and three for BCMNV serologically related to BCMV (PG-3, PG-6, PG-8) [21]. Recent molecular studies have shown high genetic diversity among all these strains and a large number of identified recombinants [10, 22-25]. The strains are distinguished due to symptoms of infection they cause in vegetable beans with various combinations of seven currently known resistance genes, a single, the main dominant strain-nonspecific gene *I* and six strain-specific recessive genes *bc-u*, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2}, and *bc-3* from four independent loci [17, 26, 27].

Breeding vegetable beans with multiple resistance to BCMV is a priority, since such varieties are resistant to various BCMV pathogroups [17, 26]. In order to pyramid genes conferring bean resistance to BCMV in beans, foreign scientists are increasingly combining the *I* and *bc*-3 genes via marker-assisted selection (MAS), which provides wider range of nonspecific protection [9, 28-31].

Alleles of the recessive genes *bc-1* and *bc-2* prevent the systemic spread of the virus. Their combination in plant genome, even in the absence of the dominant gene *I*, can be effective against many BCMV pathotypes. Transfer of resistance genes to susceptible genotypes using indirect selection under the control of DNA markers was performed for loci *I* [32, 33], *bc-3* [31], *bc-1²* [34, 35] *bc-1*, and *bc-u* [36]. For introduction of the resistance gene *I* into susceptible genotypes, the SW13 marker was used [28, 37-39]. SW13 and SBD5 were used in to pyramid the *bc-1²* and *I* genes in a susceptible genotype [35]. For the *bc-3* gene, markers ROC11 and SG6 have been developed, which can also be used in combinations with other markers [31, 40]. Thus, the SW13 marker is linked to the *I* gene, the SBD5 marker to the *bc-1²* gene, the ROC11 and SG6 markers indicate the presence and absence of the *bc-3* allele, respectively. In recent years, the recessive gene *bc-1²* conferring resistance to the most common BCMV and BCMNV pathotypes, the PG-1, PG-2, PG-3, and PG-5 has been involved in breeding programs [13, 34]. It should be noted that molecular methods for marking *R*-genes are deemed auxiliary. MAS application is successful when combined of requires a combination of molecular markers with classical phytopathological methods for assessing plant resistance to artificial and natural infections.

In Russia, bean crop breeding started in 1920 at the Gribovskaya Vegetable Breeding Experimental Station, the precursor of the Federal Research Center for Vegetable Growing (FRCVG). Over the past 100-year period, the breeders have created 45 varieties of beans, or 32% of the total assortment of the State Register of Breeding Achievements approved for use. Most of the previously created bean varieties are universal. In recent years, the creation of asparagus-type green beans that meet market requirements and are highly resistant to diseases is given a priority.

Because of climate change and uncontrolled import and logistics of virus-infected seeds, BCMV was noticed in the more northern regions of the Non-Black Earth Zone and Western Siberia of the Russian Federation [41-45]. After the first epiphytoty in the Moscow region registered in 2014-2015, a local study of the BCMV biology have been initiated in which *Ph. vulgaris* accessions from extensive Genetic collection of the Federal Research Center for Vegetable Growing were involved.

This paper is the first report on BCMV isolates affecting beans in the Moscow region, and on climatic factors influencing the severity of the infection. BCMV resistance of a wide variety of plant beans has been assessed using molecular markers. New genetic sources of bean resistance to BCMV have been identified and involved in breeding programs. The reported DNA marker-based screening of the gene pool of domestic beans for BCMV resistance is the first in the Russian Federation.

The work aimed to search for sources of resistance to bean common mosaic virus (BCMV) to involve these donor cultivars in common bean breeding for asparagus-type with a desirable combination of characteristics.

Materials and methods. Vegetable bean cultivars of different origin, mostly from the USA, the Netherlands, Germany, and the Russian Federation, including cultivars bred at FRCVG were screened for BCMV infection (207 accessions of the FRCVG collection, 45-60 plants per accession, the Federal Research Center for Vegetable Growing, Moscow Province, 2014-2019). Bean common mosaic virus was isolated from the infected plants.

BCMV was detected in plant leaves by sandwich enzyme-linked immunosorbent assay (ELISA Reagent Set for Bean common mosaic virus, Agdia, Inc., USA). The extinction coefficients were recorded (a semi-automatic Stat Fax® 2100 microplate reader, Awareness Technology, Inc., USA, λ = 480 nm). ImmunoStrip® express test (Agdia, Inc., USA) was used to avoid mixed infection with other plant viruses, in particular *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* (TMV) causing symptoms similar to BCMV.

To describe symptoms of BCMV infection and assess phenotypic resistance to BCMV in 30 promising bean accessions, pea (*Pisum sativum* L.) variety Zhegalovets plants and bean (*Phaseolus vulgaris* L.) variety Gribovskaya 92 plants were artificially infected with BCMV (a film greenhouse, ten plants of each cultivar in 3 replicates). Seeds were sown in the third decade of April when the average daily temperature in the greenhouse was 20-22 °C, and in the first decade of June when the average daily temperature was 26-29 °C. At the primordial leaf phase plants were inoculated by rubbing crude juice from leaves of the infected plant in 0.1 M phosphate buffer (pH 7.0) with carborundum, as per Mills et al. [46]. The viral infection was confirmed visually and by ELISA test.

In 2014-2019, the accessions were grown in the field (Moscow Province, Odintsovsky District) under natural infection conditions. A scheme of randomized plots allowed plants of each accession to have approximately the same chance to be infected. The severity of viral infection was assessed visually according to a modified scale where 0 means no symptoms on leaves, 0.5 means weak symptoms on some leaves, 1 means less than 10% leaves affected, 2 points stand for 10-30% affected, 3 points for 30-50%, and 4 points mean that more than 50% of the entire leaf surface of the plant is affected. The BCMV resistance of accessions was assessed by the prevalence (disease incidence) P (%), lesion index I (an average score), and disease severity R (%). The estimates were recorded 3 times, at the third true leaves, flowering and technical ripeness. The aggregated estimates were used to differentiate the accessions in susceptibility to BCMV and stability of manifestation of the infection. The accessions were deemed resistant at $R = 0$, relatively stable at $0 < R \leq 10\%$, weakly susceptible at $10 < R \leq 25\%$, and susceptible at $R > 25\%$. The accessions in group I had no signs of lesion, in group II, symptoms of BCMV infection appeared only in the years of epiphytoties, in group III, symptoms were unstable in different years, and in group IV, stable BCMV lesion occurred in all years of investigation.

During the growing season, the valuable traits of the accessions were assessed [47, 48]. Promising accessions were identified based on complex breeding values by accounting for all studied traits.

Among the accessions involved in field trials in 2014-2019, 30 most promising, with varying degrees and stability of field resistance to BCMV, were selected and screened for genes *I*, *bc-1*^{2}, and *bc-3* to reveal donor cultivars for breeding BCMV resistant common bean varieties. In PCR analysis, markers SW13, SBD5, and ROC11 were used.

Tissues of young leaves of individual plants were disrupted in 200 µl CTAB buffer (a ball mill TissueLyser II, Qiagen, Germany) at 26 Hz (1560 oscillations/min) for 1.7 min to a suspension followed by addition of 15 µl of proteinase K. DNA extraction by CTAB method was performed using a set of Sorb-GMO-B reagents (OOO Syntol, Russia) according to the manufacturer's protocol. The final purity and total DNA concentration were determined spectrophotometrically (a Smart Spec Plus, Bio-Rad, USA) ($OD_{260/280}$ = 1.6-1.8 corre-

sponded to a pure DNA preparation).

For 25 μl PCR, 2.5 μl of 10× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM individual dNTPs, 0.3 μM of each primer, 1.5 U SynTaq polymerase (Syntol LLC, Russia), and 50 ng of individual DNA template were mixed. The primer sequences for the three resistance markers were taken from Hegay et al. [49]: for SW13, the forward primer 5′-CACAGCGACATTAATTTTCCTTTC-3′, the reverse primer 5′-CACAGCGACAGGGGAGCTTATTA-3′; for SBD5, the forward primer 5′-GTGCGGAGAGGCCATCCATTGGTG-3′, the reverse primer 5′-GTGCGGAGAGTTTCAGTGTTGACA-3′; for ROC11, the forward primer 5′-CCAATTCTCTTTCACTTGTAACC-3′, the reverse primer 5′-GCATGTTCCAGCAAACC-3′. PCR was run according to the following program: 2-5 min at 95 °C (initial denaturation); 30 s at 95 °C (denaturation), 30 s at 59 °C, 64.6 °C, and 53 °C (annealed for SW13, SBD5, and ROC11, respectively), 30 s at 72 °C (elation) (35 cycles); 7 min at 72 °C (final elongation) (a C1000 Touch Thermal Cycler, Bio-Rad, USA) The primer annealing temperature was adjusted so that clear single and reproducible fragments were obtained.

Amplification products were separated in 1.7% agarose gel in 0.5× TBE buffer (a Wide Mini-Sub Cell GT horizontal electrophoresis chamber, Bio-Rad, USA). The resulting gels were stained with ethidium bromide and photographed (a ChemiDoc XRS + system, Bio-Rad, USA) followed by image processing (ImageLab software, Bio-Rad, USA). The sizes of the amplified fragments were determined using the GeneRuler100 bp Plus DNA Ladder molecular weight marker (Thermo Fisher Scientific, Inc., USA).

Data were processed using LightCycler® 480 SW 1.5.1 software (Roche Molecular Systems, Inc., USA) and Microsoft Excel 2010. Mean values (*M*), their standard deviations (±SD) and standard mean error (±SEM), the level of significance of differences (p) was assessed, regression, variance, correlation analysis, and Chi-square goodness-of-fit test ($\chi 2$) were performed [51].

Results. The origin of the common bean samples used in the work is shown in Figure 1.

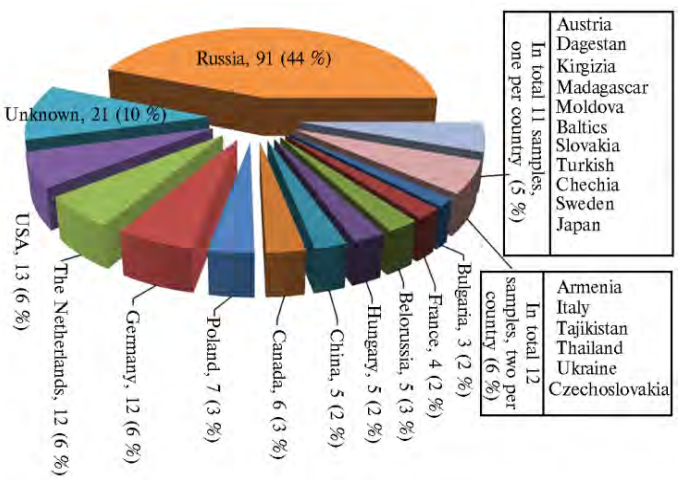

Fig. 1. Origin and number of common bean (*Phaseolus vulgaris* L.) cultivars tested for resistance to Bean common mosaic virus (Genetic collection of the Federal Research Center for Vegetable Growing).

The weather conditions in the years of research differed significantly in the combination of the main climatic factors over growing seasons (Fig. 2), which influenced incidence and severity of the BCMV infection damage.

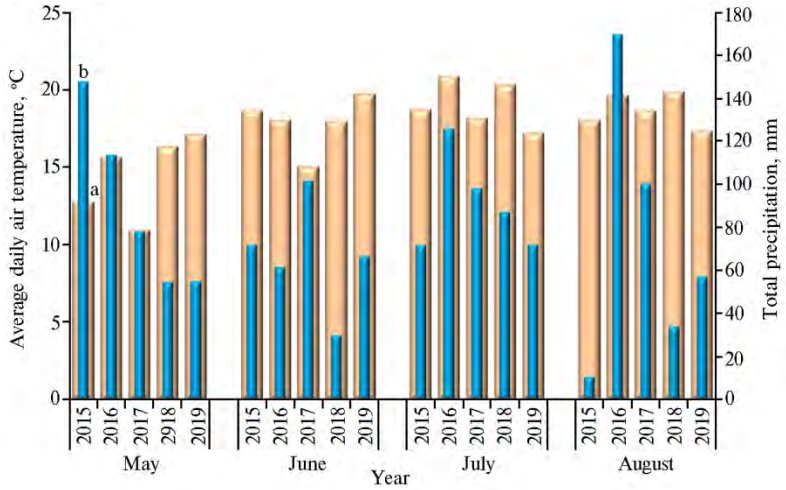

Fig. 2. Average daily air temperature (a) **and total precipitation** (b) **over the years of investigation** (Moscow Province, Odintsovsky District).

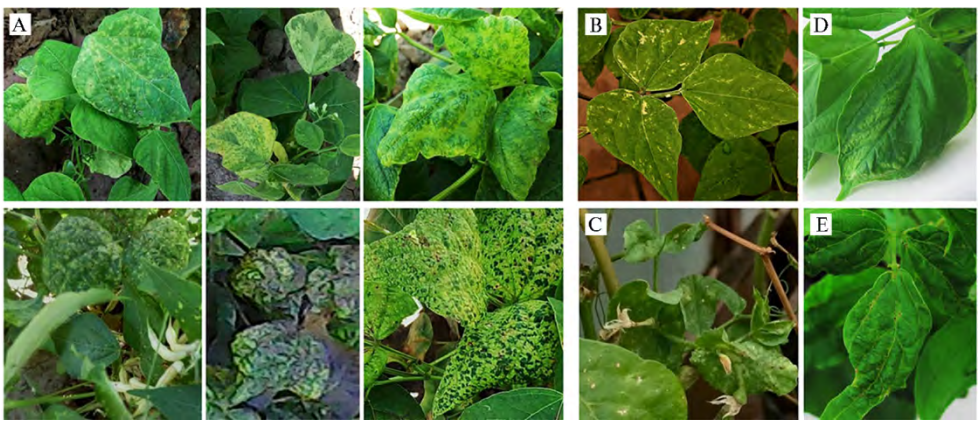

Fig. 3. Symptoms of Bean common mosaic virus (BCMV) **infection in naturally infected common bean (*Phaseolus vulgaris* L.) plants** (A) (Genetic collection of the Federal Research Center for Vegetable Growing, Moscow Province, Odintsovsky District, field surveys, 2014-2019), **and in inoculated test plants of Gribovskaya 92 bean cultivar (**B, C) **and pea (*Pisum sativum* L.) Zhegalovets cultivar** (D, E) (a greenhouse, 2015-2016).

In the Moscow region, bean plants infected by BCMV had typical symptoms of dark green or light green mosaic and leaf deformation (wrinkling and twisting) (Fig. 3, A). ELISA test confirmed the presence of BCMV in plants with different scores of lesions.

In biotests, at temperatures below 26 °C, inoculation with the Moscow BCMV isolate caused weak mosaic of young bean plants. During vegetative phase, a latent (asymptomatic) course of the disease was also noted. However, BCMV had a notable effect on the development of reproductive organs thus reducing plant productivity. At elevated air temperatures (26-29 °C), symptoms typical for BCMV (dark green mosaic, leaf twisting or wrinkling) appeared on susceptible adult plants of Gribovskaya 92 variety followed by leaf necrosis (see Fig. 3, B, C). At the early development, plants of vegetable pea variety Zhegalovets had no symptoms, but, prior the plants entered the flowering stage, a mosaic appeared with subsequent necrosis (see Fig. 3, D, E).

Annual monitoring revealed a significantly increased BCMV prevalence in the Moscow region, starting from 2014. In 2015, 2016 and 2019, the disease

reached epiphytotic levels. The maximum number of affected accessions (90% of the total) was noted in 2016. In 2015 and 2019, the proportion was about 50%. The disease prevalence in these years exceeded the threshold of harmfulness, averaging more than 30% of plants with symptoms of damage (34% in 2015, 81% in 2016, and 36% in 2019), with the highest average damage index (2.7 points) registered in 2019.

In 2017 and 2018, there was a sharp decline in the disease incidence and harmfulness. The prevalence did not exceed 10%, the affected accessions amounted to 22 and 13%, respectively, however, the infectious load in 2018 was higher and the lesion index averaged 2.2 points, whereas in 2017 it was less than 1.0 points (Table 1).

1. Characterization of Bean common mosaic virus (BCMV) **infection of common bean (*Phaseolus vulgaris* L.) accessions in the years of investigation** (Genetic collection of the Federal Research Center for Vegetable Growing, field tests, Moscow Province, Odintsovsky District)

Parameters	Year and a sample							
	2016		2017		2018		2019	
	A	B	A	B	A	B	A	B
Infection parameters and loads								
P, %	81	90	9	42	4	33	36	54
I, points	2,2	2,5	0,2	0,9	0,3	2,2	1,0	2,7
R, %	51	57	2	12	2	18	15	40
Proportion of total number of cultivars, %								
Without symptoms	9		78		87		48	
With symptoms	91		22		13		52	
including:			22		13		52	
0 < R < 10%	4		9		6		6	
R = 10-25%	9		11		4		24	
R > 25%	78		1		3		22	

Note. A — the complete sample mean (for all accessions), B — the affected sample mean (infection load); P (%) — prevalence (disease incidence), I — lesion index (an average score), and R (%) — disease severity.

The development of any disease in a specific cultivation zone is known to depend on plant resistance and the environmental conditions. A five-year study of a constant set of accessions revealed a more significant role of weather conditions in BCMV spreading and harmfulness in the Moscow region. Two-way ANOVA showed the 41% contribution of the climatic factor to total variability of the disease development (the aggregate indicator R), with only 17% for the genotype. Moreover, the weather conditions of the year to a greater extent determined BCMV prevalence (73%) as compared to severity (50%).

Based on the effect of climatic factors on BCMV infection, combinations of favorable and unfavorable conditions for the disease development the Moscow region were revealed. The dependences between the average values P and I, on the one hand, and the average daily air temperature (T, °C) and the amount of precipitation ($\sum p$), on the other hand, for each month of the growing season had a complex character and were described by polynomial functions of second and third orders (Fig. 4). However, a number of patterns could be identified. Moderate rainfall together with moderate temperatures in the first half of the growing season ($\sum p \sim 110$ mm, T ~ 15-16 °C in May, $\sum p \sim 70$ mm, T ~ 18 °C in June) and heavy rainfall combined with increased daily air temperatures in the second half ($\sum p > 120$ mm, T > 20 °C) facilitated intensive spread of the virus on bean crops. Decreased precipitation during all periods of plant growth in combination with higher temperatures ($\sum p < 60$ mm, T > 16 °C in May, $\sum p < 70$ mm, T > 18 °C in June, $\sum p < 85$ mm, T > 20 °C in July, and $\sum p < 60$ mm, T > 19 °C in July) as a whole restrained the pathogen spread. However, this combination of factors contributed to more apparent symptoms of

the virus affecting leaves, especially in early growth (see Fig. 4). Thus, a high score of BCMV lesion was noted in years with May average daily temperatures of 16-18 °C and minimum amount of precipitation (2018 and 2019), or with June air temperature of 18-20 °C and moderate rains (2016 and 2019). In the second half of the growing season, the combination of contributing factors was less apparent. It was both cool (T ~ 17.2 °C) and dry ($\sum p$ ~ 72 mm) weather in July 2019 (as compare to average annual rates of 18.8 °C and 81.6 mm), and elevated temperatures (T > 20 °C) with heavy precipitation (Σ~87-126 mm) in 2016 and 2018.

Dry and hot weather in certain periods, mainly in the middle of the growing season, could decrease lesion intensity in relatively stable accessions, as it was in 2015, 2017, and 2019. In 2019, clear symptoms of BCMV lesion appeared in 52% of samples at the beginning of growth (early June), in 36% at the beginning of fruiting, in mid-July when the weather was hot and dry, and in 47% after a cold snap at the end of the growing season. These results are consistent with the observed "masking" of symptoms due to high temperatures. [6, 22]. Therefore, the BCMV infection should be assessed at least twice, in the first and second half of the growing season, and it is the maximum seasonal values of P and I that should be used to rank the BCMV resistance of the sample.

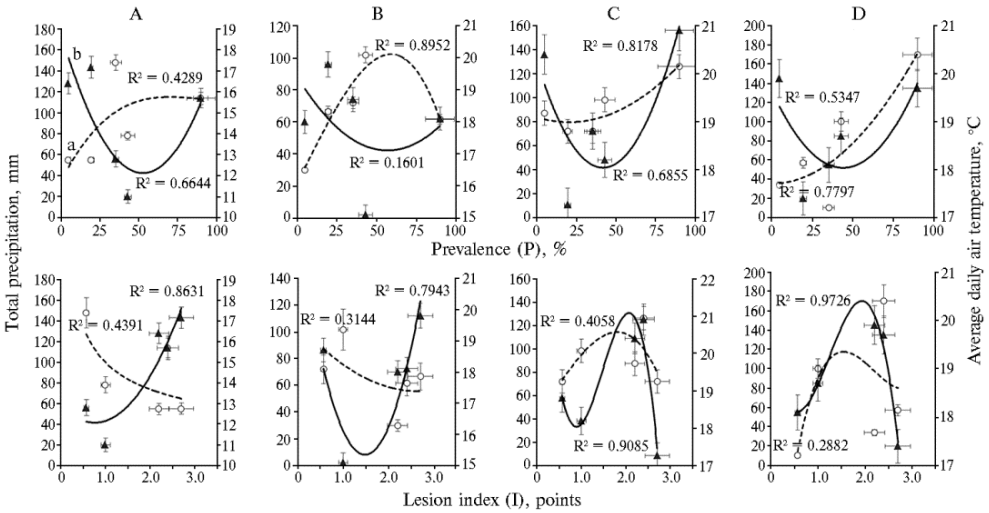

Fig. 4. A relationship between parameters (*M*±SD) **of Bean common mosaic virus** (BCMV) **infection of common bean (*Phaseolus vulgaris* L.) plants and climatic conditions** (the complete sample of accessions, Genetic collection of the Federal Research Center for Vegetable Growing)**:** A — May, B — June, C — July, D — August; a, b — polynomial functions of incidence and severity of BCMV infection plotted against the average daily air temperature (°C, triangles) and the total amount of precipitation (mm, circles) (Moscow Province, Odintsovsky District, 2015-2019).

Stability of plant viral resistance in years varying in combination of environmental factors and natural infectious loads should be accounted in searching for sources of resistance. According to this criterion, the collection samples were divided into four groups of stability (Table 2).

The accessions of group I had no BCMV symptoms in all years of investigation. The resistant forms constituted 6% of the complete sample. Most of them were varieties of domestic selection: Zabava (Russia, VIR k-15356), Izunrudnaya (Russia, VIR k-15593), Khavskaya universalnaya (Russia), Mulatka (Russia), Local vegetable beans (Russia, VIR k-15673), Zolotoi nectar (Russia), Zapadnaya Sibir (Russia), Oktava (Belarus), Ryabushka (Russia), Zit 551 RS (the Neth-

erlands, VIR k-15375), Cade 128 (the Netherlands, VIR k-15261), and Alice Sunshine (USA, VIR k-15599).

Susceptible cultivars (group IV, 2% of the complete sample) were stably infected by the virus regardless of the conditions of the year. These were Kirgizskaya Sakharnaya (Kyrgyzstan), Kustovaya (Russia), Zatus (Poland), and Gribovskaya 92 (Russia, VIR k-12200). The prevalence of BCMV in this group averaged 78% over four years (an average lesion index of 2.5 points) and reached 100% in the epiphytotic years (an average lesion index of 3-4 points).

2. Stability of resistance of common bean (*Phaseolus vulgaris* L.) accessions to Bean common mosaic virus (BCMV) (Genetic collection of the Federal Research Center for Vegetable Growing, field tests, Moscow Province, Odintsovsky District, 2015-2019)

Parameters	Stability group				LSD_{05}
	I	II	III	IV	
Averaged infection parameters					
P, %	0	26 (0-100)	38 (0-100)	78 (1-100)	9
I, points	0	0.8 (0-2.6)	1.2 (0-4.0)	2.5 (0.5-4.0)	0.3
R, %	0	17	21	45	6
Proportion of total number of cultivars, %					
Without symptoms	6	0	0	0	6
With symptoms	0	62	30	2	94
including:					
0 < R < 10 %	0	14	1	0	15
R = 10-25 %	0	41	7	1	49
R > 25 %	0	7	22	1	30

Note. I — no symptoms, II — symptoms appeared only in the epiphytotic years, III — unstable symptoms over years, IV — stable lesion occurred in all years of investigations (the range of variation of the indicator between years and samples within a group is indicated in brackets, min-max,); P (%) — prevalence (disease incidence), I — lesion index (an average score), and R (%) — disease severity.

In the largest group II, BCMV infection was recorded in the epiphytotic years 2016 and 2019, with 30% accessions affected in both years, 61% in 2016, and 9% in 2019. Group III showed unstable BCMV resistance. Viral symptoms appeared regardless of the year, including years when the infectious load was low (in 2017 and 2018).

It is worth noting that the four-year mean values of prevalence and lesion indexes in group III were significantly higher compared to group II ($\chi 2 = 3.8$ at $p = 0.05$ and $\chi 2 = 6.6$ at $p = 0.01$, respectively) due to the greater proportion of accessions with R > 25% (see Table 2). However, the average values for 2016 and 2019 indicated that approximately 1% and 4% of the complete sample (in group II and group III, respectively) were highly susceptible to BCMV (R > 75%) during epiphytoties. That is, only in epiphytotic years, it is possible to correctly assess the resistance to BCMV, provided that the combinations of weather conditions in these years differ. Indicators averaged even over several years do not provide reliable estimates. This should be taken into account when selecting valuable samples. In the years with a low infectious load, only negative selection (i.e. culling of susceptible specimens) must be applied.

Primers to the SW13 marker closely linked to the dominant gene *I* generated a 690 bp PCR amplification product, as described [50]. Primers to the SBD5 marker gave a 1300 bp PCR amplification product, indicating the *bc-1²* gene [28, 39]. Primers to the ROC11 marker linked to the *bc-3* gene amplified DNA fragments of 300 bp, whereas in earlier studies it was noted that the size of the product should reach 420 bp [31, 40]. However, other researchers also found deviations in the size of the amplified PCR product when working with this marker [52]. Genetic marking of three resistance genes, *I*, *bc-1²*, and *bc-3* revealed the *bc-3* gene in most of the 30 studied accessions (Table 3).

3. DNA marking of *R*-genes of common bean (*Phaseolus vulgaris* L.) accessions (Genetic collection of the Federal Research Center for Vegetable Growing) **different in stability and field resistance to Bean common mosaic virus** (BCMV)

Stability group	Accessions	Disease severity R, %	Marker (gene)			Extinction coefficient
			SW13 (*I*)	SBD5 (*bc-1²*)	ROC11 (*bc-3*)	
I	Cade 128	0	+	+	+	0
	SP-232	0	–	+	+	0
	Khavskaya universalnaya	0	–	+	+	0
II	Vestochka	2,5	–	+	+	0.115
	Rant	2,9	–	+	+	0.118
	Kit-79	3,3	+	+	+	nt
	Veritsa	7,7	+	+	+	nt
	KP-84	10,7	+	+	+	nt
	Fatima	13,5	+	+	–	0.115
	Montdor	14,0	–	+	+	0.100
	Purpurnaya	20,7	+	+	+	nt
	Holberg	23,3	+	–	–	0.281
	Poroto Evestad	30,0	–	+	+	0.280
	Arion	31,7	+	+	+	nt
	Niagara 776	37,2	+	–	+	0.352
III	SP-164	4,0	–	+	+	nt
	SP-220	5,0	+	+	+	nt
	Zolushka	6,0	–	+	+	0.112
	MBZ 556	11,1	–	–	–	0.250
	Sparzhevaya	11,7	–	+	+	0.114
	Secunda	12,3	–	+	+	0.230
	Rubin	16,2	–	+	+	nt
	Pluto	17,7	+	+	+	0.114
	Kentuky Wander	19,3	–	+	+	nt
	Dilano	20,7	+	+	+	nt
	Rannyaya voskovaya	25,0	+	+	+	nt
IV	Lika	7,8	–	+	+	0.114
	Kustovaya	17,1	–	+	+	0.240
	gribovskaya 92	52,1	–	–	–	0.561

Note. I — no symptoms, II — symptoms appeared only in the epiphytotic years, III — unstable symptoms over years, IV — stable lesion occurred in all years of investigations; nt — not tested. The extinction coefficient is given according to the ELISA test performed to confirm the presence of BCMV in the specimens.

In lab tests, on day 14 after inoculation of 30 promising accessions of various origin with BCMV, typical symptoms (mosaic and leaf deformation) appeared only in plants lacking resistance genes *I* and *bc-1²*. In genotypes harboring these genes, depending on their combination, a hypersensitivity-like response occurred as punctate dry necrosis, yellowing and wilting of infected leaves. Moreover, the gene *I* enhanced wilting which proceeded faster, and on day 10, the infected leaves were practically dry and easily separated from the plant. This was confirmed by significant correlation coefficients between the presence of the gene *I* and the scoring of leaf wilting, *r* from +0.64 to +0.74 depending on the gene combinations. The *bc-1²* in the genome, even in the absence of gene *I*, prevented wrinkling (*r* from –0.59 to –0.73), and reduced the number of necrosis along the veins (r from –0.35 to –0.53).

When comparing the data of field and lab resistance tests, a negative relationship was traced between the degree of BCMV field harmfulness and the intensity of leaf yellowing in seedlings (*r* from –0.52 to –0.85, depending on the gene combinations), as well as a positive relationship with wrinkling (*r* from +0.69 to +0.72). However, we did not reveal a consistently close relationship between the presence of one or another resistance gene and the field resistance of genotypes in different years. Depending on the intensity of the infectious load, it varied from weak to medium or was absent. In some years, there was a relationship between the average score of plant damage and the genes *I* (r from –0.33 to –0.71) and *bc-1²* (*r* from –0.35 to –0.57). Analysis of the average parameters of BCMV infection over all the years of investigation using $\chi 2$ test revealed a significant effect of only the *bc-1²* gene on the field resistance of vege-

table beans in the Moscow region (Table 4).

For all correlation coefficients we obtained, $r_{critical} = 0.36$ at $p = 0.05$.

4. Chi-square-based correspondence between *R*-genes of resistance to Bean common mosaic virus (BCMV) **and field susceptibility to BCMV common bean (*Phaseolus vulgaris* L.) accessions** (Genetic collection of the Federal Research Center for Vegetable Growing, Moscow Province, Odintsovsky District, 2016-2019)

Parameters	*I*	*bc-1²*	*bc-3*	*I* + *bc-1²*	*I* + *bc-1²* + *bc-3*
Number of degrees of freedom	3	3	3	6	8
$\chi^2_{fact.}$ value	0,312	8,334	3,111	10,437	14,844
$\chi^2_{crit.}$ at $p < 0.05$	7,815	7,815	7,815	12,592	15,507
Correspondence at p level	Not found p = 0.958	Found p = 0.040	Not found p = 0.375	Not found p = 0.475	Not found p = 0.875

Nevertheless, analysis of various combinations of *I*, *bc-1²*, and *bc-3* genes with respect to BCMV infection severity (R) clearly indicates that the resistant forms, which showed no signs of BCMV infection during epiphytoty of 2019, can only be detected among genotypes +/+/+ and –/+/+ (Fig. 5, A, B). They amounted to 40 and 13%, respectively, which confirms the data available in the literature on the enhanced protective functions in plants with a combination of resistance genes *I* and *bc-1²*, although not in all cases this combination guarantees 100% efficiency. In half of the accessions tested, the infection severity was lower (R < 10%); 10% of +/+/+ genotypes and 33% of –/+/+ genotypes showed moderate resistance (R = 10-25%). In the absence of the *bc-3*, combinations +/+/– and +/–/– conferred little field protection. These genotypes turned out to be moderately susceptible, and in the absence of all resistance genes genotypes were moderately and highly susceptible to BCMV.

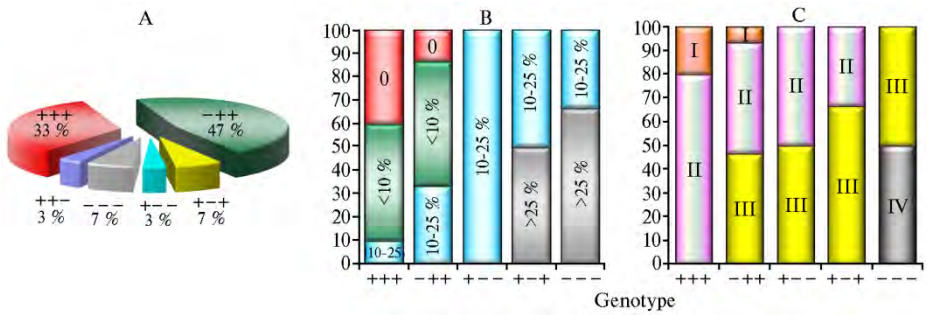

Fig. 5. Combinations of genes *I*, *bc-1²*, and *bc-3* ("+" for presence, "—" for absence the gene) **among 30 promising genotypes of common bean (*Phaseolus vulgaris* L.)** (Genetic collection of the Federal Research Center for Vegetable Growing) (A)**, and their distribution with respect to severity of Bean common mosaic virus** (BCMV) **infection** (R) **in 2019** (B) **and BCMV resistance stability in 2016-2019 in field conditions** (C)**:** R = 0 — resistant, 0 < R ≤ 10 % — relatively resistant, 10 < R ≤ 25 % — low-susceptible, R > 25 % — susceptible; I — no symptoms, II — symptoms appeared only in the epiphytotic years, III — unstable symptoms over years, IV — stable lesion occurred in all years of investigations (Moscow Province, Odintsovsky District).

Involvement of nonspecific resistance genes *I* and *bc-3* in breeding programs worldwide resulted in the emergence of new BCMV strains that overcoming plant resistance that these genes confer, also, the closely related BCMNV virus is spreading which affects BCMV-resistant varieties [9, 22]. In 2018, Feng et al. [22] showed that the use of the recessive resistance genes *bc-1* and *bc-2*, alone or in combination (even without the dominant gene I) is effective in breeding for resistance to a number of BCMV pathotypes. These genes do not affect replication and cell-to-cell movement of the virus, but they affect its systemic spread. We have found that a particular combination of genes does not always

5. Characterization of agronomically valuable genotypes of common bean (*Phaseolus vulgaris* L.) (Genetic collection of the Federal Research Center for Vegetable Growing) **resistant to Bean common mosaic virus** (BCMV) **in the conditions of Moscow region** (2016-2019 годы)

Name (origin)	A	B	C	Pods (technical maturity), *M*±SEM						Seeds (biological maturity), *M*±SEM			
				D	E	F	G	H	I	J	K	L	M
Resistant (group I)													
Izumrudnaya (Russia)	0	3	10.5±0.8	dg	0	12.5±1.2	0.9±0.1	0.85±0.07	90±4.1	w	4.5±0.2	250±14.5	20±1.5
Zapadnaya Sibir (Russia)	0	3	13.0±1.2	y	0	12.5±1.1	0.9±0.1	0.82±0.08	96±4.5	w	4.3±0.2	289±13.7	19±1.2
Zabava (Russia)	0	3	10.5±1.0	g	0-1	10.0±0.9	0.8±0.0	0.65±0.05	82±3.7	w	4.1±0.2	279±15.2	18±1.3
Khavskaya universalnaya (Russia)	0	3	10.1±0.9	g	0-1	9.8±0.8	1.0±0.1	0.90±0.07	82±3.4	w	3.9±0.2	235±13.4	15±1.1
Mulatks (Russia)	0	2	14.5±1.3	v	0-1	13.5±1.2	1.0±0.1	0.85±0.06	91±3.6	lb	5.1±0.3	302±17.5	26±2.1
Oktava (Belarus)	0	2	11.5±0.9	g	0	13.8±1.2	1.4±0.1	1.05±0.09	95±3.9	bl	4.4±0.2	204±12.3	16±1.3
Ryabushka (Russia)	0	3	10.5±0.8	y	0-1	14.2±1.3	1.5±0.1	0.70±0.05	71±3.2	bl	4.3±0.2	250±15.4	15±1.2
Zit 551 RS (the Netherlands)	0	3	11.5±0.8	g	0-1	9.5±0.7	1.3±0.1	0.80±0.04	74±3.5	w	3.9±0.2	260±17.5	14±1.1
Cade 128 (the Netherlands)	0	4	14.5±1.2	g	0-1	9.5±0.8	0.9±0.1	0.65±0.04	80±4.1	w	3.7±0.2	271±12.7	16±1.3
Zolotoi nektar[c] (Russia)	0	5	9.5±0.7	g	0	17.4±1.5	1.4±0.1	0.85±0.05	178±9.5	w	5.5±0.3	284±13.8	34±2.7
Fasol mestnaya ovoshchnaya[c] (Russia)	0	5	11.5±0.8	g	0-1	10.5±0.8	0.8±0.1	0.70±0.03	160±8.7	w	5.3±0.2	207±9.7	22±1.4
Relatively resistant (groups II and III)													
Zinuly (Belarus)	5.6	3	12.2±0.9	g	0	10.5±0.8	1.0±0.1	0.93±0.08	74±4.1	w	3.8±0.2	337±18.3	15±0.9
Bertires (the Netherlands)	0.6	3	9.5±0.7	g	0-1	9.5±0.7	0.8±0.1	0.75±0.06	88±4.7	w	4.1±0.2	285±15.9	15±1.0
Fanacnos (Poland)	2.0	5	10.5±0.8	g	0-1	9.5±0.8	1.0±0.1	0.76±0.05	74±4.5	w	3.9±0.2	275±14.3	13±0.8
Верица (Bulgaria)	9.7	3	10.5±0.9	g	0-1	9.5±0.7	1.1±0.1	0.70±0.04	75±4.3	w	3.9±0.2	319±16.4	19±1.1
Purpiat (Poland)	8.8	2	13.5±1.1	v	0-1	10.5±0.7	1.3±0.1	0.72±0.04	92±5.1	b	3.9±0.2	427±17.2	18±1.0
Kit-№ 79 (China)	6.1	2	17.5±1.3	g	0-1	15.5±0.9	1.3±0.1	0.95±0.07	115±6.7	bl	4.9±0.2	367±15.8	29±2.2

Note. A — BCMV resistance (R, %); B — ripening groups (2 — precocious, 3 — early-ripening, 4 — mid-early ripening, 5 — mid-ripening), C — lower pod attachment height, cm; D — color, E — stringiness, points; F — length, cm; G — width, cm; 3 — thickness, cm; H — yield, g per plant; I — color (dg — dark green, g — green, y — yellow, v — violet фиолетовая, w —white, lb — light brown, b — beige, bl — black); J — number per pod; K — 1000-seed weigh, g; L — yield, g per plant); [c] — climbing bean plants.

provide field resistance to the virus. The resistance stability of a cultivar in different years is influenced by climatic conditions which largely determine the physiological state of the plant, changing expression of *R*-genes responsible for the immune response and appearance of symptoms during infection. Both the dominant gene *I* combination with recessive genes *bc-1²* and *bc-3*, and the recessive combination without dominant gene provide high BCMV resistance. The Cade 128, SP-232, Veritsa, Kit-89, KP-84, Khavskaya universalnaya, Vestochka, and Rant cultivars and breeding samples of resistance stability groups I and (+/+/+ and –/+/+, see Fig. 5, B) are of practical interest for breeding.

When selecting the initial material for target selection, it is important that the samples have not only the necessary genes and high field resistance to the pathogen, but other economically important traits. From this point of view, relatively stable accessions (R < 10% in the epiphytotic years) from groups II and III, which possess a number of valuable characters, in addition to resistant forms from stability group I, are of interest. Among them, 17 accessions have the highest breeding value (Table 5).

Selection of parents for breeding commercial varieties for industrial cultivation are based on following characteristics: early maturity, stable yield, sugar beans, high technological parameters (Izumrudnaya, Khavskaya universalnaya, Oktava, Mulatka as genetic sources); high pod attachment, sugar beans, multiple resistance to diseases (Mulatka, Kit-№ 79, Purpiat, Cade 128); straight long sugar beans (Golden nectar, Mulatka, Ryabushka); suitability for freezing and canning (Octave, Mulatto, Emerald), as well as for some other characteristics

Among the farmers, beans of climbing type are in demand, which allow obtaining fresh young pods during growing season, or varieties of the determinant type but with a long season of pods picked repeatedly yield productions. From this point of view, the samples of interest are Zolotoi nectar, Kit-№ 79, Fasol mestnaya ovoshchnayac, and two varieties bred at FRCVG, Ulyasha and Malume (the latter of climbing type, was submitted for State testing in 2019). The most popular varieties bred at FRCVG are highly resistant to bacterial, fungal and viral diseases [43, 44], including BCMV. These are Mariinka, Svetlyachok, Kreolka, Rant, and Pagoda of groups II and III. In survey of crops of these varieties, severity of BCMV infection over the years averaged 10% at most. Possessing a complex of other economically valuable traits [53], these varieties can also be genetic sources of resistance to BCMV.

Thus, global climate change has intensified the spread of Bean common mosaic virus (BCMV) to the northern regions of the Russian Federation. In the Moscow region, the prevalence of the virus is more influenced by the amount of precipitation, and the lesion index by the air temperature. The BCMV epiphytoties were recorded in 2015, 2016, and 2019. In searching for genetic sources of BCMV resistance, combined estimates of a particular sample must be used. These are disease severity and resistance stability evaluated over several years with different combinations of weather conditions that determine BCMV infectious load. The $\chi 2$ test revealed a significant effect of the *bc-1²* gene on field resistance of bean plants to BCMV. In the local climatic conditions, genotypes *I/bc-1²/bc-3* and *–/bc-1²/bc-3* are of the greatest value as sources of *R*-genes. Seventeen most promising breeding samples of various origins, five varieties (Khavskaya universalnaya, Rant, Zolushka, Mariinka, and Svetlyachok), and two promising cultivars possessing a set of economically valuable traits (SP-232, KP-84, bred at FRCVG) are suggested as genetic sources for breeding asparagus-type beans highly resistant to BCMV.

REFERENCES

1. De Ron A.M. Grain legumes. In: *Handbook of plant breeding*. A.M. De Ron (ed.). Pontevedra, 2015 (doi: 10.1007/978-1-4939-2797-5).
2. Broughton W.J., Hernández G., Blair M., Beebe S., Gepts P., Vanderleyden J. Beans (*Phaseolus* spp.) — model food legumes. *Plant and Soil*, 2003, 252: 55-128 (doi: 10.1023/A:1024146710611).
3. *FAOSTAT — Food and Agriculture Organization*. Available: http://faostat.fao.org. No date.
4. Vishnyakova M.A., Bulyntsev S.V., Burlyaeva M.O., Buravtseva T.V., Egorova G.P., Semenova E.V., Seferova I.V. *Ovoshchi Rossii*, 2013, 1(18): 16-25 (doi: 10.18619/2072-9146-2013-1-16-25) (in Russ.).
5. Verhoeven Th.J., Roenhorst J.W., Lesemann D.E., Segundo E., Velasco L., Ruiz L., Janssen D., Cuadrado I.M. Southern bean mosaic virus the causal agent of a new disease of *Phaseolus vulgaris* beans in Spain. *European Journal of Plant Pathology*, 2003, 109: 935-941 (doi: 10.1023/B:EJPP.0000003673.10046.2f).
6. Gnutova R.V. Virusnye infektsii ovoshchnykh bobovykh kul'tur i soi na Dal'nem Vostoke. *Zashchita i karantin rastenii*, 2013, 1: 14-17 (in Russ.).
7. Reddick D., Stewart V.B. Transmission of the virus of bean mosaic in seed and observations on thermal death point of seed and virus. *Phytopathology*, 1919, 9: 445-450.
8. Flores-Estévez N., Acosta-Gallegos J.A., Silva-Rosales L. *Bean common mosaic virus* and *Bean common mosaic necrosis virus* in Mexico. *Plant Disease*, 2003, 87: 21-25 (doi: 10.1094/PDIS.2003.87.1.21).
9. Singh S.P., Schwartz H.F. Breeding common bean for resistance to diseases: a review. *Crop Science*, 2010, 50(6): 2199-2223 (doi: 10.2135/cropsci2009.03.0163).
10. Zhou G.-C., Wu X.-Y., Zhang Y.-M., Wu P., Wu X.-Z., Liu L.-W., Wang Q., Hang Y.-Y., Yang J.-Y., Shao Z.-Q., Wang B., Chen J.-Q. Genomic survey of thirty soybean-infecting bean common mosaic virus (BCMV) isolates from China pointed BCMV as a potential threat to soybean production. *Virus Research*, 2014, 191: 125-133 (doi: 10.1016/j.virusres.2014.07.029).
11. Biddle A.J. *Peas and beans. Crop production science in horticulture*. R. Russel, A. Lainsbury (eds.). Boston, 2017.
12. Verma P., Gupta U.P. Immunological detection of bean common mosaic virus in French bean (*Phaseolus vulgaris* L.) leaves. *Indian J. Microbiol.*, 2010, 50: 263-275 (doi: 10.1007/s12088-010-0019-8).
13. Worrall E.A., Wamonje F.O., Mukeshimana G., Harvey J.J.W., Carr J.P., Mitter N. *Bean common mosaic virus* and *Bean common mosaic necrosis virus*: relationships, biology and prospects for control. In: *Advances in virus research. V. 93*. M. Kielian, K. Maramorosch, T.C. Mettenleiter (eds.). Academic Press, NY, 2015: 1-46 (doi: 10.1016/bs.aivir.2015.04.002).
14. Polivanova T.A., Krylov A.V. V knige: *Vzaimootnosheniya virusov s kletkami rasteniya-khozyaina*. [In: The relationship of viruses with cells of the host plant]. Vladivostok, 1985: 87-93 (in Russ.).
15. Tolkach V.F., Gnutova R.V. *Doklady RASKHN*, 1998, 5: 18-19 (in Russ.).
16. Schippers B. Transmission of bean common mosaic virus by seed of *Phaseolus vulgaris* L. cv. Beka. *Acta Botanica Neerlandica,* 1963, 12(4): 433-497 (doi: 10.1111/j.1438-8677.1963.tb00130.x).
17. Drijfhout E. *Genetic interaction between Phaseolus vulgaris L. and bean common mosaic virus with implications for strain identification and breeding for resistance*. Wageningen, 1978.
18. Gnutova R.V., Zolotareva E.V. *Bolezni ovoshchnykh kul'tur i kartofelya na Dal'nem Vostoke Rossii* [Diseases of vegetable crops and potatoes in the Russian Far East]. Vladivostok, 2011 (in Russ.).
19. Chekalin N.M. *Geneticheskie osnovy selektsii zernobobovykh kul'tur na ustoichivost' k patogenam* [Genetic basis for breeding leguminous crops for resistance to pathogens]. Poltava, 2003 (in Russ.).
20. *Diagnosis of plant virus diseases*. R.E.F. Matthews (ed.). CRC Press, NY, 2018 (doi: 10.1201/9781351071352).
21. Larsen R.C., Miklas P.N., Druffel K.L., Wyatt S.D. NL-3 strain is a stable and naturally occurring interspecific recombinant derived from *Bean common mosaic necrosis virus* and *Bean common mosaic virus*. *Phytopatholog*y, 2005, 95(9): 1037-1042 (doi: 10.1094/phyto-95-1037).
22. Feng X., Orellana G.E., Myers J.R., Karasev A.V. Recessive resistance to *bean common mosaic virus* conferred by the *bc-1* and *bc-2* genes in common bean (*Phaseolus vulgaris*) affects long-distance movement of the virus. *Phytopathology*, 2018, 108(8): 1011-1018 (doi: 10.1094/phyto-01-18-0021-R).
23. Feng X., Guzmán P., Myers J.R., Karasev A.V. Resistance to *bean common mosaic necrosis virus* conferred by the *bc-1* gene affects systemic spread of the virus in common bean. *Phytopathology*, 2017, 107(7): 893-900 (doi: 10.1094/phyto-01-17-0013-R).
24. Flasinski S., Gunasinghe U.B., Gonzales R.A., Cassidy B.G. The cDNA seqence and infectious transcripts of peanut stripe virus. *Gene,* 1996, 171(2): 299-308 (doi: 10.1016/0378-1119(96)00010-8).
25. Li Y., Cao Y., Fan Z., Wan P. Identification of a naturally ocurring *Been common mosaic virus* recombinant isolate infecting azuki bean. *Journal of Plant Pathology*, 2016, 98: 129-133 (doi:

10.4454/JPP.V98I1.071).
26. Kelly J.D. A review of varietal response to bean common mosaic potyvirus in *Phaseolus vulgaris*. *Plant Varieties & Seeds*, 1997, 10(1): 1-6.
27. Li Y.Q., Liu Z.P., Yang Y.S., Zhao B., Fan Z.F, Wan P. First report of bean common mosaic virus infecting azuki bean (*Vigna angularis*) in China. *Plant Disease*, 2014, 98: 1017 (doi: 10.1094/PDIS-01-14-0064-PDN).
28. Kelly J.D., Afanador L., Haley S.D. Pyramiding genes for resistance to bean common mosaic virus. *Euphytica*, 1995, 82: 207-212 (doi: 10.1007/BF00029562).
29. Naderpour M., Johansen I. E. Visualization of resistance responses in *Phaseolus vulgaris* using reporter tagged clones of *Bean common mosaic virus*. *Virus Research,* 2011, 159(1): 1-8 (doi: 10.1016/j.virusres.2011.04.004).
30. Naderpour M., Lund, O. S., and Johansen, I. E. Sequence analysis of expressed cDNA of Bean common mosaic virus RU1 isolate. *Iran J. Virus*, 2009, 3: 41-43.
31. Mukeshimana G., Pañeda A., Rodríguez-Suárez C., Ferreira J.J., Giraldez R., Kelly J.D. Markers linked to the bc-3 gene conditioning resistance to bean common mosaic potyviruses in common bean. *Euphytica*, 2005, 144: 291-299 (doi: 10.1007/s10681-005-7397-8).
32. Haley S.D., Afanador L., Kelly J.D. Identification and application of a random amplified polymorphic DNA marker for the *I* gene (potyvirus resistance) in common bean. *Phytopathogy*, 1994, 84: 157-160 (doi: 10.1094/phyto-84-157).
33. Melotto M., Afanador L. Kelly J.D. Development of a SCAR marker linked to the *I* gene in common bean. *Genome*, 1996, 39(6): 1216-1219 (doi: 10.1139/g96-155).
34. Miklas P.N., Larsen R.C., Riley R., Kelly J.D. Potential marker-assisted selection for *bc-1*^{2} resistance to bean common mosaic potyvirus in common bean. *Euphytica*, 2000, 116(3): 211-219 (doi: 10.1023/a:1004006514814).
35. Vandemark G.J., Miklas P.N. Genotyping common bean for the potyvirus resistance alleles *I* and *bc-1*^{2} with a multiplex real-time polymerase chain reaction assay. *Phytopathology*, 2005, 95: 499-505 (doi: 10.1094/phyto-95-0499).
36. Strausbaugh C.A., Myers J.R., Forster R.L., McClean P.E. *Bc-1* and *bc-u* — two loci controlling bean common mosaic virus resistance in common bean are linked. *Journal of the American Society for Horticultural Science*, 1999, 124(6): 644-648 (doi: 10.21273/JASHS.124.6.644).
37. Miklas P.N., Hang A.N., Kelly J.D., Strausbaugh C.A., Forster R.L. Registration of three kidney bean germplasm lines resistant to bean common mosaic and necrosis potyviruses: USLK-2 light red kidney, USDK-4 dark red kidney, and USWK-6 white kidney. *Crop Science*, 2002, 42(2): 674-675 (doi: 10.2135/cropsci2002.6740).
38. Pastor-Corrales M.A., Kelly J.D., Steadman J.R., Lindgren D.T., Stavely J.R., Coyne D.P. Registration of six great Northern bean germplasm lines with enhanced resistance to rust and bean common mosaic and necrosis potyviruses. *Plant Registrations,* 2007, 1(1): 77-79 (doi: 10.3198/jpr2005.12.0517crg).
39. Sharma P.N., Pathania A, Kapil R., Sharma P., Sharma O.P., Patial M., Kapoor V. Resistance to bean common mosaic potyvirus strains and its inheritance in some Indian land races of common bean. *Euphytica*, 2008, 164: 173-180 (doi: 10.1007/s10681-008-9689-2).
40. Johnson W.C., Guzmán P., Mandala D., Mkandawire A.B.C., Temple S., Gilbertson R.L., Gepts P. Molecular tagging of the *bc-3* gene for introgression into Andean common bean. *Crop Science*, 1997, 37(1): 248-254 (doi: 10.2135/cropsci1997.0011183X003700010044x).
41. Engalycheva I.A., Kozar' E.G., Antoshkin A.A., Pronina E.P., Volkov Yu.G., Kakareka N.N., Shchelkanov M.Yu., Gapeka A.V. *Ovoshchi Rossii*, 2018, 6(44): 77-83 (doi: 10.18619/2072-9146-2018-6-77-83 (in Russ.).
42. Rakina M.S. *Bioresursnyi potentsial zernobobovykh kul'tur iz kollektsii mirovogo genofonda vserossiiskogo nauchno-issledovatel'skogo instituta rastenievodstva im. N.I. Vavilova. Avtoreferat kandidatskoi dissertatsii* [Bioresource potential of leguminous crops from the world gene pool collection of the Vavilov All-Russian Research Institute of Plant Industry. PhD Thesis]. Novosibirsk, 2011 (in Russ.).
43. Lazareva E.K. *Morfobiologicheskie i biokhimicheskie osobennosti sortoobraztsov fasoli obyknovennoi (Phaseolus vulgaris L.) v usloviyakh Orlovskoi oblasti. Avtoreferat kandidatskoi dissertatsii* [Morphobiological and biochemical characteristics of common bean varieties (*Phaseolus vulgaris* L.) in the conditions of the Orel region. PhD Thesis]. Ramon', 2006 (in Russ.).
44. Pletneva M.M. *Otsenka obraztsov fasoli obyknovennoi po khozyaistvenno-tsennym priznakam i kachestvu zerna dlya selektsii v yuzhnoi lesostepi Zapadnoi Sibiri. Avtoreferat kandidatskoi dissertatsii* [Evaluation of common bean samples for economically valuable traits and grain quality for breeding in the southern forest-steppe of Western Siberia. PhD Thesis]. Omsk, 2019 (in Russ.).
45. Engalycheva I.A., Pleshakova T.I., Gapeka A.V., Timina L.T. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii* molodykh uchenykh i spetsialistov «*Povyshenie effektivnosti sel'skokhozyaistvennoi nauki v sovremennykh usloviyakh materialy mezhdunarodnoi nauchno-prakticheskoi konferentsii molodykh uchenykh i spetsialistov*» [Proc. Int. Conf. «Improving the ef-

ficiency of agricultural science in modern conditions materials of the international scientific and practical conference of young rsearchers and practitioners»]. Krasnodar, 2015, 41-44 (in Russ.).
46. Mills L.J., Silbernagel M.J. A rapid screening technique to combine resistance to halo blight and bean common mosaic virus in *Phaseolus vulgaris* L. *Euphytica*, 1991, 58: 201-208 (doi: 10.1007/BF00025251).
47. *Metodicheskie ukazaniya i rekomendatsii po selektsii i semenovodstvu ovoshchnykh bobovykh i kapustnykh kul'tur* /Pod redaktsiei V.F. Pivovarova, N.S. Tsyganka [Methodical instructions and recommendations for the selection and seed production of vegetable legumes and cabbage crops. V.F. Pivovarov, N.S. Tsyganok (eds.)]. Moscow, 2001 (in Russ.).
48. *Metodicheskie ukazaniya po selektsii i pervichnomu semenovodstvu ovoshchnykh bobovykh* [Guidelines for selection and primary seed production of vegetable legumes]. Moscow, 1985 (in Russ.).
49. Hegay S., Ortiz R., Garkava-Gustavsson L., Hovmalm H.P, Geleta M. Marker-aided breeding for resistance to *bean common mosaic virus* in Kyrgyz bean cultivars. *Euphytica*, 2013, 193(1): 67-78 (doi: 10.1007/s10681-013-0928-9).
50. Vallejos C.E., Astua-Monge G., Jones V., Plyler T.R., Sakiyama N.S., Mackenzie S.A. Genetic and molecular characterization of the I locus of Phaseolus vulgaris. *Genetics*, 2006, 172(2): 1229-1242 (doi: 10.1534/genetics.105.050815).
51. Dospekhov B.A. *Metodika polevogo opyta* [Methods of field trials]. Moscow, 1975 (in Russ.).
52. Pasev G., Kostova D., Sofkova S. Identification of genes for resistance to *bean common mosaic virus* and *bean common mosaic necrosis virus* in snap bean (*Phaseolus vulgaris* L.) breeding lines using conventional and molecular methods. *Journal of Phytopathology*, 2014, 162(1): 19-25 (doi: 10.1111/jph.12149).
53. Antoshkin A.A., Degovtsov V.E., Pronina E.P., Antoshkina M.S. *Zernobobovye i krupyanye kul'tury*, 2014, 4(12): 86-89 (in Russ.).

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THE METABOLITES OF AUTOTROPHIC AND HETEROTROPHIC LEAVES OF *Amaranthus tricolor* L. EARLY SPLENDOR VARIETY

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Abstract

An important area of systemic biology (metabolomics) is the study of the composition and properties of low-molecular metabolites of agricultural plants with different modes of nutrition. The use of metabolic technologies expands the possibilities of analyzing biochemical changes in the composition and structural modifications of metabolites occurring during the transition from autotrophic to heterotrophic nutrition. Most photosynthetic plants are capable of autotrophic nutrition, but in their lifetime, there are periods of appearance of the achlorophyllic organs which receive nutritients from the organic substances stored earlier. Thus, among *Amaranthus tricolor* L. plants there are varieties with leaves which differ from each other in the way of nutrition. For example, Early Splendor variety plants form brightly colored red heterotrophic leaves along with green photosynthesis leaf blades at the end of the vegetative phase. The comparative study of the low-molecular metabolites composition in these leaves is important for understanding the relationship between heterotrophic and autotrophic nutrition in the whole plant. In this paper, significant qualitative differences in metabolites composition between autotrophic and heterotrophic leaves were stated for the first time during the metabolome analysis of water and alcohol extracts of heterotrophic and autotrophic amaranth leaves of Early Splendor variety using the method of gas chromato-mass spectrometry. It was found that the low-molecular metabolites of autotrophic and heterotrophic leaves contained both non-specific metabolites common for both type of nutrition and specific metabolites characteristic for each of the ways separately. On the one hand, it indicates the close interaction between two ways of nutrition and, on the other hand, the ability to synthesize and modify the metabolites which demonstrates partial autonomy of heterotrophic leaves. The purpose of the work is to study the composition of low-molecular metabolites and to identify new biologically active metabolites antioxidants in heterotrophic and autotrophic amaranth leaves of Early Splendor variety. Experiments were carried out in 2017-2019 with amaranth plants of the Early Splendor variety at the end of flowering—the beginning of seed formation phase. The plants were grown in a film greenhouse (the Federal Research Center for Vegetable Growing). The fresh red-colored heterotrophic leaves formed at the top of the main stem and the underlying photosynthetic leaves with a fully formed leaf blade were collected for analysis. The leaves were homogenized (T18 homogenizer, IKA, Germany) and extracted for 30 min at 24 °C with either 96 % ethanol or distilled water (leaves weighing batch: extragent 1:10). The metabolites were profiled by gas chromato-mass spectrometry method (GH-MC) with a chromograph GH-MC JMS-Q1050GC (JEOL Ltd., Japan). According to the mass spectra library of the NIST-5 National Institute of Standards and Technology (USA), a total of 87 metabolites were totally identified. Heterotrophic leaves contained 19 substances in water extracts and 38 metabolites in alcohol extracts, while photosynthetic leaves contained 21 substances in the water extract and 57 metabolites in alcohol extracts. Twenty-nine identical metabolites were found in water and alcohol extracts. In heterotrophic and autotrophic amaranth leaves of Early Splendor variety squalene ($C_{30}H_{50}$), a biologically active compound with antioxidant properties was identified for the first time. Also, in heterotrophic leaves monopelargonine (mononononanoin) ($C_{15}H_{11}O_7$) was identified. Mon-

opelargonine is an intermediate product of flavonoid o-glycosylation, is referred to phenolic compounds and possesses high antioxidant activity. Metabolites have been identified that are present in both autotrophic and heterotrophic amaranth leaves, which suggests a close interaction of the two types of nutrition during the appearance, growth and development of heterotrophic leaves. At the same time, photosynthesizing leaves serve as donors of key metabolites for heterotrophic leaves, while the latter are not only acceptors, but also can synthesize and modify metabolites necessary for cell formation. Due to revealed rich composition of carbohydrates, essential amino acids, lipids and organic acids, the photosynthesizing leaf biomass is a source of antioxidants and biologically active substances. It should be stressed that not all metabolites were identified. Nevertheless, the set of metabolites that we identified in the photosynthetic leaves allows us to suggest these substances to be key and sufficient compounds for the construction and functioning of cells and tissues in heterotrophic leaves.

Keywords: *Amaranthus tricolor*, low-molecular antioxidants, autotrophic leaf, heterotrophic leaf, gas chromatography, mass spectrometry

Photosynthetic plants are mostly autotrophs, however, their chlorophyll-less organs (flowers, leaves, bulbs, etc.) should utilize organic nutrients accumulated earlier. As all non-green organs and plant cells in the dark are heterotrophic, disclosing the mechanisms of heterotrophic nutrition is of fundamental importance.

Amaranth plants (*Amarantus tricolor* L.) are widespread throughout the world and recognized as a source of essential nutrients [1-3]. Healthy nutrition is an important element of human life. Deficiency of essential nutrients, bioactive compounds and minerals poses a serious threat to health [4]. Gaining more knowledge about the nutritional and pharmacological metabolites derived from amaranth leaves allows for a better development of safe functional foodstuff. Amaranth leaves contain vitamins, minerals [5-7], red betacyanins and yellow betaxanthins, flavonoids and other physiologically active compounds with antioxidant properties [8-11].

The amaranth (*A. tricolor*) Early Splendor variety is convenient for profiling organic substances in photo-synthesizing and chlorophyll-less leaves. The variety considered as leafy vegetable is so decorative that also decorate flower beds. During their life cycle, plants pass through stages characteristic only for the Early Splendor variety, i.e., the emergence of bright red heterotrophic leaves at shoot tops after growth completed, the formation of a green spot at the tip of each red leaf, and a gradual spread of the green zone throughout the leaf masking the red color.

Our previous findings have shown that the total content of antioxidants, photo-synthetic pigments and betacyanins in photosynthetic and heterotrophic leaves of Early Splendor plants differs. Moreover, the heterotrophic tissue of red leaves contains trace amounts of chlorophyll and is incapable of photosynthesis [12]. A number of studies have been reported on chlorophyll accumulation in red leaves [13, 14]. When assessing the expression of nine genes encoding eight enzymes that are involved in chlorophyll biosynthesis, a decrease was observed in the expression of the NADP-H-protochlorophyllide oxidoreductase gene. It catalyzes one of the stages of chlorophyll biosynthesis, therefore, a decrease in the NADP-H-protochlorophyllide oxidoreductase gene expression causes a loss of the ability to synthesize this photosynthetic pigment which plays a key role in the formation of red leaves in *A. tricolor* and its nutrition. Overproduction of the red-colored pigment amarantine can also be regulated by altering gene expression [15, 16].

The autotrophic leaves use inorganic substrates to photosynthesize while heterotrophic leaves of the same plant utilize pre-synthetized organic compounds [17]. However, scant data are available on the patterns of the main low-molecular-weight metabolites in auto- and heterotrophic leaves.

This paper is the first to report significant differences in the metabolite profiles between auto- and heterotrophic leaves of the Early Splendor amaranth plants. Gas chromatography-mass spectrometry (GC-MS)-based metabolomic analysis of water and ethanol leaf extracts has identified both nonspecific (common for different trophic types) and specific (characteristic of each type) low-molecular-weight metabolites. These findings broaden current knowledge of the adaptive changes in cells during the transition from phototrophic to heterotrophic nutrition type through modulation of the set and properties of low-molecular-weight metabolites.

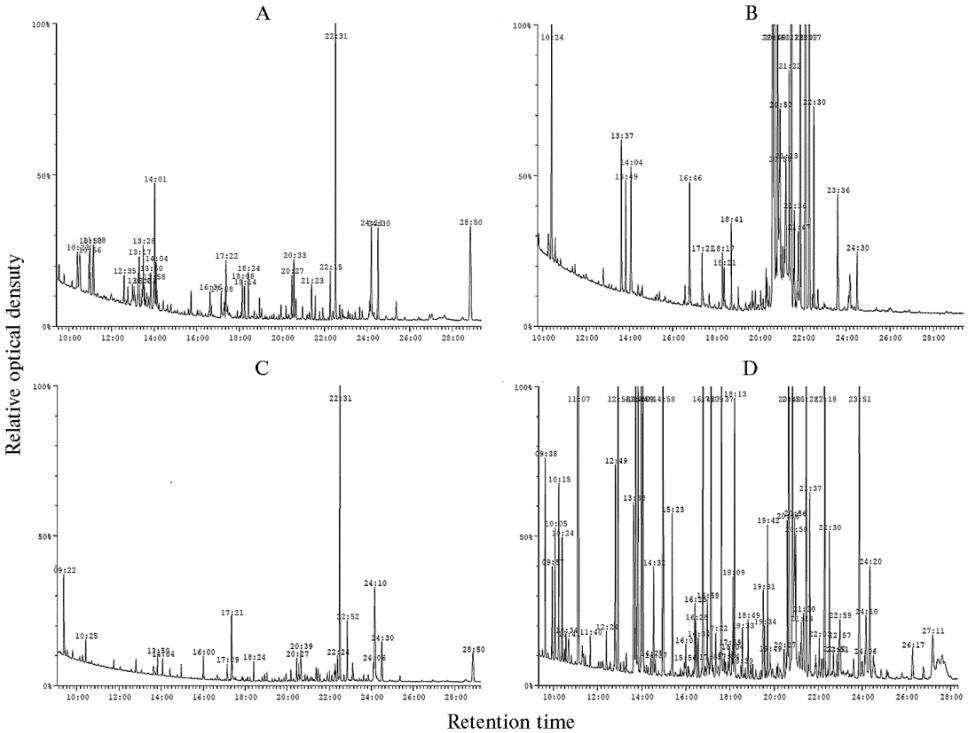
This work aimed to comparatively profile the low-molecular-weight metabolites from heterotrophic and autotrophic leaves of the amaranth plant and to identify new bioactive antioxidant metabolites.

Material and methods. The amaranth (*Amarantus tricolor* L.) Early Splendor cv. plants were grown in a film greenhouse (the Federal Scientific Center for Vegetable Growing, 2017-2019). During the period from the end of flowering to the beginning of seed formation, fully formed red-colored heterotrophic leaves from the top of the main shoot and the underlying photosynthetic leaves were collected. The fresh leaves were crushed (an A11 basic homogenizer, IKA, Germany) and extracted with 96% ethanol or distilled water at a ratio of 1:10 (leaves:extractant) at 24 °C for 30 min. Metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS) using a JMS-Q1050GC chromatograph (JEOL Ltd, Japan) equipped with a DB-5HT capillary column (Agilent, USA; 30 m×0.25 mm, film thickness 0.52 μm). The temperature gradient ranged from 40 to 280 °C (250 °C for the injector and interface, 200 °C for the ion source). The helium carrier gas flow rate in the column was 2.0 ml/min, the analysis time was 45 min, the injection mode was split-flow, the volume of the injected sample was 1-2 μl of the evaporated extract. For derivatization, the silylation reagent N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was used as described by Robbins [18]. The scanning range was 33-900 m/z. Substances were identified by retention parameters and mass spectra according to the of the library NIST-5 (National Institute of Standards and Technology, https://www.nist.gov, USA). The reliable probability of identification ranged within 75-98%.

Results. The Figure (A, B) shows typical GC-MS profiles of water and ethanol extracts from heterotrophic amaranth leaves. The analysis identified 87 metabolites of which 33 were found in water extracts and 74 in ethanol extracts (Table).

A total of 22 compounds were organic acids, including aliphatic, cyclic and high molecular weight acids which contain one or more carboxyl groups (see Table). In cells, organic acids can be free or bound. The physiological role of organic acids lies in their protective antibacterial activity, antioxidant properties, and the ability to buffer cell sap and participate in the Krebs cycle. Five organic acids were identified in the water extracts from heterotrophic amaranth leaves and nine in the ethanol extracts. Bromsebacic, monoamidoethylmalonic and succinic acids were found in both extracts of the heterogeneous leaves. Phenolic acids (i.e., benzoic, phenylacetic, and phthalic) were identified only in autotrophic leaves.

An important metabolite found in the ethanol extract was pantothenic acid (vitamin B_5), an amide of the amino acid β-alanine and pantothenic acid. Pantothenic acid as a coenzyme in CoA is involved in more than 130 metabolic reactions, participating in the synthesis of fatty acids, sterols, glycerides, citric acid, etc. [19].



Chromatographic profiles of water (left) **and ethanol** (right) **extracts from heterotrophic** (A, B) **and photosynthesizing** (C, D) **leaves of amaranth (*Amaranthus tricolor* L.) cv. Early Splendor.** Gas chromatography-mass spectrometer JMC-Q1050GC (JEOL Ltd, Japan).

Metabolites identified in extracts from heterotrophic and photosynthesizing tissues of amaranth (*Amarantus tricolor* L.) cv. Early Splendor leaves by gas chromatography-mass spectrometry

Rt, min	Compounds	Extract
	Organic acids	
20:02	D-arabinonic acid, $C_5H_{10}O_5$	EH, EP
19:57	Azelainic acid, $C_9H_{16}O_4$	WH, WP
22:42	2-Bromosebacic acid, $C_{10}H_{17}O_4Br$	WH, EH
10:23	Butanoic acid, $C_4H_8O_2$	EP
27;25	Dodecanedioic acid, $C_{12}H_{22}O_2$	EP
17:59	Glutaric acid, $C_5H_8O_4$	EH, EP
22:23	2-Hydroxyoctanoic acid, $C_4H_8O_3$	WP
12:09	Monoamidoethylmalonic acid, $C_{14}H_{33}NO_3$	WH, EH, EP
16:33	Malic acid, $C_4H_6O_5$	EH, EP
14:59	Malonic acid, $C_3H_4O_4$	EH, EP
10:24	Lactic acid, $C_3H_6O_3$	EH, EP
20:47	Pentonic acid, $C_5H_{10}O_2$	EH, EP
20:11	10-Undecynoic acid, $C_{11}H_{22}O_2$	WP
14:58	Erythronic acid, $C_{18}H_{30}O_5$	EP
22:59	Erythro-pentonic acid, $C_6H_{12}O_2$	EP
13:09	DL-malic (Butanedioic) acid, $C_4H_4O_5$	EP
14:04	Succinic acid, $C_4H_4O_4$	WH, EH
17:47	2-Isopropylmalic acid, $C_4H_4O_4$	EH
22:04	Pantothenic acid, $C_9H_{17}O_5$	EH
	Phenolic acids	
19:37	Benzoic acid, $C_7H_6O_2$	WP
21:09	Phenylacetic acid, $C_8H_8O_2$	EP
22:51	Isoferulic acid, $C_{10}H_{13}O_4$	WP
21:23	Phthalic acid, $C_8H_{12}O_4$	WP
	Fatty acids	
18:25	Dodecanoic acid, $C_{10}H_{20}O_2$	EH
19:07	Myristic acid, $C_{14}H_{28}O_2$	EH
21:15	Hexadecanoic acid, $C_{16}H_{32}O_2$	WH, EH, WP, EP
17:51	Octadecanoic (Stearic) acid, $C_{17}H_{35}COOH$	WH, EH, WP, EP
21:10	Linoleic acid (essential), $C_{12}H_{14}O_2$	WH, EH, WP, EP

Continued Table

	Carbohydrates	
19:43	L-(-)-Arabitol, $C_5H_{12}O_5$	EP
21:22	β-DL-Arabinopyranose, $C_5H_{12}O_5$	WH, EH, EP
19:03	β-L-Galactopyranose, $C_6H_{13}O_8$	EH, WP, EP
19:26	D-Galactose, $C_6H_{16}O_6$	EH, WP, EP
20:01	β-D-Xylopyranose, $C_6H_{10}O_5$	EH
22:07	D-Mannitol, $C_6H_{12}O_6$	EP
19:43	Ribitol, $C_5H_{12}O_5$	WP, EP
17:57	β-Erythrotetrofuranose, $C_5H_{14}O_5$	EP
22:41	Ribonic acid, pentakis, $C_5H_{12}O_5$	EP
22:17	β-D-Glucopyranose, $C_6H_6O_6$	EP
20:46	β-(DL)-Lyxopyranose, $C_5H_{10}O_5$	EP
20:00	Levoglucosan, $C_6H_{10}O_5$	WP, EP
36:53	3-α-Mannobiose, $C_{12}H_{22}O_{11}$	EP
20:11	D-(-)-Tagatofuranose, $C_6H_{12}O_6$	EH, EP
20:15	L-(-)-Sorbose, $C_6H_{12}O_6$	EP
20:36	α-l-(-)Sorbofuranose, $C_6H_{12}O_6$	EH, WP, EP
18:36	D-(-)-Ribofuranose, $C_5H_{10}O_5$	WH, EH, EP
20:36	D-(-)-Fructofuranose, $C_5H_8O_6$	EP
20:51	D-(-)-Fructopyranose, $C_5H_8O_6$	WP, EP
30:48	D-(+)-Turanose, $C_6H_{12}O_6$	EP
20:55	D-(+)-Talofuranose, $C_5H_{12}O_6$	EH
19:43	Ribitol, $C_5H_{12}O_5$	EH
21:17	Glyceryl glycoside	EP
20:18	Glucofuranoside, $C_6H_{12}O_6$	EP
19:43	Gluconic acid, γ-lacton, $C_6H_{12}O_6$	EP
21:51	Inositol, $C_6H_{12}O_6$	EP
19:30	D-(+)-Ribono-1,4-lactone, $C_5H_{12}O_5$	EH, EP
19:43	Gluconic acid, γ-lacton, $C_6H_{10}O_6$	WH, EH
13:50	Clycerol, $C_3H_8O_3$	WH, EH, WP, EP
14:57	2(3H)-Furanone, $C_4H_4O_2$	EH
	Amino acids and their derivates	
11:07	L-Alanin, $C_3H_7NO_2$	EH, WP, EP
12:06	L-Leucine, $C_6H_{13}NO_2$	EH, EP
14:03	L-Isoleucine, $C_6H_{13}NO_2$	EP
16:58	L-Aspartic acid, $C_4H_7NO_4$	EP
18:40	L-Asparagin, $C_4H_8N_2O_3$	EP
16:48	L-Proline, $C_3H_9NO_2$	EH, EP
13:47	L-Homoserine, $C_4H_9NO_3$	EH
13:20	L-Serin, $C_3H_7N_1O_3$	EP
12:55	L-Valin, $C_5H_{11}NO_2$	EH, EP
15:23	L-Treonin, $C_4H_9NO_3$	EH, EP
21:37	L-Tyrosine, $C_9H_{17}NO_2$	EP
18:12	L-Phenylalanin, $C_9H_{18}NO_2$	EP
22:38	L-Cistatione, $C_7H_{14}N_2O_2$	EH
	Terpenes	
36:06	Squalen, $C_{30}H_{50}$	WH, EH, WP, EP
23:01	Borneol, $C_{10}H_{18}O$	EH
20:10	Izoborneol, $C_{10}H_{18}O$	EH
22:42	Dehydroabietic acid, $C_{20}H_{29}O$	EH
	Others	
40:20	(+)-α-Tocopherol, $C_{29}H_{50}O_2$	EP
16:00	Niacinamide, $C_6H_5NO_2$	WP, EP
22:25	Stigmasterol, $C_{30}H_{18}O$	EH
20:29	Mononononanoin, $C_{15}H_{18}O_7$	EH
11:45	Carbamothioic acid, CH_3NO_2	EH, WP
20:08	Adenine, $C_5H_5N_5$	EH, WP
14:25	Uracil, $C_4H_6N_2O_2$	EP
11:18	Mono-ethylmalonate, $C_4H_4O_5$	EH
16:25	Trigonelline, $C_7H_7NO_2$	EP
16:08	2-Pyrrolidone-5-carboxylic acid, $C_9H_{27}N_2O_4$	EH
16:28	2,4(1H,3H)-Pyromidinedione, $C_4H_4N_2O_3$	EP

Note. Rt — retention time, WH — water extract from heterotrophic tissues, EH — ethanol extract from heterotrophic tissues, WP — water extract from photosyntesizing tissues, EP — ethanol extract from photosyntesizing tissues.

The next group of identified compounds refers to fatty acids that contain an acidic group —COOH (carboxyl) in their molecule. Fatty acids are the lipid components and have protective functions. In addition to fatty acids, lipids include alcohol glycerol which has been identified in water and ethanol extracts of

heterotrophic leaves. In the course of plant adaptation to changing ambient conditions, an important role is assigned to an increase in the content of saturated and unsaturated fatty acids and their ratio, which determines the stability of the membrane lipids [20, 21].

In heterotrophic and autotrophic leaves, two saturated fatty acids (palmitic and stearic) and one unsaturated fatty acid (linoleic) were identified. In addition, in the aqueous extract of heterotrophic leaves, we found two saturated fatty acids, the myristic ($C_{14}H_{28}O_2$) and lauric ($C_{10}H_{20}O_2$), which indicates a high adaptability of photosynthetic leaves.

Low-molecular-weight sugars and polyhydric alcohols perform protective functions in the plant, exhibiting osmoprotective and antioxidant properties, and can also be part of signaling systems [22]. Monosaccharides serve as sources of energy and nutrients and are used for the synthesis of polysaccharides. The group of carbohydrate derivates is the largest and represented by 30 compounds, including simple carbohydrates, sugar alcohols, and lactones. Analysis of these compounds from the heterotrophic leaves revealed 12 substances, including two polyhydric alcohols, the glycerol and ribitol. The water extracts contained only four carbohydrate metabolites, the gluconic acid lactone, arabinopyranose, galactopyranose, and ribose

Amino acids are bioactive compounds with physiological activity. They are structural elements of synthesized protein molecules and participate in various metabolic events, in formation of plant resistance to stresses of various natures, and in detoxification of xenobiotics [23]. In the ethanol extract from heterotrophic leaves, we identified six amino acids of which three, the valine, threonine, and leucine are essential. It should be noted that cystathione serves as an intermediate in the biosynthesis of methionine.

Terpene metabolites, the borneol, isoborneol, dehydroabietic acid, and squalene were identified in the water extracts, while phytosterols and stigmasterol involved in the synthesis of cholesterol were identified in the ethanol extracts. The unique phytosterol squalene ($C_{30}H_{50}$) found in the heterotrophic leaves exhibits anticarcinogenic activity and wound-healing effects in humans and, being a powerful antioxidant, promotes intensive metabolism in cells [24]. As a food ingredient or as a special dietary supplement, squalene lowers cholesterol. Earlier, we first identified squalene in an aqueous extract from leaves of amaranth *Amaranthus tricolor* L. cv. Valentina [25]. Squalene is the compound from which steroids are formed. Currently, a number of steroid compounds have been found in plants that were previously considered typical for animal organisms, for example, cholesterol pregnenolone and progesterol [26].

In heterotrophic amaranth leaves, monopelargonin (mononanoin) ($C_{15}H_{11}O_7$) was identified for the first time. Mononanoin is a phenolic compound, an intermediate of o-glycosylation of flavonoids, and has high antioxidant activity [27, 28].

The Figure (C, D) shows typical profiles of water and ethanol extracts of autotrophic leaves of amaranth cv. Early Splendor. GC-MS analysis revealed 67 low-molecular-weight compounds in photosynthesizing green-violet leaves, of which 59 metabolites from autotrophic tissues were present in the ethanol extracts, and only 21 metabolites were identified in the water extracts (see Table 1).

It should be noted that more organic acids were present in autotrophic leaves than in heterotrophic. We found five organic acids in the water extracts from autotrophic leaves. It is known that natural antimicrobial substances with antioxidant activity synthesized in the leaves of amaranth *A. tricolor* (malate, oxalacetate, ferulic, benzoic, gallic and other organic and phenolic acids) con-

tribute to food preservation [29-32]. Among natural metabolites of amaranth, these organic and phenolic acids involved in cell metabolism attract special attention [33-35].

Organic acids of different chemical nature (i.e., polyoxycarboxylic and phenylcarboxylic acids) were found in the ethanol extracts of green amaranth leaves. For example, the bioactive phenylacetic acid ($C_8H_8O_2$) serves as a plant hormone (auxin).

Of the fatty acids, we found palmitic, stearic, and linolenic acids both in water and ethanol extracts. In addition, lauric and myristic saturated fatty acids were detected in the water extracts.

In green leaves, five monosaccharides and a polyhydric alcohol ribit were identified in the water extracts, erythrofuranose and ribonic acid were additionally found in the ethanol extracts (see Table). The ethanol extracts of autotrophic leaves contained the largest number of monosaccharides and their derivatives, a total of 24, of which five, the mannitol, ribitol, glycerin, arabitol, and inositol (a vitamin-like substance cyclohexane-1,2,3,4,5,6-hexol, the vitamin B_8) belong to polyhydric alcohols.

In water and ethanol extracts of autotrophic amaranth leaves, we found essential amino acids valine, leucine, isoleucine, threonine, and phenylalanine, as well as stress-protective amino acids serine, proline, aspartic acid, asparagine, alanine, and tyrosine. Six amino acids were detected in heterotrophic leaves.

Ore earlier studies have shown that the leaves of the vegetable amaranth *A. tricolor* cv. Valentina contain a full set of free and protein-bound essential amino acids, as well as a large number of bioactive metabolites with antioxidant activity which determine the pharmacological property of a bioactive supplement (herbal tea) Amarantil [7, 36].

Trigonelline (betaine) which is formed by methylation of nicotinic acid and plays a significant role in nitrogen metabolism [31, 37] was also found in the ethanol extracts from photosynthesizing amaranth leaves.

The E group vitamins identified in the ethanol extracts of photosynthesizing leaves, being strong antioxidants, regulate free radical activity in the cell and thus protect unsaturated fatty acids of membrane lipids from oxidation. Tocopherols reduce the risk of chronic free radical pathologies and suppress the enzyme responsible for cholesterol synthesis [38]. The profiles of bioactive metabolites in water and ethanol extracts from amaranth leaves identified in this work and obtained earlier [24] indicate that amaranth can be used to develop safe functional products and drugs exhibiting antioxidant [39], hepatoprotective [40, 41], and antidiabetic [42, 43] effects.

The biochemical composition of low-molecular-weight bioactive substances, including those with anti-stress and pharmacological effects, is being actively studied in various organs of amaranth, but especially in the leaves [44, 45]. With the advent of metabolomic technologies, the list of identified low-molecular-weight metabolites in various amaranth species has increased [46]. In leaves of green- and red-colored vegetable and grain amaranth species, compounds were found that are valuable not only for the food, but also for the pharmaceutical industry (squalene, inositol, glycerin, stigmasterol, linoleic acid, glucopyranose, mannose, etc.), which is similar to the data obtained in this work.

The relationship between hetero- and phototrophic leaves is of particular interest. Metabolic profiling showed that the heterotrophic leaves of cv. Early Splendor contain 13 carbohydrate derivates out of 29 those present in autotrophic leaves (see Table). It should be noted that carbohydrates in plants play a key role as energy sources and carbohydrate skeletons for organic compounds,

storage substances, signaling molecules, they also participate in plant defense response to abiotic stresses.

Of the monosaccharides, eight compounds were detected in the heterotrophic leaves, which was significantly less compared to autotrophic leaves containing 13 compounds. It should be noted that among the latter, xylose and talose which are involved in the synthesis of complex carbohydrates were not identified. The revealed ability of monosaccharides to be both water- and ethanol-extracted from heterotrophic (arabinopyranose and ribose) and autotrophic (galactopyranose, galactose, sorbofuranose and fructofuranose) leaves indicates the amphiphilic (osmoprotective) properties of their molecules.

Metabolic profiling revealed five polycyclic alcohols in the autotrophic leaves and glycerol and ribitol in heterotrophic leaves.

Monosaccharides, polycyclic alcohols and proline in amaranth leaves are compatible osmolytes that help maintain the osmotic balance of the cell, stabilize proteins and cellular structures, exhibiting a protective function. However, heterotrophic leaves contained significantly less compatible osmolytes compared to autotrophic ones. Probably, in heterotrophic leaves, carbohydrates perform the function of signaling molecules and are necessary to construct cell membranes.

The obtained results suggest the formation of three pools of monosaccharides and their derivates in hetero- and autotrophic amaranth leaves which perform different, possibly interchangeable functions. The water-soluble pool of carbohydrate metabolites plays a decisive role in cell metabolism, providing optimal metabolic activity in the cytoplasm and antioxidant protection. The ethanol-soluble pool of monosaccharides and their derivates ensures the preservation and activity of cell structures. The pool of amphiphilic monosaccharides and their derivates maintains the osmotic balance, participates in the stabilization of proteins and cell structures, and provides antioxidant protection.

In cv. Early Splendor amaranth plants, after the growth of the main shoot is completed and the formation of heterotrophic tissues are initiated at its apex, complex metabolic links arise between red and photosynthesizing leaves. We assume that they are mediated by the transport of assimilates from photosynthetic leaves (donor) to newly formed growing heterotrophic leaves (acceptor). This study indicates that the heterotrophic tissues of red leaves contain 37 low-molecular-weight metabolites found in photosynthesizing leaves. These are carbohydrate substances, organic acids, amino acids, and essential fatty acids. Identical metabolites found in heterotrophic and autotrophic leaves seem to be key for the formation of heterotrophic tissue.

Thus, gas chromatography-mass spectrometry profiling of water and ethanol extracts from photosynthesizing and heterotrophic leaves of amaranth cv. Early Splendor revealed 87 low-molecular-weight metabolites, including organic acids, monosaccharides and their derivates, fatty and amino acids, and secondary metabolites (phenolic compounds, terpenes, and glycosides). Heterotrophic leaves contain almost 1.5 times less metabolites compared to autotrophic leaves. The appearance of nonspecific and specific metabolites in both photosynthesizing and heterotrophic leaves, on the one hand, indicates a close interaction of these trophic ways, and on the other, the partial autonomy of heterotrophic leaves due to their ability to synthesize and modify metabolites. Most of the identified compounds have nutritional and pharmacological value. Therefore, leaves of the amaranth cv. Early Splendor are rich in bioactive metabolites and can be used as a raw material for production of dietary and prophylactic bioactive food additives and herbal medicines. The detection of identical metabolites

in photosynthesizing and heterotrophic tissues indicates that in-deep study of their interaction is of fundamental and practical matter.

REFERENCES

1. Hilou A., Ouedraogo I., Sombié P., Guenné S., Paré D., Compaoré M. Leafy amaranthus consumption patterns in Ouagadougou, Burkina Faso. *African Journal of Food, Agriculture, Nutrition and Development*, 2016, 16(4): 11248-11264 (doi: 10.18697/ajfand.76.13810).
2. Li H., Deng Z., Liu R., Zhu H., Draves J., Marcone M., Sun Y., Tsao R. Characterization of phenolics, betacyanins and antioxidant activities of the seed, leaf, sprout, flower and stalk extracts of three *Amaranthus* species. *Journal of Food Composition and Analysis*, 2015, 37: 75-81 (doi: 10.1016/j.jfca.2014.09.003).
3. Rastogi A., Shukla S. Amaranth: a new millennium crop of nutraceutical values. *Critical Reviews in Food Science and Nutrition*, 2013, 53(2): 109-125 (doi: 10.1080/10408398.2010.517876).
4. Gins M.S., Gins V.K., Kononkov P.F. *Vestnik Rossiiskoi sel'skokhozyaistvennoi nauki*, 2016, 2: 55-58 (in Russ.).
5. Kachiguma N., Mwase W., Maliro M., Damaliphetsa A. Chemical and mineral composition of amaranth (*Amaranthus* L.) species collected from central Malawi. *Journal of Food Research*, 2015, 4(4): 92-102 (doi: 10.5539/jfr.v4n4p92).
6. Sharma N., Gupta P. C., Rao C. V. Nutrient content, mineral content and antioxidant activity of *Amaranthus viridis* and *Moringa oleifera* leaves. *Research Journal of Medicinal Plants*, 2012, 6(3): 253-259 (doi: 10.3923/rjmp.2012.253.259).
7. Gins M., Gins V., Momyleva, S., Kulikov I., Medvedev S., Kononkov P., Pivovarov V. Mineral composition of amaranth (*Amaranthus* L.) seeds of vegetable and grain usage by ARHIVBSP selection. *Potravinarstvo Slovak Journal of Food Sciences*, 2018, 12(1): 330-336 (doi: 10.5219/863).
8. Arunachalam V., Dhargalkar S., Vaingankar J., Kevat N. Pigment rich amaranth by tri-stimulus colorimetry and progeny test. *National Academy Science Letters*, 2016, 39(6): 411-415 (doi: 10.1007/s40009-016-0491-8).
9. Noori M., Talebi M., Nasiri Z. Seven *Amaranthus* L. (*Amaranthaceae*) taxa flavonoid compounds from Tehran Province, Iran. *International Journal of Modern Botany*, 2015, 5(1): 9-17 (doi: 10.5923/j.ijmb.20150501.02).
10. Kwon H.J., Jung N.S., Han S.B., Park S.B. Evaluation of antioxidant activity of *Amaranthus hypochondriacus* L. extract using cyclic voltammetry. *Electrochemistry*, 2019, 87(6): 336-340 (doi: 10.5796/electrochemistry.18-00097).
11. Karamać M., Gai F., Longato E., Meineri G., Janiak M., Amarowicz R., Peiretti P.G. Antioxidant activity and phenolic composition of amaranth (*Amaranthus caudatus*) during plant growth. *Antioxidants*, 2019, 8(6): 173 (doi: 10.3390/antiox8060173).
12. Gins M.S., Pivovarov V.F., Gins V.K., Baikov A.A., Platonova S.Yu., Gins E.M. *Ovoshchi Rossii*, 2016, 3(32): 79-83 (doi: 10.18619/2072-9146-2016-3-79-83) (in Russ.).
13. Iwamoto K., Fukuda H., Sugiyama M. Elimination of POR expression correlates with red leaf formation in *Amaranthus tricolor*. *The Plant Journal*, 2001, 27(4): 275-284 (doi: 10.1046/j.1365-313x.2001.01082.x).
14. Wang Q.M., Cui J., Dai H., Zhou Y., Li N., Zhang Z. Comparative transcriptome profiling of genes and pathways involved in leaf-patterning of *Clivia miniata* var. *variegata*. *Gene*, 2018, 677: 280-288 (doi: 10.1016/j.gene.2018.07.075).
15. Liu S., Zheng X., Pan J., Peng L., Cheng C., Wang X., Zhao C., Zhang Z., Lin Y., XuHan X., Lai Z.-X. RNA-sequencing analysis reveals betalains metabolism in the leaf of *Amaranthus tricolor* L. *PLoS ONE*, 2019, 14(4): e0216001 (doi: 10.1371/journal.pone.0216001).
16. Khandaker L., Akond M., Oba S. Air temperature and sunlight intensity of different growing period affects the biomass, leaf color and betacyanin pigment accumulations in red amaranth (*Amaranthus tricolor* L.). *Journal of Central European Agriculture*, 2009, 10 (4): 439-448.
17. Mokronosov A.T. *Ontogeneticheskii aspekt fotosinteza* [Ontogenetic aspect of photosynthesis]. Moscow, 1981 (in Russ.).
18. Robbins R.J. Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry*, 2003, 51(10): 2866-2887 (doi: 10.1021/jf026182t).
19. Smashevskii N.D., Ionova L.P. *Astrakhanskii vestnik ekologicheskogo obrazovaniya*, 2016, 2(36): 54-66 (in Russ.).
20. Upchurch R.G. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters*, 2008, 30(6): 967-977 (doi: 10.1007/s10529-008-9639-z).
21. Yadav S.K. Cold stress tolerance mechanisms in plants. A review. *Agronomy for Sustainable Development*, 2010, 30(3): 515-527 (doi: 10.1051/agro/2009050).
22. Ibragimova S.S., Gorelova V.V., Kochetov A.V., Shumnyi V.K. *Vestnik Novosibirskogo gosudarstvennogo universiteta. Seriya: Biologiya, klinicheskaya meditsina*, 2010, 8(3): 98-103 (in Russ.).

23. Yakhin O.I., Lubyanov A.A., Kalimullina Z.F., Batraev R.A. *Izvestiya Orenburgskogo gosudarstvennogo agrarnogo universiteta,* 2012, 1(33): 38-40 (in Russ.).
24. Lutsenko M.T. *Sibirskii nauchnyi meditsinskii zhurnal*, 2010, 30(6): 152-154 (in Russ.).
25. Gins M.S., Gins V.K., Motyleva S.M., Kulikov I.M., Medvedev S.M., Pivovarov V.F., Mertvishcheva M.E. Metabolites with antioxidant and protective functions from leaves of vegetable amaranth (*Amaranthus tricolor* L.) *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(5): 1030-1040 (doi: 10.15389/agrobiology.2017.5.1030eng).
26. Vysochina G.I. *Khimiya rastitel'nogo syr'ya*, 2013, 2: 5-14 (doi: 10.14258/jcprm.1302005) (in Russ.).
27. Zaprometov M.H. *Fenol'nye soedineniya: Rasprostranenie, metabolizm i funktsii v rasteniyakh* [Phenolic compounds: localization, metabolism and function in plants]. Moscow, 1993 (in Russ.).
28. Hofer B. Recent developments in the enzymatic O-glycosylation of flavonoids. *Applied Microbiology* and *Biotechnology*, 2016, 100(10): 4269-4281 (doi: 10.1007/s00253-016-7465-0).
29. Buldagov A.S. *Pishchevye dobavki* [Nutritional supplements]. St. Petersburg, 1996 (in Russ.).
30. Shkarina E.I., Maksimova T.V., Nikulina I.N., Lozovskaya E.P., Chumakova Z.V., Pakhomov V.P., Sapezhinskii I.M., Arzamastsev A.P. *Khimiko-Farmatsevticheskii Zhurnal*, 2001, 35(6): 40-47 (in Russ.).
31. Kretovich V.L. *Biokhimiya rastenii* [Plant biochemistry]. Moscow, 1980 (in Russ.).
32. Korul'kin D.Yu., Abilov Zh.A., Muzychkina R.A., Tolstikov G.A. *Prirodnye flavonoidy* [Natural flavonoids]. Novosibirsk, 2007 (in Russ.).
33. Agarwal A.G., Khan M.I., Singh K., Sidhu O.P. Isolation, identification and quantification of bioactive compounds from *Amaranthus hypochondriacus* leaf extract: a vital source of potent natural antioxidants. *International Journal of Research in Pharmaceutical Sciences*, 2019, 10(12): 5632-5638 (doi: 10.13040/IJPSR.0975-8232.10(12).5632-38).
34. Nimbalkar M.S., Pai S.R., Pawar N.V., Oulkar D., Dixit G.B. Free amino acid profiling in grain amaranth using LC-MS/MS. *Food Chemistry*, 2012, 134(4): 2565-2569 (doi: 10.1016/j.foodchem.2012.04.057).
35. Guo S.-H., Hu N., Li Q.-S., Yang P., Wang L.-L., Xu Z.-M., Chen H.-J., He B.-Y., Zeng E.-Y. Response of edible amaranth cultivar to salt stress led to Cd mobilization in rhizosphere soil: a metabolomic analysis. *Environmental Pollution*, 2018, 241: 422-431 (doi: 10.1016/j.envpol.2018.05.018).
36. Pivovarov V.F., Gins M.S., Gins V.K. Innovative beverage production technologies based on Amaranth biomass. *IOP Conference Series: Earth and Environmental Science*, 2019, 395: 012090 (doi: 10.1088/1755-1315/395/1/012090).
37. Bubnova M.G., Aronov D.M., Perova N.V., Chudakova I.V., Mel'kina O.E., Lobikova S.V., Zhasminova V.G. *Klinicheskaya farmakologiya i terapiya*, 2002, 11(4): 26-30 (in Russ.).
38. Byshevskii A.Sh., Galyan S.L., Dement'eva I.A., Rudzevich A.V. *Tromboz, gemostaz i reologiya*, 2009, 4(40): 16-32 (in Russ.).
39. Clemente A.C., Desai P.V. Evaluation of the hematological, hypoglyces, hypolipidemic and antioxidant properties of *Amaranthus tricolor* leaf extract in rat. *Tropical Journal of Pharmaceutical Research*, 2011, 10(5): 595-602 (doi: 10.4314/tjpr.v10i5.8).
40. Al-Dosari M.S. The effectiveness of ethanolic extract of *Amaranthus tricolor* L.: a natural hepatoprotective agent. *The American Journal of Chinese Medicine*, 2010, 38(6): 1051-1064 (doi: 10.1142/S0192415X10008469).
41. Aneja S., Vats M., Aggarwal S., Sardana S. Phytochemistry and hepatoprotective activity of aqueous extract of *Amaranthus tricolor* Linn. roots. *Journal of Ayurveda and Integrative Medicine*, 2013, 4(4): 211-215 (doi: 10.4103/0975-9476.123693).
42. Biswas M., Dey S., Sen R. Betalains from *Amaranthus tricolor* L. *Journal of Pharmacognosy and Phytochemistry*, 2013, 1(8): 7-95.
43. Rahmatullah M., Hosain M., Rahman S., Rahman S., Akter M., Rahman F., Rehana F., Munmun M., Kalpana M.A. Antyhyperglycaemic and antinociceptive activity evaluation of methanolic extract of whole plant of *Amaranthus tricolor* L. (*Amaranthaceae*). *African Journal of Traditional, Complementary and Alternative Medicines*, 2013, 10(5): 408-411 (doi: 10.4314/ajtcam.v10i5.31).
44. Nimbalkar M., Pawar N., Pai S., Dixit G. Synchronized variations in levels of essential amino acids during germination in grain Amaranth. *Brazilian Journal of Botany*, 2020, 43: 481-491 (doi: 10.1007/s40415-020-00624-5).
45. Chmelík Z., Šnejdrlová M., Vrablík M. Amaranth as a potential dietary adjunct of lifestyle modification to improve cardiovascular risk profile. *Nutrition Research*, 2019, 72: 36-45 (doi: 10.1016/j.nutres.2019.09.006).
46. Rocchetti G., Tomas M., Zhang L., Zengin G., Lucini L., Capanoglu E. Red beet (*Beta vulgaris*) and amaranth (*Amaranthus* sp.) microgreens: Effect of storage and in vitro gastrointestinal digestion on the untargeted metabolomic profile. *Food Chemistry*, 2020, 332: 127415 (doi: 10.1016/j.foodchem.2020.127415).

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IMPACT OF HIGH TEMPERATURE ON GROWTH OF EMBRYO AND GERMINATION OF HETEROMORPHIC SEEDS OF *Anethum graveolens* L. (*Apiaceae*)

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Abstract

Heteromorphism is widespread in nature and manifests itself in the variation of various parameters of seeds within individual individuals and populations. Dill (*Anethum graveolens* L.) seeds are characterized by heteromorphism caused by the maternal factor. First of all, the maternal factor effects on the size of the seeds, in this case a variation in the size of the embryo can be observed. The study of the reaction of such seeds to the action of abnormal weather conditions is an urgent task. High temperature is one of the unfavorable abiotic factors that plants can be exposed to at different stages of development. In the present study, a significant thermal sensitivity to the long-term effect of suboptimal (higher than the optimal) temperature of embryos from dill seeds, formed in inflorescences of the second order of branching, was revealed for the first time. Under the influence of high temperature, the growth of embryos was inhibited. As a result, germination of intact dill seeds obtained from second-order inflorescences was observed. This work is devoted to the study of the effect of high temperature on the growth of the embryo and the germination of intact dill seeds obtained from different orders of branching. The study aimed to determine the influence of the maternal factor, as well as high temperature on the growth of the embryo during germination and on the germination of intact dill seeds formed in inflorescences of different orders of branching. The research was conducted in 2015-2016 at the All-Russian Research Institute for Vegetable Growing, Branch of the Federal Scientific Vegetable Center, with the late-ripening dill variety Centaur seeds, formed in inflorescences of the first and second orders of branching. The seeds were obtained from dill plants grown in the open field. Harvesting was carried out on day 50 after flowering of 1st order umbels. The experiments were carried out in a temperature-controlled thermostat. To determine the critical temperature for the growth of the embryo during germination and germination rate of intact seeds formed in different orders of branching, a wide temperature range was applied, 20 °C as control, and 25, 30, 35 and 40 °C. Using the morphometric method of analysis, we studied the growth of the embryo, as well as the dynamics of the germination of intact seeds against the high temperatures background. The data obtained were used to calculate the parameters and plot the embryo growth curve, as well as the germination curve of intact seeds. Logistic regression was used to calculate the maximum suboptimal temperature at which embryo growth and seed germination are possible. On the basis of experimental data, we have shown that embryos, formed at different branching orders of the mother plant, have different stages of development. The initial dimensions of the embryo of the first branching order are 30 % higher than the second ($p < 0.001$). Under the action of a temperature of 30-35 °C, differences appeared in the thermal sensitivity of the embryos and the growth rate of the embryos. The effect of high temperatures is crucial for the growth of the embryo (57.0 %; $F = 415.3$, $p < 0.001$) and germination of dill seeds (37.2 %; $F = 270.5$, $p < 0.001$). The maximum temperature at which the growth is possible is 40±0.4 °C for the first-order embryo, and 38±0.5 °C for the second-order embryo ($p < 0.001$). The maximum temperature allowing for germination of at least 50 % of

viable first-order seeds is 34±0.3 °C, for the second-order 30±0.4 °C ($p < 0.001$). The seeds are more sensitive to high temperatures than the embryos, and the growth of the embryo has a significant effect on seed germination ($r = 0.946$; $t = 25.85$; $p < 0.001$). Our studies have shown that the temperature sensitivity of second-order embryos, which is clearly manifested against the background of morphological underdevelopment, is one of the main reasons for the slow, inhomogeneous and incomplete germination of the dill seed population under suboptimal temperature conditions.

Keywords: *Anethum graveolens* L., embryo growth, heteromorphism, seed position, mother plant, seed germination, thermosensitivity

Heteromorphism which refers to the appearance on one plant of seeds that differ in size, weight, color, morphology, anatomy, germination and other characteristics is widely represented in the plant world and inherent in both wild and cultivated forms [1-3]. In the families *Asteraceae*, *Chenopodiaceae*, *Poaceae*, *Apeaceae*, and *Brassicaceae*, the seed size varies significantly [4-7]. Some species, for example *Danthonia spicata* [8] and *Heterosperma pinnatum* [9], show a discrete variability, while others, in particular *Rubus ulmifolius* [10], *Raphanus raphanistrum* [11], and *Rubus chamaemorus* [12], show permanent variation in seed size.

Between individuals [13-15] and in the population [16, 17], the variability of seeds in terms of a set of characters, including sowing qualities [18-20], is widespread. Different germination capacity and requirements, as a rule, are associated with the location of seeds formed on different metameres of the mother plant. One of the main explanations for these differences is that the nutritional resources of the mother plant are not equally distributed among the seeds [21]. In addition, seed formation differs temporally and, therefore, occurs under different weather conditions. Moreover, the age, physiological and biochemical state of the mother plant also change, which additionally affects the seed metabolism [22].

Heteromorphism caused by the maternal factor is widespread among vegetable crops of the family *Apiaceae* [23, 24]. These plants are characterized by extended flowering and seed maturation in umbels. Hendrix [25] showed that the size and weight of parsnip seeds decrease as the branching order increases. Thompson notes [26] that the variation in seed size within a single *Lomatium gray*i plant reaches 16%. The influence of heteromorphism of *Apiaceae* seeds on their quality has been studied by many researchers. Thomas et al. [27] found that carrot seeds collected from primary and secondary umbels are unequal in quality parameters under different conditions of germination. In the same work, the influence of the branching order on the manifestation of dormancy in *Apium graveolens* seeds and their sensitivity to $GA_{4/7}$ was revealed. Many papers have shown that the quality of carrot seeds decreases as the branching order increases [28-30].

Morphologically underdeveloped embryo is among the key endogenous factors which affect quality of the *Apiaceae* seeds [31-33] and impose special requirements for their use. Various conditions can inhibit embryonic growth at several critical stages and thus affect the seed germination rate and the number of germinated seeds. Plant adaptive responses are attracting urgent scientific interest due to current climatic instability and the likelihood of abnormal weather conditions. For example, plants can be exposed to high temperatures at different stages of development [34]. The influence of this factor on seed germination is considered from the point of view of cardinal temperatures [35, 36].

For seeds of most crops, the temperature optimum ranges from 15 to 30 °C with the maximum from 30 to 40 °C [35]. For embryos and intact dill seeds formed in inflorescences of the first and second branching orders, the cardinal temperature has not been determined. In most works, the influence of seed location in the umbel on seed quality and germination parameters [28-30] and the effect of high temperature on seeds [37, 38] were studied on carrot plants. Fewer

similar studies have been conducted on dill [39]. The growth of the embryo in the seeds after separation from the mother plant was assessed mainly on wild *Apiaceae* species [40, 41]. The pre-development of embryos from different branching orders in umbels and the influence of stress factors on this process in the *Apiaceae* seeds, in particular in dill, is poorly covered.

Previously, we considered the germination features and kinetic parameters at different temperatures in homogeneous lots of seeds of vegetable umbellifers [42].

In this work, for the first time, we revealed a significant thermal sensitivity of embryos from seeds of second-order umbels to the long-term exposure to superoptimal temperature. High temperatures inhibit the embryo growth and, consequently, the germination of intact dill seeds derived from the second-order inflorescences.

The work aimed at studying effects of seed position in an umbel and high temperatures on embryo growth and germination of intact dill seeds.

Materials and methods. Dill (*Anethum graveolens* L.) late-season cv. Centaur plants were grown in the field (Moscow region, 55°36′N 38°1′E, the All-Russian Research Institute of Vegetable Growing — a Branch of Federal Research Center for Vegetable Growing, 2015-2016). The cv. Centaur plants in the flowering phase are 100-110 cm in height, spreading and leafy, the umbels are large in size, convex, and multi-radial. In the conditions of the Moscow region, cv. Centaur plants form mature umbels on the axes of two orders. To produce seeds derived from first-order (1o) and second-order (2o) umbels, the sowing was performed in the second decade of May (10 m^2 plots allocated randomly, 45×10 cm rows, a 1.5 cm seeding depth, and the 1-2 g/m^2 seeding rate). Experiments were arranged in triplicate.

Harvesting was carried out on day 50 after the 1o umbel flowering began. Two-order umbels were cut off 90 selected plants. The seeds were dried and stored under natural conditions in the laboratory for 6 months. After storage, the moisture content and 1000-seed weight were measured.

The temperatures critical for embryo growth in intact 1o and 2o germinating seeds were determined within the following rang: 20 °C (control) refers to as the average optimum temperature for non-dormant seeds [43], 25, 30, 35, and 40 °C (a TC 1/80 thermostat, OJSC Smolenskoye SKTB SPU, Russia). The substrate was constantly kept moist, the air humidity in the chamber was 90-95%. Before the test, seeds were sterilized with 0.125% sodium hypochlorite for 5 min and rinsed in distilled water. The tests continued for 21 days in the dark.

To assess the embryo growth dynamics, the intact seeds were placed into 9-cm Petri dishes on a sheet of filter paper moistened with distilled water (four replicates of 10 seeds per day). Additional portion of seeds (four replicates, 100 seeds each) were provided to be randomly selected, if necessary, in case of seed death or appearance of embryo-less seeds. For each treatment, the seeds in Petri dishes selected daily at random were cut with a blade and the embryos were removed. Approximately 6,000 seeds were examined. The seeds were cut in half, the embryo length was measured. In germinated seeds, the critical embryo length parameter was used, i.e., the length of the embryo after the rupture of the seed coat but before the root appears [44]. Embryos were imaged using a Levenhuk 670T microscope (Levenhuk, USA) with a 4× achromatic objective connected to a ScopeTek DCM 300 MD video eyepiece (ScopeTek, China). The embryo length was measured using the Scope Photo image analysis software (Image Software V. 3.1.386), the endosperm length was measured with a caliper, and the embryo length (E) to endosperm length (S) (E:S) ratio was calculated. The E:S ratio was scored as follows: 1 — 0.0-0.19 (the embryo is less than $^1/_4$ of the endosperm in length, the heart stage); 2 — 0.20-0.29 (the embryo is $^1/_4$ of the endosperm in

length, the cotyledon and the root are of equal length); 3 — 0.30-0.39 (the embryo is approximately $^1/_3$ of the endosperm in length, it has pronounced cotyledons and the root); 4 — 0.40-0.59 (the embryo is $^1/_2$ of the endosperm in length, the root is longer than the cotyledons); 5 — 0.60-0.79 (the embryo is $^2/_3$ of the endosperm in length); 6 — 0.80-1.00 (the embryo and the endosperm are almost equal in length) [45].

The effect of high temperature on seed germination was assessed in a 21-day test by the radicle protrusion. Radicle emergence was considered as the completion of germination. In the test, portions of intact seeds (4 replicates, 100 seeds each) were germinated as described above. The number of seeds with a visible protrusion of radicle was counted daily. In total, approximately 4000 seeds were examined. Sprouted seeds were removed.

The data were used to construct graphs of embryo growth and seed germination. For embryo growth, the four-parameter logistic regression was applied where b was the slope of the embryo growth graph, c was the lower point of the embryo growth graph which corresponds s to the initial embryo length, d was the upper point of the embryo growth graph which corresponds to the maximum embryo length during germination, e was the period (in days) during which the embryo reached 50% length necessary for seed germination. To plot the seed germination graph, the three-parameter logistic regression was used where b was the slope of the seed germination graph, d was the upper point of the seed germination graph which corresponds to the percentage of germinated seeds during the test); e was the period (in days) during which germination of 50% of all germinated seeds occurred [46, 47]. Logistic regression was used to calculate the maximum temperature at which embryo growth and seed germination are possible.

Differences were statistically assessed using the standard error (±SEM) and criteria for the null hypothesis that the difference was 0. All data were tested for normality of distribution (W, Shapiro-Wilk test). Two-way analysis of variance with preliminary conversion to the root of the arcsine angle was used to assess the influence of the studied factors on the embryo growth and seed germination. The relationship between parameters was assessed using Pearson's correlation analysis. Differences in each pair of compared values were considered statistically significant at $p \leq 0.05$. The presented models were implemented in the drc extension package for the R software environment. All statistical analyzes were performed in R version 3.4.3 [48].

Results. For 1o and 2o umbels, the 1000-seed weigh was 1.50 and 1.32 g, respectively, in 2015 and 1.59 and 1.41 g, respectively, in 2016. The moisture content in seeds after drying was 12-13%.

An increase in the temperature to the maximum (40 °C) inhibited the embryo growth in both 10 and 20 seeds ($p < 0.001$) (Fig. 1). A less aggressive temperature (30-35 °C) disclosed differences in the thermal sensitivity of embryos. Comparison of their growth graphs clearly reveals heteromorphism of the initial length ($p < 0.001$). The inhibition of the growth was expressed during germination in a decrease in their maximum length from 2.30±0.02 mm (E:S = 0.68) at 20 °C to 1.21±0.04 mm (E:S = 0.36) at 40 °C for 1o seeds and from 1.89±0.03 mm (E:S = 0.62) at 20 °C to 0.86±0.01 mm (E:S = 0.28) at 40 °C for 2o seeds. High temperatures negatively affected the growth rate of embryos. As the temperature rose from 20 to 25 and 35 °C, the time embryos needed increased by 2.4±0.3 ($p < 0.001$) and 5.3±0.9 days ($p < 0.001$), respectively, for 1o seeds and by 2.9±0.8 ($p < 0.001$) and 3.1±1.2 days ($p = 0.007$), respectively, for 2o seed. The growth rates of 1o and 2o embryos under the same stress differed insignificantly. With an increase in temperature to 40 °C, the growth of embryos in seeds of both orders

completely stopped in 4-5 days.

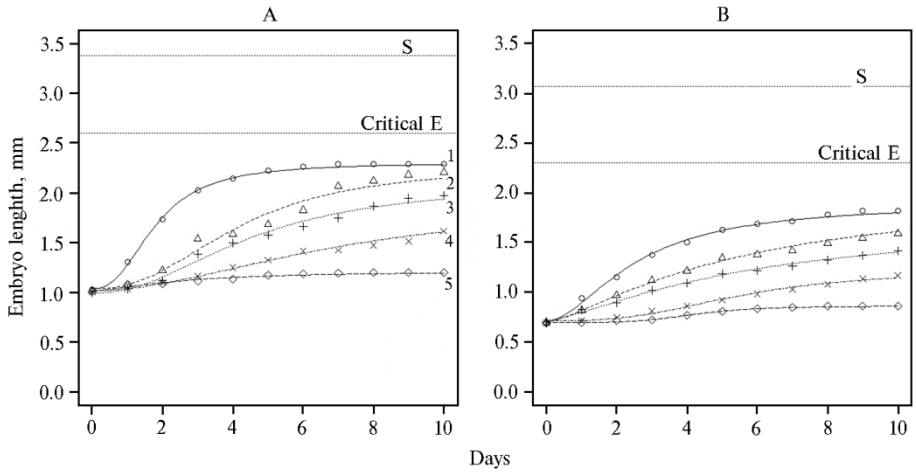

Fig. 1. Embryo growth during dill (*Anethum graveolens* L.) late-season cv. Centaur seed germination as influenced by high temperature and the umbel order on the mother plant: A — first-order umbels, B — second-order umbels; 1 — 20 °C, 2 — 25 °C, 3 — 30 °C, 4 — 35 °C, 5 — 40 °C; S — endosperm length, Critical E — critical embryo length (lab tests, 2015-2016).

An increase in temperature also resulted in inhibition of seed germination ($p < 0.001$), which was expressed in a decrease in the germination rate and the number of germinated seeds (Fig. 2). The average percentage of germination for 1o seeds decreased with an increase in temperature to 30 and 35 °C compared to 20 °C (by 16.0±0.7%, $p < 0.001$ and 46.0±0.8%, $p < 0.001$, respectively). The percentage of germination of 2o seeds also decreased significantly ($p < 0.001$). At 20 °C, the percentage of germination of 1o seeds was 19.0±0.63% ($p < 0.001$) higher than that of 2o seeds. At 30 and 35 °C, germination of 2o seeds compared to 1o seeds decreased 2.1-fold and 5.2-fold, respectively ($p < 0.001$). The 50% germination period for 1o seeds at 30 and 35 °C was 2.3±0.1 ($p < 0.001$) and 3.5±0.2 days ($p < 0.001$) longer than at 20 °C. For 2o seeds at 30 °C, this time increased by 3.7±0.2 days ($p < 0.001$) compared to 20 °C.

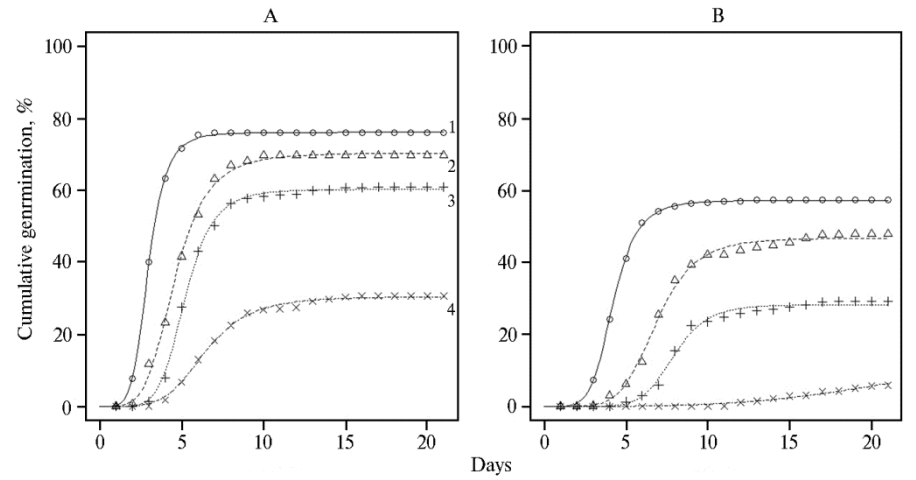

Fig. 2. Germination of dill (*Anethum graveolens* L.) late-season cv. Centaur seeds as influenced by high temperature and the umbel order on the mother plant: A — first-order umbels, B — second-order umbels; 1 — 20 °C, 2 — 25 °C, 3 — 30 °C, 4 — 35 °C (at 40 °C, no germination occurred; lab tests, 2015-2016).

The maximum embryo length in germinating seeds had a significant effect

on the number of germinated seeds (the Pearson correlation coefficient $r = 0.946$, $t = 25.85$; $p < 0.001$). Two-way analysis of variance revealed a significant influence of several factors, i.e., the high temperature (57%, $F = 415.3$, $p < 0.001$), the location on the mother plant (37.2%, $F = 270.5$, $p < 0.001$), the year of growing (5.3%, $F = 38.5$; $p < 0.001$), and their interaction (0.37%, $F = 2.7$, $p = 0.004$) on the maximum embryo length during seed germination. The data show that the year had the smallest influence. The high-temperature factor (71.0%, $F = 1013.1$, $p < 0.001$), the location on the mother plant (27.6%, $F = 395.1$, $p < 0.001$), the year of growing (0.9%, $F = 12.9$, $p = 0.001$), and the interaction of these factors (0.7%, $F = 10.1$, $p < 0.001$) had a significant effect on the percentage of germinated seeds. As seen, the effect of high temperature was of decisive importance for the embryo growth and germination of intact dill seeds.

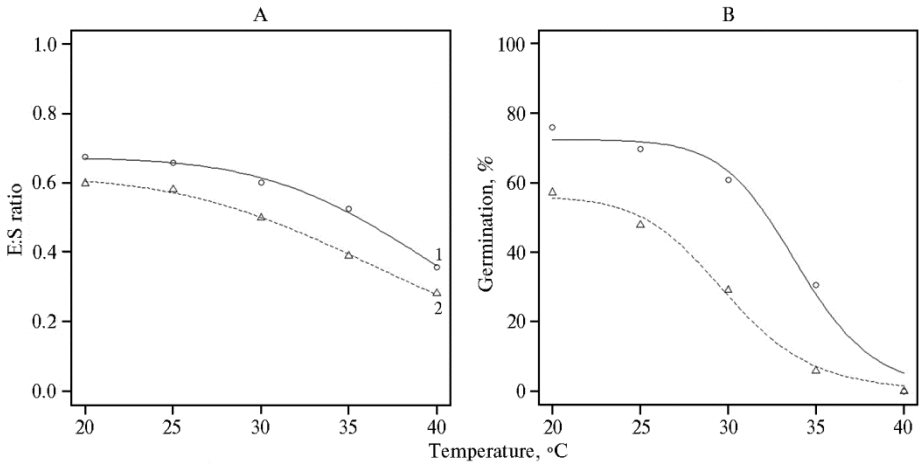

Fig. 3. Dose—response relationship between temperature and maximum embryo length in dill (*Anethum graveolens* L.) late-season cv. Centaur seeds during germination (for more correct interpretation, embryo length to the endosperm length E:S ratio was used for recalculation) (A) **and percentage of germinated seeds** (B) **depending on the umbel order:** A — first-order umbels, B — second-order umbels lab tests, 2015-2016).

The maximum temperature which allows embryos growth is 40±0.4 °C, for 1o seed and 38±0.5 °C for 2o seeds ($p < 0.001$). The maximum temperature, allowing for radicle emergence in at least 50% of viable seeds, was 34±0.3 °C for 1o and 30±0.4 °C for 2o ($p < 0.001$) (Fig. 3).

Differentiated embryos in mature seeds is characteristic of the *Apiaceae* crops, however, a certain proportion of seeds have embryos underdeveloped to varying degrees. Their further development occurs after the seed separation from the mother plant, i.e., during germination, and has its own characteristics [49, 50]. We have shown that embryos from seeds of different orders differ not only in length, but also in E:S ratios. The initial E:S value for the 1o embryos was 0.31±0.01. They had pronounced cotyledons and root. For the 2o embryos, the initial ratio was 0.23±0.01, and the cotyledons and root were of equal length and weakly developed. That is, already at the early germination, the 1o embryos had an advantage over the 2p embryos. Larger lo seeds accumulated more nutrients and had a potential resource for maintaining higher growth rate of the embryo. According to the data obtained, in order the radicle to be emerged, the embryo should lengthen at least by 40-50% of its original length. In this case, the growth rates of the embryo (see Fig. 1) under conditions of optimal and stress temperatures significantly differed depending on seed location. As a result, the number of germinated 1o seeds was higher than that of 2o seeds (see Fig. 2).

Under unpredictable growing conditions, temporal separation of seed

ripening or germination effectively reduces the risk of offspring death and increases reproductive success. It can be expected that the role of varying the morphometric parameters of seeds within individual plants will increase if the environment is unpredictable [51-53]. Consequently, from an evolutionary point of view, seed heteromorphism is an adaptively positive phenomenon. However, in agronomic practice, as a rule, it becomes the cause of a decrease in the number and rates of seed germination, thinness of stands, heterogeneity of seedlings and plants [54, 55]. The problem of heteromorphism can be addressed by improving the production and processing of seeds.

Heat stress is one of the most significant abiotic factors that determine the productivity of many agricultural crops [56-58], in particular celery [59, 60]. It is believed that exceeding the optimal temperature for a particular plant species by 10-15 °C causes a cascade of responses aimed at transmitting a stress signal and increasing resistance, which is expressed in a shift in metabolic reactions and physiological processes [61-63]. Non-dormant seeds can usually germinate over a wide range of temperatures. However, a constant temperature of about 40 °C during the swelling period turns out to be critical for seeds of many species and makes germination difficult [64].

We revealed a significant sensitivity of dill embryos to high temperatures, depending on the order of umbels. The physiological response to the continuous action of elevated temperatures (30-40 °C) was a progressive inhibition of embryonic growth and seed germination. At temperatures from 25 to 35 °C, differences were observed in the response of embryos and seeds to stressors. The growth of the embryo and germination slowed down to one degree or another with an increase in the swelling temperature to 30-35 °C for 1o seeds ad up to 25-30 °C for 2o seeds.

The effect of temperature on seed germination has been studied in several works [65-67]. In particular, the combination of factors of salinity, water and temperature regimes for seed germination was studied [68-70]. However, the influence of the embryo diversity due to location on the mother plant on the resistance to high temperatures during germination was not considered. In our experiments, the maximum germination temperature, allowing for radicle emergence in at least 50% of viable seeds and embryo growth, differed significantly depending on seed orders. As the critical temperature (40 °C) was approached, the differences leveled off. Embryos in 1o and 2o seeds exhibited significant sensitivity to prolonged and continuous exposure to elevated temperatures during swelling and were not capable of germination at 40 °C. Intact seeds were found to be more sensitive to high temperatures than embryos.

Germination rate is another important aspect of the seed germination process which can be temperature dependent. Our results showed that, for dill, the germination rate of intact seeds increased linearly in the temperature range to their optimum value, and then decreased. Similar linear relationships between the germination rate and temperature were observed in millet [71], *Kochia scoparia* [72], cuphea [73], *Plantago ovata* [74], and some medicinal plants [75]. We found significant differences in the germination rate of 10 and 2o dill seeds under high temperature stress.

The thermosensitivity of embryos, due to their morphological underdevelopment, is one of the main reasons for the slow, inhomogeneous and incomplete germination of the seed population under superoptimal temperature conditions. Modeling the process of germination of seeds with an underdeveloped embryo is of interest both for breeding practice and for improving seed pre-sowing processing. Methods based on the kinetics of embryo growth and germination of

heteromorphic dill seeds under a wide range of temperature can be useful in breeding for heat resistance

Thus, the initial development of dill embryos of the cv. Centaur during formation of seeds on the mother plant affects their germination. Embryos from seeds derived from umbels of different orders (first-order — 1o, second-order — 2o) differ in morphometric parameters. The 1o embryos were significantly larger than 2o embryos in initial size. The 1o embryos are better developed and less sensitive to high temperatures during germination. High temperatures significantly influence the embryo growth rate, seed germination, and the number of germinated seeds. The seeds are more sensitive to high temperatures than the embryos. Significant differences in the thermal sensitivity of embryos and intact seeds due to location on the mother plant that we revealed during germination occur already at 20-30 °C. The temperature which rises to 35-40 °C inhibits embryo growth and seed germination regardless of the umbel orders. In dill cv. Centaur, the maximum temperature for the 1o embryos was significantly higher than for the 2o embryos. Our study has shown that the temperature factor largely determines embryo development prior to seed germination and can be an effective for pre-sowing seed treatment.

REFERENCES

1. Sun H.Z., Lu J.J., Tan D.Y., Baskin J.M., Baskin C.C. Dormancy and germination characteristics of the trimorphic achenes of *Garhadiolus papposus* (*Asteraceae*), an annual ephemeral from the Junggar Desert, China. *South African Journal of Botany*, 2009, 75(3): 537-545 (doi: 10.1016/j.sajb.2009.05.001).
2. Yao S., Lan H., Zhang F. Variation of seed heteromorphism in *Chenopodium album* and the effect of salinity stress on the descendants. *Annals of Botany*, 2010, 105(6): 1015-1025 (doi: 10.1093/aob/mcq060).
3. Cao J., Lv X.Y., Chen L., Xing J.J., Lan H.Y. Effects of salinity on the growth, physiology and relevant gene expression of an annual halophyte grown from heteromorphic seeds. *AoB PLANTS*, 2015, 7: plv112 (doi: 10.1093/aobpla/plv112).
4. Harper J.L. *Population biology of plants.* London, Academic Press, 1977.
5. Mandák B. Seed heteromorphism and the life cycle of plants: a literature review. *Preslia-Prague*, 1997, 69: 129-159.
6. Imbert E. Ecological consequences and ontogeny of seed heteromorphism. *Perspectives in Plant Ecology, Evolution and Systematics*, 2002, 5(1): 13-36 (doi: 10.1078/1433-8319-00021).
7. Moles A.T., Westoby M. Seed size and plant strategy across the whole life cycle. *Oikos*, 2006, 113(1): 91-105 (doi: 10.1111/j.0030-1299.2006.14194.x).
8. Clay K. The differential establishment of seedlings from chasmogamous and cleistogamous flowers in natural populations of the grass *Danthonia spicata* (L.) Beauv. *Oecologia*, 1983, 57: 183-188 (doi: 10.1007/BF00379579).
9. Venable D.L., Burquez A., Corral G., Morales E., Espinosa F. The ecology of seed heteromorphism in *Heterosperma pinnatum* in Central Mexico. *Ecology*, 1987, 68(1): 65-76 (doi: 10.2307/1938805).
10. Jordano P. Seed weight variation and differential avian dispersal in blackberries *Rubus ulmifolius*. *Oikos*, 1984, 43(2): 149-153 (doi: 10.2307/3544762).
11. Stanton M.L. Seed variation in wild radish: effect of seed size on components of seedling and adult fitness. *Ecology*, 1984, 65(4): 1105-1112 (doi: 10.2307/1938318).
12. Agren J. Seed size and number in *Rubus chamaemorus*: between-habitat variation, and effects of defoliation and supplemental pollination. *Journal of Ecology*, 1989, 77(4): 1080-1092 (doi: 10.2307/2260824).
13. Venudevan B., Srimathi P., Natarajan N., Vijayakumar R.M. Influence of fruit polymorphism on seed and seedling quality characters of bael (*Aegle marmelos*) the endangered medicinal tree. *Asian Journal of Crop Science*, 2013, 5(4): 452-458 (doi: 10.3923/ajcs.2013.452.458).
14. Bhatt A., Santo A. Germination and recovery of heteromorphic seeds of *Atriplex canescens* (*Amaranthaceae*) under increasing salinity. *Plant Ecology*, 2016, 217: 1069-1079 (doi: 10.1007/s11258-016-0633-6).
15. García-Beltrán J.A., Barrios D., Cuza-Pérez A. Heteromorphism in seeds of *Leptocereus scopulophilus* (*Cactaceae*) from Pan de Matanzas, Cuba. *Seed Science Research*, 2017, 27(4): 311-320 (doi: 10.1017/s0960258517000289).

16. Leverett L.D., Jolls C.L. Cryptic seed heteromorphism in *Packera tomentosa* (*Asteraceae*): differences in mass and germination. *Plant Species Biology*, 2014, 29(2): 169-180 (doi: 10.1111/1442-1984.12011).
17. Souza M.L., Fagundes M. Seed size as key factor in germination and seedling development of *Copaifera langsdorffii* (*Fabaceae*). *American Journal of Plant Sciences*, 2014, 5(17): 2566-2573 (doi: 10.4236/ajps.2014.517270).
18. van Mölken T., Jorritsma-Wienk L.D., van Hoek P.H.W., de Kroon H. Only seed size matters for germination in different populations of the dimorphic *Tragopogon ratensis* subsp. *pratensis* (*Asteraceae*). *American Journal of Botany*, 2005, 92(3): 432-437 (doi: 10.3732/ajb.92.3.432).
19. Baskin J.M., Lu J.J., Baskin C.C., Tan D.Y. The necessity for testing germination of fresh seeds in studies on diaspore heteromorphism as a life-history strategy. *Seed Science Research*, 2013, 23(2): 83-88 (doi: 10.1017/s096025851300010x).
20. Tongshun W., Hongling W., Lei W., Baoping S. Germination of heteromorphic seeds of Atriplex aucheri and its hormonal explanation. *Vegetos — An International Journal of Plant Research*, 2014, 27(1): 103-107 (doi: 10.5958/j.2229-4473.27.1.017).
21. Halloran G.M., Collins W.J. Physiological predetermination of the order of hardseededness breakdown in subterranean clover (*Trifolium subterraneum* L.). *Annals of Botany*, 1974, 38(5): 1039-1044 (doi: 10.1093/oxfordjournals.aob.a084894).
22. Baskin C.C., Baskin J.M. *Seeds: ecology, biogeography, and evolution of dormancy and germination*. San Diego, Academic Press, 1998.
23. Gray D., Steckel J.R.A. Parsnip (*Pastinaca sativa*) seed production: effects of seed crop plant density, seed position on the mother plant, harvest date and method, and seed grading on embryo and seed size and seedling performance. *Annals of Applied Biology*, 1985, 107(3): 559-570 (doi: 10.1111/j.1744-7348.1985.tb03172.x).
24. Bianco V.V., Damato G., Defilippis R. Umbel position on the mother plant: «seed» yield and quality of seven cultivars of Florence fennel. *Acta Horticulturae*, 1994, 362: 51-58 (doi: 10.17660/actahortic.1994.362.5).
25. Hendrix S.D. Variation in seed weight and its effects on germination in *Pastinaca sativa* L. (*Umbelliferae*). *American Journal of Botany*, 1984, 71(6): 795-802 (doi: 10.1002/j.1537-2197.1984.tb14144.x).
26. Thompson J.N. Variation among individual seed masses in *Lomatium grayi* (*Umbelliferae*) under controlled conditions: magnitude and partitioning of the variance. *Ecology*, 1984, 65(2): 626-631 (doi: 10.2307/1941425).
27. Thomas T.H., Gray D., Biddington N.L. The influence of the position of the seed on the mother plant on seed and seedling performance. *Acta Horticulturae*, 1978, 83: 57-66 (doi: 10.17660/actahortic.1978.83.7).
28. Szafiroska A.I. The correlation between mother plant architecture, seed quality and field emergence of carrot. *Acta Horticulturae*, 1994, 354: 93-98 (doi: 10.17660/actahortic.1994.354.10).
29. Corbineau F., Picard M.A., Bonnet A., Côme D. Effects of production factors on germination responses of carrot seeds to temperature and oxygen. *Seed Science Research*, 1995, 5(3): 129-135 (doi: 10.1017/s0960258500002749).
30. Panayotov N. Heterogeneity of carrot seeds depending on their position on the mother plant. *Folia Horticulturae*, 2010, 22(1): 25-30 (doi: 10.2478/fhort-2013-0147).
31. Scholten M., Donahue J., Shaw N.L., Serpe M.D. Environmental regulation of dormancy loss in seeds of *Lomatium dissectum* (Apiaceae). *Annals of Botany*, 2009, 103(7): 1091-1101 (doi: 10.1093/aob/mcp038).
32. Hawkins T.S., Baskin C.C., Baskin J.M. Morphophysiological dormancy in seeds of three eastern North American *Sanicula species* (*Apiaceae* subf. *Saniculoideae*): evolutionary implications for dormancy break. *Plant Species Biology*, 2010, 25(2): 103-113 (doi: 10.1111/j.1442-1984.2010.00273.x).
33. Vandelook F., Janssens S.B., Probert R.J. Relative embryo length as an adaptation to habitat and life cycle in *Apiaceae*. *New Phytologist*, 2012, 195(2): 479-487 (doi: 10.1111/j.1469-8137.2012.04172.x).
34. Alvarado V., Bradford K.J. A hydrothermal time model explains the cardinal temperature for seed germination. *Plant, Cell and Environment*, 2002, 25(8): 1061-1069 (doi: 10.1046/j.1365-3040.2002.00894.x).
35. Sarmadnia G.H. *Seed technology*. Mashhad University Press, 1997.
36. Yan W., Hunt L.A. An equation for modeling the temperature response of plants using only the cardinal temperatures. *Annals of Botany*, 1999, 84(5): 607-614 (doi: 10.1006/anbo.1999.0955).
37. Pereira R.S., Nascimento W.M., Vieira J.V. Carrot seed germination and vigor in response to temperature and umbel orders. *Scientia Agricola*, 2008, 65(2): 145-150 (doi: 10.1590/s0103-90162008000200006).
38. Nascimento W.M., Pereira R.S., Vieira J.V., Cantliffe D.J. Carrot seed germination at high temperature conditions. *Acta Horticulturae*, 2012, 936: 133-138 (doi: 10.17660/actahortic.2012.936.15).

39. Hołubowicz R., Morozowska M. Effect of umbel position on dill (*Anethum graveolens* L.) plants growing in field stands on selected seed stalk features. *Folia Horticulturae*, 2011, 23(2): 157-163 (doi: 10.2478/v10245-011-0024-3).
40. Vandelook F., Bolle N., Van Assche J.A. Seed dormancy and germination of the European *Chaerophyllum temulum* (*Apiaceae*), a member of a trans-Atlantic genus. *Annals of Botany*, 2007, 100(2): 233-239 (doi: 10.1093/aob/mcm090).
41. Vandelook F., Bolle N., Van Assche J.A. Morphological and physiological dormancy in seeds of *Aegopodium podagraria* (*Apiaceae*) broken successively during cold stratification. *Seed Science Research*, 2009, 19(2): 115-123 (doi: 10.1017/s0960258509301075).
42. Baleev D.N., Bukharov A.F. *Ovoshchi Rossii*, 2012, 3(16): 38-46 (in Russ.).
43. Nikolaeva M.G., Lyanguzova I.V., Pozdova L.M. *Biologiya semyan* [Seed biology]. St. Petersburg, 1999 (in Russ.).
44. Vandelook F., Van Assche J.A. Temperature, requirements for seed germination and seedling development determine timing of seedling emergence of three monocotyledonous temperate forest spring geophytes. *Annals of Botany*, 2008, 102(5): 865-875 (doi: 10.1093/aob/mcn165).
45. Necajeva J., Ievinsh G. Seed dormancy and germination of an endangered coastal plant *Eryngium maritimum* (*Apiaceae*). *Estonian Journal of Ecology*, 2013, 62(2): 150-161 (doi: 10.3176/eco.2013.2.06).
46. Ritz C., Pipper C.B., Streibig J.C. Analysis of germination data from agricultural experiments. *European Journal of Agronomy*, 2013, 45: 1-6 (doi: 10.1016/j.eja.2012.10.003).
47. Ritz C., Baty F., Streibig J.C., Gerhard D. Dose-response analysis using R. *PLoS ONE*, 2015, 10(12): e0146021 (doi: 10.1371/journal.pone.0146021).
48. R Development Core Team. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria, 2011.
49. Martin A.C. The comparative internal morphology of seeds. *The American Midland Naturalist*, 1946, 36(3): 513-660 (doi: 10.2307/2421457).
50. Stokes P.A physiological study of embryo development in *Heracleum sphondylium* L.: I. The effect of temperature on embryo development. *Annals of Botany*, 1952, 16(3): 441-447 (doi: 10.1093/oxfordjournals.aob.a083326).
51. Galloway L.F., Etterson J.R., McGlothlin J.W. Contribution of direct and maternal genetic effects to life-history evolution. *New Phytologist*, 2009, 183(3): 826-838 (doi: 10.1111/j.1469-8137.2009.02939.x).
52. Dyer A.R., Brown C.S., Espeland E.K., McKay J.K., Meimberg H., Rice K.J. Synthesis: the role of adaptive trans-generational plasticity in biological invasions of plants. *Evolutionary Applications*, 2010, 3(2): 179-192 (doi: 10.1111/j.1752-4571.2010.00118.x).
53. Lerner P.D., Bai Y., Morici E.F.A. Does seed heteromorphism have different roles in the fitness of species with contrasting life history strategies? *Botany*, 2008, 86(12): 1404-1415 (doi: 10.1139/b08-106).
54. Gharoobi B. Effects of seed size on seedlings characteristics of five barley cultivars. *Iranian Journal of Plant Physiology*, 2011, 1(4): 265-270.
55. Nik M.M., Babaeian M., Tavassoli A. Effect of seed size and genotype on germination characteristic and seed nutrient content of wheat. *Scientific Research and Essays*, 2011, 6(9): 2019-2025 (doi: 10.5897/sre11.621).
56. Maraghni M., Gorai M., Neffati M. Seed germination at different temperatures and water stress levels, and seedling emergence from different depths of *Ziziphus lotus*. *South African Journal of Botany*, 2010, 76(3): 453-459 (doi: 10.1016/j.sajb.2010.02.092).
57. Wen B. Effects of high temperature and water stress on seed germination of the invasive species Mexican sunflower. *PLoS ONE*, 2015, 10: e0141567 (doi: 10.1371/journal.pone.0141567).
58. Chitwood J., Shi A., Evans M., Rom C., Gbur E.E., Motes D., Chen P., Hensley D. Effect of temperature on seed germination in spinach (*Spinacia oleracea*). *HortScience*, 2016, 51(12): 1475-1478 (doi: 10.21273/hortsci11414-16).
59. Nascimento W.M., Huber D.J., Cantliffe D.J. Carrot seed germination and respiration at high temperature in response to seed maturity and priming. *Seed Science and Technology*, 2013, 41(1): 164-169 (doi: 10.15258/sst.2013.41.1.19).
60. Nascimento W.M., Huber D.J., Cantliffe D.J. Carrot seed germination and ethylene production at high temperature in response to seed osmopriming. *Horticultura Brasileira*, 2013, 31(4): 554-558 (doi: 10.1590/s0102-05362013000400008).
61. Wehmeyer N., Vierling E. The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. *Plant Physiology*, 2000, 122(4): 1099-1108 (doi: 10.1104/pp.122.4.1099).
62. Wang W., Vinocur B., Shoseyov O., Altman A. Role of plant heat-shock proteins and molecular shaperones in the abiotic stress response. *Trends in Plant Science*, 2004, 9(5): 244-252 (doi: 10.1016/j.tplants.2004.03.006).
63. Mahmood T., Safdar W., Abbasi B.H., Naqvi S.M.S. An overview on the small heat shock

proteins. *African Journal of Biotechnology*, 2010, 9(7): 927-939 (doi: 10.5897/ajb09.006).
64. Bewley J.D., Black M. *Physiology and biochemistry of seeds in relation to germination*. Berlin, Heidelberg, Springer, 1982.
65. Cicek E., Tilki F. Effects of temperature, light and storage on seed germination of *Ulmus glabra* Huds. and *U. laevis* Pall. *Pakistan Journal of Biological Sciences*, 2006, 9(4): 697-699 (doi: 10.3923/pjbs.2006.697.699).
66. Martínez-Sánchez J.J., Conesa E., Vicente M.J., Jiménez A., Franco J.A. Germination responses of *Juncus acutus* (*Juncaceae*) and *Schoenus nigricans* (*Cyperaceae*) to light and temperature. *Journal of Arid Environments*, 2006, 66(1): 187-191 (doi: 10.1016/j.jaridenv.2005.11.004).
67. Zehtab-Salmasi S. Effects of salinity and temperature on germination of dill (*Anethum graveolens* L.). *Plant Sciences Research*, 2008, 1(1): 27-29.
68. Khan M.A., Ungar I.A. Effect of thermoperiod on recovery of seed germination of halophytes from saline conditions. *American Journal of Botany*, 1997, 84(2): 279-283 (doi: 10.2307/2446089).
69. Khan M.A., Gul B., Weber D.J. Seed germination in relation to salinity and temperature in *Sarcobatus vermiculatus*. *Biologia plantarum*, 2001, 45(1): 133-135 (doi: 10.1023/a:1015133515568).
70. Pompelli M.F., Fernandes D., Guerra M.P. Germination of *Dyckia encholirioides* (Gaudichaud) Mez var. *encholiriodies* under saline conditions. *Seed Science and Technology*, 2006, 34(3): 759-763 (doi: 10.15258/sst.2006.34.3.24).
71. Kamkar B., Koochaki A., Mahallati M.N., Moghaddam P.R. Cardinal temperatures for germination in three millet species. *Asian Journal of Plant Sciences*, 2006, 5(2): 316-319 (doi: 10.3923/ajps.2006.316.319).
72. Jami Al-Ahmadi M., Kafi M. Cardinal temperatures for germination of *Kochia scoparia* (L.). *Journal of Arid Environments*, 2007, 68(2): 308-314 (doi: 10.1016/j.jaridenv.2006.05.006).
73. Berti M.T., Johnson B.L. Seed germination response of cuphea to temperature. *Industrial Crops and Products*, 2008, 27(1): 17-21 (doi: 10.1016/j.indcrop.2007.05.004).
74. Tabrizi L., Nasiri M., Kouchaki A. Investigations on the cardinal temperatures for germination of *Plantago ovata* and *Plantago psyllium*. *Iranian Journal of Field Crops Research*, 2005, 2(2): 143-150.
75. Bannayan M., Nadjafi F., Rastgoo M., Tabrizi L. Germination properties of some wild medicinal plants from Iran. *Seed Technology*, 2006, 28(1): 80-86.

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HORMONE BALANCE AND SHOOT GROWTH IN WHEAT (*Triticum durum* Desf.) PLANTS AS INFLUENCED BY SODIUM HUMATES OF THE GRANULATED ORGANIC FERTILIZER

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Abstract

Humic acids are formed in soil during decomposition of organic residues and are capable of increasing plant productivity. Plant growing widely utilizes application of the most soluble preparations on the base of sodium and potassium humates possessing hormone-like activity. Humic substances are capable of stimulating plant growth in very low concentrations that determines the necessity of targeting their uptake by plants. Such a possibility is provided by placing fertilizers in the direct vicinity of the seeds. The effect of incrustation of Nitrofosque granules with sodium humate (SH) on the crop yield of spring durum wheat has been shown previously. In the present experiments, the data on the changes in growth and hormone content of wheat plants (Bashkirskaya 27 cv.) treated with SH included into the granules of organic-mineral fertilizer (OMF) are reported for the first time. Granules of fertilizers without SH and those containing 1.25×10^{-2} %, 2.5×10^{-2} % and 5×10^{-2} % of humic preparation (of the granule mass) were placed at the distance of 2-3 cm from the 1-day seedlings planted into the soil. OMF was obtained from poultry waste (chicken manure) and dolomite and SH was extracted from brown coal. Plants that obtained neither humates nor OMF served as the control. Leaf length and transpiration (according to the decline in the mass of vessels with plants) were measured starting from the third day after sowing. Leaf samples for determination of hormones with enzyme-linked immunoassay were sampled on day 9 and leaf area and plant mass were measured on day 21 after sowing. OMF granules without SH stimulated leaf elongation resulting in their significantly longer length compared with the control (42, 156, 187 and 274 mm against 47, 167, 199 and 294 mm, $p \le 0.05$) detected in the first leaves 3, 6 and 8 days after sowing and 14 days after sowing in the second leaves. Meanwhile an increase in the OMF dosage did not increase their length significantly compared to the lower dose. Addition of humic substances to the OMF granules increased the promotive effect of the preparation on the leaf elongation most clearly manifested in the case of intermediate SH concentration ($2{,}5\times10^{-2}$ %), which significantly increased the leaf length during the time of registration (3, 6, 8, and 14 days after sowing) compared to the application of OMF without SH (6, 11, 13 mm increment of the first leaves and 9.4, 9, 22 mm of the second leaves, $p \le 0.05$). Unlike OFM without SH, which application did not change the shoot mass and leaf area, combination of OFM with intermediate SH concentration increased shoot mass (from 538 to 583 mg, $p \le 0{,}05$). Leaf area was significantly greater than in the control in the case of intermediate and maximal SH concentration (increments were 385 and 283 mm^2, $p \le 0.05$). Thus, addition of SH to OFM increased the effectiveness of the OFM action on growth characteristics. Accumulation of nitrogen in the shoots of the wheat plants

supplied only with OFM without SH did not differ from the control, while combination of OFM with SH resulted in accumulation of nitrogen at the level (by 8-15 %, $p \leq 0.05$) higher than in the control. Application of OFM without SH did not influence the content of studied hormones (auxins, cytokinins and abscisic acid — ABA), while addition of SH to the granules of fertilizers resulted in significantly higher concentration of abscisic acid (ABA) and cytokinins in the shoots, the 1.6-2.8 $ng \cdot g^{-1}$ increment (1.5-1.8 times) and 3.8-4.9 $ng \cdot g^{-1}$ increment (1.5-1.7 times) ($p \leq 0.05$). Concentration of indole acetic acid (IAA) with intermediate SH concentration was higher than in the control (40 against 15 $ng \cdot g^{-1}$, $p \leq 0.05$). The results obtained by us allow attributing increased effectiveness of fertilizers in the case of their supply with humates to their effect on the hormonal content. Increased content of hormones with promotive type of action is likely to enable activation of plant growth, while accumulation of ABA limits water losses by transpiration. The results of these laboratory experiments indicate perspectives of application of OMF containing sodium humates according to technology developed by us. Furthermore, the obtained data are of importance for revealing fundamental mechanisms, by which the action of humic compounds is manifested, in particular at the level of hormonal balance in plants.

Keywords: *Triticum durum,* organo-mineral fertilizer, sodium humate, auxins, cytokinins, abscisic acid, plant growth

Humic substances, mostly humic acids and fulvic acids synthesized in the soil during the decomposition of organic residues, can stimulate plant growth and yield [1]. Sodium and potassium humates manufactured commercially by alkaline extraction of caustobiolites (brown coal, peat, sapropel) are increasingly used in crop production. It is assumed that humates affect plant productivity both directly and indirectly. The indirect effect is associated with a modification of the soil structure in the rhizosphere and an increase in the availability of mineral elements for plants, while the direct effect lies in a change in the metabolism and development of plants [2, 3].

The mechanism underlying the effect of humic substances on physiological and biochemical processes in plants is not fully understood [4]. Humic substances of a relatively low molecular weight can form supramolecular associations consisting of hydrophilic and hydrophobic domains [5]. The hydrophobic components of humic substances resulted from destruction of plant residues capture the hydrophilic components of the soil and protect them from degradation [6). The compounds captured in this way can be further released due to changes in the structure of these associates under the influence of low-pH root exudates [4]. The direct effect of humic acids on the growth and development of plants is a manifestation of their hormone-like activity [7-9]. Auxins (mainly indoleacetic acid) [7, 10] and cytokinins (in the form of isopentenyladenine) [11] are identified in preparations of humic acids. Humic acids affect enzyme activity, gene expression, and proton pump activity in the same way as the plant hormone auxin [7-10]. There are reports that humic substances change the root architecture and metabolism due to such activity [12]. The hormone-like properties of humic preparations determine one of the advantages of organic fertilizers over chemical ones. However, the effects of humic acids on the levels of auxins and cytokinins in plants have not yet been studied.

The practical advantage of humic compounds is that they can stimulate plant growth in very low concentrations, from 20 mg / l^{-1} [7], which provides their cost-effectiveness. However, in case of low concentrations, the problem of targeting and entry into plants arises. The strip till technology and special seeders which allow fertilizers to be incorporated in the immediate vicinity of the seeds facilitate the problem.

Previously, we have shown the positive effect of sodium humate incorporated into nitrophoska granules on the yield of spring durum wheat [13]. This work is the first to report on the effect of different doses of sodium humate-based organic mineral fertilizers (OMF) on the growth and hormonal balance of wheat plants.

Our main purpose was to confirm or disprove the hypothesis that the effectiveness of the organomineral fertilizers combined with sodium humate is due to the sodium humate effect on the hormonal system of plants.

Materials and methods. Sodium humate (SH) was obtained according to a procedure similar to that described [14, 15]: 100 ml of 3% NaOH was added to 5 g of brown coal (Kumertau deposit, Republic of Bashkortstan) and stirred for 2 h at 60 °C, the undissolved precipitate (humin) was separated by centrifugation and rinsed by distilled water, 1% HCl was added to the resulting solution to pH = 2, the precipitate of humic acids was separated (a PE 6910 centrifuge, OOO EkrosKhim, Russia, 10 min, 4000 rpm/2325 g), rinsed by distilled water, dried in air, and dissolved in 1% NaOH (0.1 g per 10 ml).

The concentration of 0.01-0.001% solutions of humic acids in a 0.4% NaOH solution was measured by the optical density (OD) (Shimadzu UV 2600, Shimadzu, Japan, λ = 465 nm) as described [14, 15].

The organomineral fertilizers (OMF) were 60-65% fresh chicken manure and 54-33% dolomite (calcium and magnesium carbonates). SH was added to OMF at 1.25×10 2%, 2.5×10 2%, and 5×10 2% (SH1, SH2, and GN3, respectively) of the weight of the OMF granules. A portion of granules were not added with SH.

OMF granules, approximately 80 mg in weight with different contents of humic acids, were placed in pots filled with soil (about 0.82 kg, with a 2 cm layer of pebbles drainage) at a 5-6 cm depth from the soil surface at the rate of one or three granules per seed (80 or 240 mg). The seeds of durum spring wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 after 1-day germination on filter paper were placed at a depth of 3 cm from the soil surface (3 cm from the granules), 10 seeds per pot, 8 pots per treatment. Plants that did not receive additional OMF nutrition served as control. Plants were grown at PAR 400 μmol / m^{-2} (14 h daylight hours, 20/24 °C). The 60-80% soil moisture of the full moisture capacity was maintained with irrigation (2-3 times a week). From day 3 after planting the seedlings, the lengths of the 1st and 2nd leaves were measured. Twenty-one days after planting, the shoots were weighed, and the area of scanned leaf images were measured using the ImageJ software (National Institute of Health, USA; https://imagej.nih.gov).

The hormone concentration was determined on day 9 after planting. Phytohormones were extracted from homogenized shoots with 80% ethyl alcohol at 4 °C overnight. Purification and concentration of indoleacetic acid (IAA) and abscisic acid (ABA) from an aliquot of the aqueous residue after evaporation of the alcohol extract (from Petri dishes in a stream of air) was performed using ether extraction according to a modified scheme with a decrease in volume as described [16). IAA and ABA were twice extracted with diethyl ether from an aliquot of the aqueous residue acidified to pH 2-3 in a ratio of 1:3. Then they were returned to the aqueous phase (1% sodium bicarbonate solution, the ratio of the aqueous to organic phase is 1: 2), acidified again to pH 2-3, and, after 2-fold back-extraction with diethyl ether, methylated with diazomethane derived from nitrosomethylurea and added to the samples. The dried samples were dissolved in a small amount of 80% ethanol immediately before the immunoassay or in 100 μl of 80% ethyl alcohol before the enzyme-linked immunosorbent assay. Cytokinins from an aliquot of the aqueous residue (a sample previously purified by centrifugation) were concentrated in a C18 cartridge (Waters Corporation, USA) equilibrated with distilled water. The column with the sample was washed with 20 ml of distilled water. Cytokinins were eluted with 70% ethyl alcohol, then the alcohol was evaporated to dryness and, after dissolving the residue in 20 μl of 80% ethanol, the sample was analyzed by thin layer chromatography (TLC) in a silufol plate. TLC was

performed using a butanol:ammonia:water (6:1:2) solvent system as described [17]. After UV-detection (TCP-15.MC transilluminator, 4×8 W 312 nm lamps, Vilber Lourmat, France) of the zeatin, zeatin nucleotide and zeatin riboside markers, the zones were eluted with 0.1 M phosphate buffer (pH 7.2-7.4). To sediment silufol, the eluate was centrifuged for 10 min at 10000 rpm (Eppendorf MiniSpin, Eppendorf, US). The IAA, ABA and cytokinins were quantified by enzyme-linked immunosorbent assay (ELISA) with specific antibodies to hormones [16-18]. Antibodies to zeatin riboside were also used for the determination of zeatin and its nucleotide since they have cross-reactivity to these cytokinins.

ELISA test was performed in the wells of Castar polystyrene plates (Corning Incorparated, USA). The hormone-protein conjugate was immobilized on polystyrene. A 200 μl aliquots of the conjugate pre-diluted in 0.05 M immobilization buffer (9% NaCl) were poured into each well and kept at 4 °C for 18-20 h in a refrigerator or 2 h in a thermostat at 37 °C. The plates were washed thrice by physiological saline (pH 7.2-7.4) with 0.05% Tween 20 (PT solution). In all subsequent washings, the same solution was used. In some wells, 10-fold dilutions of hormone standards were poured to obtain a calibration curve. In the remaining wells, the aliquots of alcohol solutions of methylated hormones (IAA, ABA) or aliquots of phosphate buffer (pH 7.2-7.4) used to elute cytokinins from silufol were poured. Antisera to the corresponding hormone (100 μl per well) obtained as described [18, 19] and diluted with saline + 0.3% bovine serum albumin + 0.05% Tween 20 (PTB) were added to all wells. The plates were incubated at 37 °C for 1 h and rinsed with PT solution. To visualize the reaction of serum antibodies with the immobilized hormone conjugates, the peroxidase-conjugated bovine anti-rabbit antibodies were used. The secondary antibodies were diluted in PTB, 200 portions were poured into wells, incubated for 1 h at 37 °C, and rinsed by PT. The color reaction with the substrate ortho-phenylenediamine (0.4 mg/ml in 0.06 M phosphate buffer, pH 5.2, with 0.006% hydrogen peroxide) was stopped in 15-30 min using 4 N sulfuric acid. After measuring the optical density (photometer AIFR-01 UNIPLAN, ZAO PIKON, Russia; $\lambda = 492$ nm), the hormone concentration was calculated using the calibration curve.

On day 21, the total nitrogen concentration was measured according to Kjeldahl. Evapotranspiration of plants was evaluated throughout the experiment as pot weight losses.

Statistical processing was performed by standard methods using the MS Excel software. The means (M) and their standard errors (±SEM) were calculated. Differences were assessed by Student's t-test and considered statistically significant at $p \leq 0.05$. The number of replicates were 5 for nitrogen content assessment, 9 for hormones, 30 for water consumption, and 50 for assessment of plant weight, area and length of leaves.

Results. Mixing chicken manure with dolomite leads to formation of magnesium and calcium salts of uric and other organic acids contained in chicken manure, which reduces their solubility and a negative effect on plant growth when used in high concentrations. In addition, heating OMF to 100 °C due to friction during mixing and extrusion kills pathogenic microorganisms from chicken droppings.

As seen (Fig. 1), the OMF granules increased the length of the leaves by 5-17% compared to the control depending on the leaf age and the treatment option. In 3 days after sowing, the leaves were significantly longer than in the control ($p \leq 0.05$) only at a lower dose of OMF (one granule instead of three) and an intermediate concentration of humates (SH2) (see Fig. 1, A). As the plants grew, the effects of OMF and SH were also manifested at other doses and concentrations, i.e., the 1st leaves on day 8 and the 2nd leaves on day 14 were significantly

longer ($p \le 0.05$) than in the control for all treatments with OMF (with and without humate) (see Fig. 1). An increase in the dose of OMF did not lead to a significant increase in the length of leaves as compared to the minimum OMF dose, i.e., the increase in length compared to the control averaged 7.5 and 6.0% for one and three granules per seed, respectively.

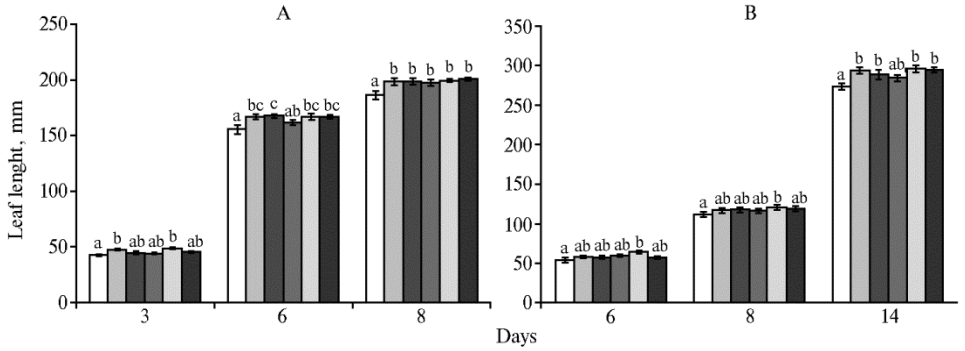

Fig. 1. The 1st (A) and 2nd (B) leaf length in spring durum wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 depending on the dosage pf organomineral fertilizers (OMF granules, mg per seed) **along and with different doses of sodium humate** (SH) (SH1 — $1{,}25\times10^{-2}$ %, SH2 — $2{,}5\times10^{-2}$ %, SH3 — 5×10^{-2} % of OMF granule weight). Bars from left to right are control, OMF 80 mg, OMF 240 mg, OMF 80 mg + SH1, OMF 80 mg + SH2, OMF 80 mg + SH3. Differences between the bars for which there is no coincidence in any letter designation are statistically significant at $p \le 0.05$ ($M\pm$SEM, $n = 50$, pot tests).

SH added to OMF granules increased their stimulating effect on the leaf elongation which was the clearest for SH2 (2.5×10 2% of the granule weight) as compared to the control. Upon this treatment, the average increase in leaf length over the entire observation period (3, 6, 8, and 14 days after planting) averaged 10% (differences as compared to the OMF effect without SH were statistically significant, $p \le 0.05$, paired *t*-test).

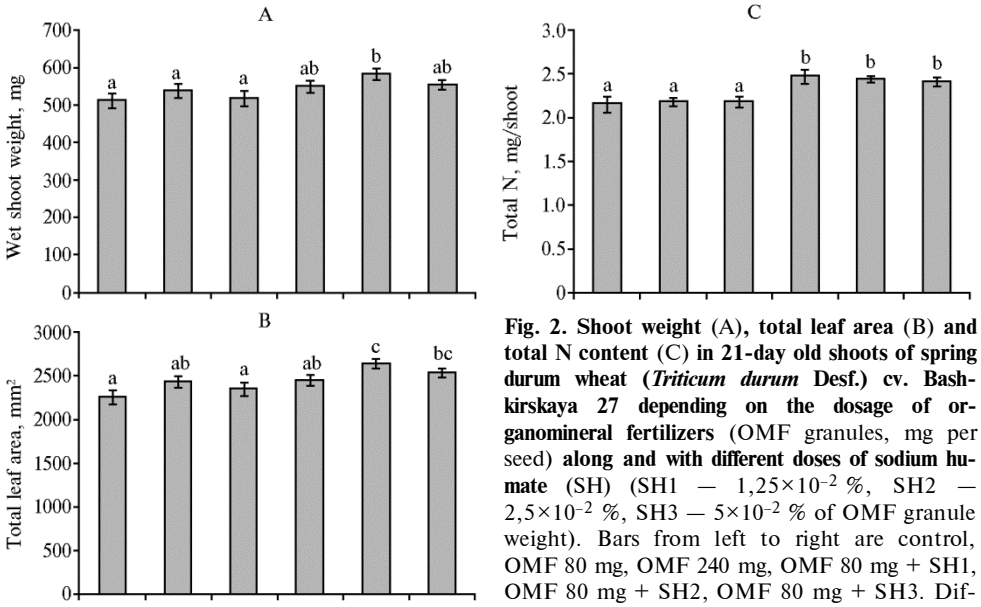

Fig. 2. Shoot weight (A)**, total leaf area** (B) **and total N content** (C) **in 21-day old shoots of spring durum wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 depending on the dosage of organomineral fertilizers** (OMF granules, mg per seed) **along and with different doses of sodium humate** (SH) (SH1 — $1{,}25\times10^{-2}$ %, SH2 — $2{,}5\times10^{-2}$ %, SH3 — 5×10^{-2} % of OMF granule weight). Bars from left to right are control, OMF 80 mg, OMF 240 mg, OMF 80 mg + SH1, OMF 80 mg + SH2, OMF 80 mg + SH3. Differences between the bars for which there is no coincidence in any letter designation are statistically significant at $p \le 0.05$ ($M\pm$SEM, for A and B $n = 50$, for C $n = 5$, pot tests).

Three weeks after planting, the OMF with SH2 led to a significant increase

in the fresh weight of the shoots compared to the control (Fig. 2, A). OMF without SH did not cause a significant increase in the shoot weight compared to the control. For the OMF supplemented with SH1 and SH3, the plants occupied an intermediate position between the control and the use of SH2, that is, their fresh weight did not differ statistically significantly from those in either the first or the second case. Thus, we revealed a tendency to increase the biomass of wheat plants under the influence of a OMF combination with SH, which was statistically significant at an intermediate SH concentration. For SH2 and SH3, the total leaf area was significantly greater than in the control (2637 and 2535 vs. 2252 mm^2, $p \le 0.05$), while OMF without SH and OMF plus the minimum SH1 concentration did not have a significant effect on the indicator (the differences from the control are not significant) (see Fig. 2, B).

The level of total nitrogen in shoots for OMF without humate did not differ from the control (see Fig. 2, C), being significantly higher ($p \le 0.05$) than in the control when OMF with SH were applied.

Evapotranspiration over the entire experiment did not differ between the control plants and those treated with fertilizers (1.6 g of water per plant per day, data not shown).

Upon application of OMF without SH, the concentration of hormones (auxins, cytokinins, and ABA) in wheat plants was at the control level (Fig. 3). The addition of sodium humate increased the content of hormones. To confirm the results of enzyme-linked immunosorbent assay, a comparison of ELISA and gas chromatography-mass spectrometry data was previously performed [17].

The total concentration of cytokinins was stably higher than in the control for all SH concentrations (see Fig. 3, A, $p \le 0.05$), and OMF without SH did not increase the cytokinin concentrations as compared to the control. The accumulation of auxins was pronounced at an intermediate concentration of humate, with the IAA level almost 3 times higher than in the control ($p \le 0.05$) (see Fig. 3, B). The ABA content was higher under the action of all tested concentrations of SH (differences from the control and the OMF without SH are significant at $p \le 0.05$) (see Fig. 3, C).

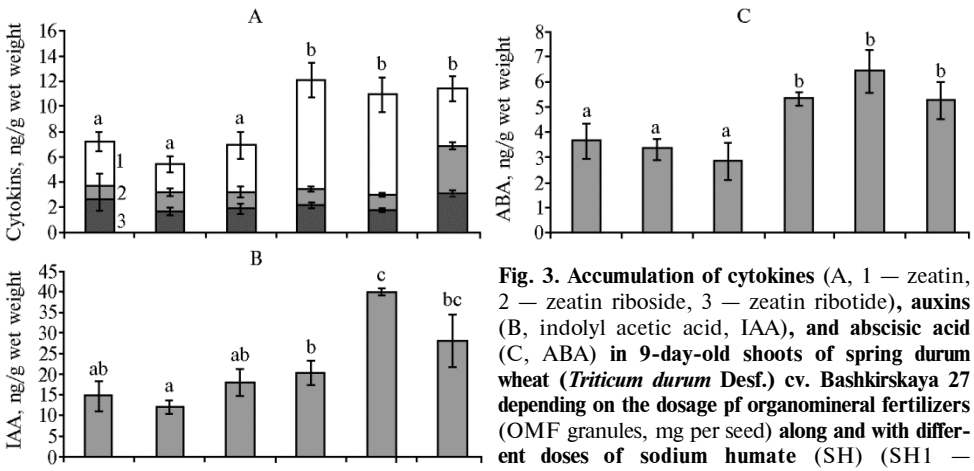

Fig. 3. Accumulation of cytokines (A, 1 — zeatin, 2 — zeatin riboside, 3 — zeatin ribotide)**, auxins** (B, indolyl acetic acid, IAA)**, and abscisic acid** (C, ABA) **in 9-day-old shoots of spring durum wheat (*Triticum durum* Desf.) cv. Bashkirskaya 27 depending on the dosage pf organomineral fertilizers** (OMF granules, mg per seed) **along and with different doses of sodium humate** (SH) (SH1 — $1{,}25\times10^{-2}$ %, SH2 — $2{,}5\times10^{-2}$ %, SH3 — 5×10^{-2} % of OMF granule weight). Bars from left to right are control, OMF 80 mg, OMF 240 mg, OMF 80 mg + SH1, OMF 80 mg + SH2, OMF 80 mg + SH3. Differences between the bars for which there is no coincidence in any letter designation are statistically significant at $p \le 0.05$ ($M\pm$SEM, $n = 9$, pot tests).

In general, the results on the length and area of leaves and the shoot weigh

showed that the humate added to organomineral fertilizers enhances their ability to stimulate the wheat plant growth. It was also revealed that OMF acts in low doses (one granule per seed), and an increase in the dosage to three granules does not enhance the positive effect.

An increase in nitrogen accumulation in shoots in response to application OMF with SH indicates an improvement in the ability of wheat plants to absorb mineral elements under the influence of humate, which corresponds to some reports [20]. Since the nitrogen has been repeatedly shown to stimulate the synthesis of cytokinins [21], their accumulation in the shoots of plants treated with SH can be associated with an increase in the nitrogen content. However, it is worth noting that the total nitrogen in shoots increased under the influence of SH to a lesser extent (to a maximum of 15%) than cytokinins the content which was 1.5 times higher than in the control, regardless of the SH concentrations. The lack of proportionality between the accumulation of nitrogen and cytokinins suggests the existence of additional mechanisms for stimulating the cytokinins accumulation in plants due to the action of sodium humates, e.g., their direct effect on plant hormones.

An alternative explanation may lie in the admixtures of hormone-like compounds in the SH preparation, which corresponds to the literature data [1]. Since physiologically active substances, including hormones, act in low concentrations, it is the presence of hormone-like substances that can explain the ability of small doses of SH to affect the growth and development of plants, as it was shown in our experiment. Auxin-like substances were found in humic preparations [4], which corresponds to our findings which revealed an increase in the content of auxins in plants under the influence of SH. It was also found that the use of a humic preparation increases the availability of nitrogen, phosphorus, and potassium [11].

The content of humic acids was 20-23% in fresh droppings and 15-17% in granulated OMF based on chicken manure and dolomite. However, the solubility of free humic acids is very low, while the solubility of their sodium salts is quite high. Therefore, the high efficiency of granular OMF containing readily soluble sodium humates on plant growth can be ensured by its high biodegradability.

Demin et al. [22] consider the direct penetration of humates into the cell unlikely, since this should be prevented by the formation of hydrogen bonds with the components of the cell walls, but it is possible for humic substances to enter the cell due to endocytosis and their further digestion in lysosomes. It is known that receptors for many hormones are located on the cell surface [23], which makes it possible to explain how hormone-like components contained in humates can affect plants by interacting with receptors on the cell surface without penetrating into the cell.

Our experiments do not allow us to conclude whether the increased levels of hormones are associated with their uptake from the SH preparation. The probability of this process is low, given the large sizes of humate molecules [24]. Nevertheless, the very fact of the accumulation of hormones in plants treated with the preparation we used clearly indicates the physiological activity of SH. It is important that in plants exposed to OMF without humate no increase in the hormone levels was recorded, that is, an increased content of hormones in plants is a characteristic response precisely to the SH in the preparations. The ability of cytokinins and auxins to stimulate shoot growth by activating cell division and elongation is well known [25]. This allows us to explain the activation of growth of wheat shoots under the influence of SH shown in our experiments by the accumulation of auxins and cytokinins.

The accumulation of ABA we found upon the introduction of granules

with sodium humate into the plant rhizosphere corresponds to some data [26]. The authors of the cited communication associate the ABA accumulation with the peculiarities of water exchange in plants treated with humate. We revealed a significantly greater accumulation of ABA under the influence of humates, i.e., almost 2-fold compared to the control, with the reliable differences from the control and OMF without SH ($p \leq 0.05$). As known, the water evaporation by leaves increases with an increase in total leaf area. In our experiment, the leaf area was significantly larger compared to the control at the intermediate and maximum doses of SH. However, we did not reveal a significant increase in transpiration losses (despite the large leaf area), which indicates a decrease in stomatal conductance. Since the ability of ABA to close stomata is well known, it is possible to explain the limitation of transpiration losses by an increase in the ABA content in plants under the influence of SH. We have previously shown that the activation of leaf growth by bacterial plant stimulants was accompanied by the accumulation of ABA which also limited evapotranspiration [18]. Thus, the change in the ABA accumulation under the influence of humate can be an adaptive response aimed at optimizing water relations when the humate acts on plants.

Thus, our findings show that sodium humate (SH) in the formulation of organic fertilizer (OMF) granules activates plant growth, which is most likely due to the ability of SH to influence the plant hormonal status. The accumulation of hormones possessing stimulating effects ensures the activation of plant growth, while the ABA accumulation normalizes water exchange. Our tests in which granulated fertilizers were placed at a distance of several centimeters from the seeds, simulating the strip till technology application, indicate the prospects of using SH-containing OMF in this technology.

REFERENCES

1. García A.C., Olaetxea M., Santos L.A., Mora V., Baigorri R., Fuentes M., Zamarreco A.M., Berbara R.L., Garcia-Mina J.M. Involvement of hormone- and ROS-signaling pathways in the beneficial action of humic substances on plants growing under normal and stressing conditions. *BioMed Research International*, 2016, 2016: 3747501 (doi: 10.1155/2016/3747501).
2. Mora V., Olaetxea M., Bacaicoa E., Baigorri R., Fuentes M., Zamarreco A.M., Garcia-Mina J.M. Abiotic stress tolerance in plants: exploring the role of nitric oxideand humic substances. In: *Nitric oxide in plants: metabolism and role in stress physiology*. M.N. Khan, M. Mobin, F. Mohammad, F.J. Corpas (eds.). Springer, Cham, 2014: 243-264 (doi: 10.1007/978-3-319-06710-0_15).
3. Chen Y., Aviad T. Effects of humic substances on plant growth. In: *Humic substances in soil and crop sciences: selected readings*. P. MacCarthy, C.E. Clapp, R.L. Malcolm and P.R. Bloom (eds.). American Society of Agronomy and Soil Sciences, Madison, 1990: 161-186 (doi: 10.2136/1990.humicsubstances.c7).
4. Canellas L.P., Dantas D.J., Aguiar N.O., Peres L.E.P., Zsogon A., Olivares F.L., Dobbss L.B., Facanha A.R., Nebbioso A., Piccolo A. Probing the hormonal activity of fractionated molecular humic components in tomato auxin mutants. *Annals of Applied Biology*, 2011, 159(2): 202-211 (doi: 10.1111/j.1744-7348.2011.00487.x).
5. Piccolo A. The supramolecular structure of humic substances. A novel understanding of humus chemistry and implications in soil science. *Advances in Agronomy*, 2002, 75: 57-134 (doi: 10.1016/S0065-2113(02)75003-7).
6. Spaccini R., Piccolo A., Conte P., Haberhauer G., Gerzabek M.H. Increased soil organic carbon sequestration through hydrophobic protection by humic substances. *Soil Biology and Biochemistry*, 2002, 34: 1839-1851 (doi: 10.1016/S0038-0717(02)00197-9).
7. Canellas L.P., Olivares F.L., Okorokova-Façanha A.L., Façanha A.R. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H+-ATPase activity in maize roots. *Plant Physiology*, 2002, 130: 1951-1957 (doi: 10.1104/pp.007088).
8. Zandonadi D.B., Santos M.P., Busato J.G., Peres L.E.P., Faзanha A.R. Plant physiology as affected by humified organic matter. *Theoretical and Experimental Plant Physiology*, 2013, 25(1): 12-25 (doi: 10.1590/S2197-00252013000100003).
9. Aguirre E., Leménager D., Bacaicoa E., Fuentes M., Baigorri R., Zamarreco A.M., García-Mina

J.M. The root application of a purified leonardite humic acid modifies the transcriptional regulation of the main physiological root responses to Fe deficiency in Fe-sufficient cucumber plants. *Plant Physiol. Biochem.*, 2009, 47(3): 215-223 (doi: 10.1016/j.plaphy.2008.11.013).
10. Jindo K., Martim S.A., Navarro E.C., Perez-Alfocea F., Hernandez T., Garcia C., Aguiar N.O., Canellas L.P. Root growth promoting by humic acids from composted and non-composted urban organic wastes. *Plant and Soil*, 2012, 353: 209-220 (doi: 10.1007/s11104-011-1024-3).
11. Pizzeghello D., Francioso O., Ertani A., Muscolo A., Nardi S. Isopentenyladenosine and cytokinin-like activity of different humic substances. *Journal of Geochemical Exploration*, 2013, 129: 70-75 (doi: 10.1016/j.gexplo.2012.10.007).
12. Trevisan S., Pizzeghello D., Ruperti B., Francioso O., Sassi A., Palme K., Quaggiotti S., Nardi S. Humic substances induce lateral root formation and expression of the early auxin-responsive IAA19 gene and DR5 synthetic element in *Arabidopsis*. *Plant Biology*, 2010, 12: 604-614 (doi: 10.1111/j.1438-8677.2009.00248.x).
13. Trapeznikov V.K., Ivanov I.I., Tal'vinskaya N.G., Anokhina N.L. Vliyanie inkrustatsii granul nitrofoski gumatom natriya na urozhai i kachestvo zerna tverdoi pshenitsy. *Agrokhimiya*, 2000, 9: 48-49.
14. Orlov D.S., Grishina L.A. *Praktikum po khimii gumusa* [Workshop on humus chemistry]. Moscow, 1981 (in Russ.).
15. Hofrichter M., Steinbüchel A. *Biopolymers, lignin, humic substances and coal.* Wiley VCH, 2001.
16. Yurkov A.P., Veselova S.V., Yakobi L.M., Stepanova G.V., Kudoyarova G.R., Shishova M.F. Effect of inoculation with arbuscular mycorrhizal fungus *Rhizophagus irregularis* on auxin content in highly mycotrophic black medick under low phosphorus in soil. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(4): 830-838 (doi: 10.15389/agrobiology.2017.4.830eng).
17. Kudoyarova G.R., Korobova A.V., Akhiyarova G.R., Arkhipova T.N., Zaytsev D.Yu., Prinsen E., Egutkin N.L., Medvedev S.S., Veselov S.Yu. Accumulation of cytokinins in roots and their export to the shoots of durum wheat plants treated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP). *Journal of Experimental Botany*, 2014, 65(9): 2287-2294 (doi: 10.1093/jxb/eru113).
18. Arkhipova T.N., Prinsen E., Veselov A.Yu., Martinenko E.V., Melentiev A.I., Kudoyarova G.R. Cytokinin producing bacteria enhance plant growth in drying soil. *Plant and Soil,* 2007, 292(1-2): 305-315 (doi: 10.1007/s11104-007-9233-5).
19. Veselov S.Yu., Kudoyarova G.R., Egutkin N.L., Gyuli-Zade V.Z., Mustafina A.R., Kof E.M. Modified solvent partitioning scheme providing increased specificity and rapidity of immunoassay for indole-3-acetic acid. *Physiologia Plantarum*, 1992, 86(1): 93-96 (doi: 10.1111/j.1399-3054.1992.tb01316.x).
20. Calvo P., Nelson L., Kloepper J.W. Agricultural uses of plant biostimulants. *Plant and Soil*, 2014, 383(1-2): 3-41 (doi: 10.1007/s11104-014-2131-8).
21. Kudoyarova G.R., Dodd I.C., Veselov D.S., Rothwell S.A., Veselov S.Y. Common and specific responses to availability of mineral nutrients and water. *Journal of Experimental Botany*, 2015, 66(8): 2133-2144 (doi: 10.1093/jxb/erv017).
22. Demin V.V., Biryukov M.V., Semenov A.A., Zavgorodnyaya Yu.A. *Doklady po ekologicheskomu pochvovedeniyu*, 2006, 1(1): 72-79 (in Russ.).
23. Spartz A.K., Gray W.M. Plant hormone receptors: new perceptions. *Genes and Development*, 2008, 22(16): 2139-2148 (doi: 10.1101/gad.1693208).
24. Baalousha M., Motelica-Heino M., Le Coustumer P. Conformation and size of humic substances: Effects of major cation concentration and type, pH, salinity, and residence time. *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 2006, 272(1-2): 48-55 (doi: 10.1016/j.colsurfa.2005.07.010).
25. Schaller G.E., Bishopp A., Kieberc J.J. The Yin-Yang of hormones: cytokinin and auxin interactions in plant development. *The Plant Cell*, 2015, 27: 44-63 (doi: 10.1105/tpc.114.133595).
26. Olaetxea M., Mora V., Bacaico E., Garnic M., Fuentes M., Casanova E., Zamarreco A.M., Iriarte J.C., Etayo D., Ederra I., Gonzalo R., Baigorri R., García-Mina J.M. Abscisic acid regulation of root hydraulic conductivity and aquaporin gene expression is crucial to the plant shoot growth enhancement caused by rhizosphere humic acids. *Plant Physiology*, 2015, 169: 2587-2596 (doi: 10.1104/pp.15.00596).

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POLYMORPHISMOF ESTERASE ISOENZYMES OF RIPE SEEDS OF ACCESSIONS OF RADISH (*Raphanus sativus* L.)

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Abstract

A biochemical approach was used to assess the genetic variability of the seed radish (*Raphanus sativus* L.) accessions which are distinguished by a wide variety of morphological characters. It is known that the esterase complex in plants has intraspecific specificity; in addition, these enzymes are characterized by tissue specificity. Earlier, the accessions of the collections of the genetic resources of the radish were never evaluated for the presence of isozyme forms of esterases in mature seeds of this crop. The establishment of the general variability of isoenzyme systems and the identification of their genetic control make it possible to reveal the subtle mechanisms of the organism's relationship with the environment and homeostasis, which is especially important for long-term storage of accessions in genetic seed collections. The development of effective biochemical markers for the rapid assessment of collection, as well as genetically and breeding significant material is also essential. This work allows us to fill the gap that exists in relation to the accessions of genetic resources of the radish. From the collection of the Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources (VIR), 49 radish accessions were selected, belonging to three subspecies, divided according to geographic principle as Chinese, Japanese and European radish. All esterase isozymes of seeds were separated using native vertical electrophoresis in polyacrylamide gel followed by processing for nonspecific esterase. According to their esterase composition, all accessions were subdivided into 7 zymotypes, differing from each other by the presence or absence of certain zones. In total, in the esterase complex of radish seeds, 5 main isozymes with different molecular weights varying from 45.3 kDa to 35.0 kDa were found. All five zones were characterized by a high level of polymorphism among the accessions. Based on the composition of isozymes, all genotypes formed 7 zymotypes. Zymotype No. 1, represented by the maximum number of esterases (5 zones), comprised of 43 % of the total number of genotypes. Zymotype No. 2 constituted 33 % of all accessions. The rarest zymotypes No. 5 and No. 7 (4 %) differed in the minimum amount of esterase enzymes (2 zones each). Zymotypes No. 2 and No. 4 were characterized by 4 zones. Representatives of two groups, No. 3 and No. 6 had 3 zones in their esterase complex. The quantitative ratio of all esterase zones varied greatly in the studied accessions. The minimum content (4.78 %) was found for the B5 zone, the maximum amount (67.44 %) was found for the B1 zone. The prevalence of each zone among all studied accessions ranged from 13 to 23 %. Zones B3 (Mr = 39.7 kDa) and B4 (Mr = 37.1 kDa) were the most common among all esterase isozymes; these zones were observed in 23 % of genotypes. For 22 % of representatives, the B2 zone was characteristic (Mr = 42.9 kD). Zones B1 (Mr = 45.3 kD) and B5 (Mr = 35 kD) were less common, 19 % and 13 %, respectively. The average heterozygosity of isozygous forms of esterases of the studied radish accessions was H_{total} = 0.212, with variance for the same accessions $Var(H_{total})$ = 0.0007. Cluster analysis of esterase enzymes divided the studied set of radish accessions into European and Asian subspecies and varieties, and together with phenotypic traits, it allowed constructing a dendrogram corresponding to the botanical, agrobiological and geographical location of the accessions. It should be noted that the accessions of the European subspecies radish are located in two clusters, and the accessions of Russian origin form a separate group in the first

cluster, and the accessions of European origin are grouped in the third cluster which also includes Japanese radishes of European origin. Perhaps this division is associated with the peculiarities of the selection process in creating these accessions. Based on the data obtained, esterase enzymes are recommended as biochemical markers in genetic selection experiments.

Keywords: *Raphanus sativus* L., morphological characters, phenological characters, seeds, esterases, isoforms, zymotypes, polymorphism, biochemical markers, clustering

Radish *Raphanus sativus* L. possesses a wide morphological variety of leaf rosette and root-crops. The existing intraspecific classifications used in Russia [1-4] divide the species geographically depending on the region of origin (Europe, China, and Japan). These classifications are based mainly on the highly variable and conditions-dependent morphological traits of the root-crop (color, shape) of heterogeneous and heterozygous cross-pollinated specimens. Therefore, it is important to find additional characters to more clearly determine the course of evolution and phylogenetic relationships within the species.

Over the past two decades, DNA markers [5, 6] and genetic maps [7-9] have been developed, and the entire genome of Japanese and Chinese radish has been sequenced [10-12]. A number of works note the effectiveness and prospects of using RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeats) molecular markers and some biochemical markers for assessing the genetic variability of radish varieties [13, 14]. Nonspecific esterases (NE, a complex of enzymes that hydrolyze ether bonds (EC 3.1.1.) can be such biochemical genetic markers [15-17]. It is known that the esterases complex in plants has intraspecific specificity, and, in addition, these enzymes are characterized by tissue specificity [18-20].

Due to the ability to hydrolyze cross-bounds of polysaccharides, NEs are important in the cell wall constructing and restructuring. The activity and isoenzyme composition of NEs play a significant role in certain mechanisms of interaction between the host plant and the pathogen [21-23], in the metabolism of fatty acids [24] and choline esters [25]. Nonspecific esterases are also involved in plant resistance to herbicides [26].

It has been shown that NE activity closely related to the physiological and metabolic states of the cell serves as an indicator of the toxic effect of pollutants [27, 28]. Environmental factors, in particular the temperature [29] and water stress [30], also affect the activity of NE, that is, esterases can be considered as potential stress markers.

In the collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR), there are 1200 *R. sativus* radish accessions from 75 countries of all continents (including 573 accessions in the permanent catalog), representing all subspecies, varieties and types of the crop.

This paper is the first report on isozyme forms of esterases in mature seeds of the radish accessions from the VIR World Collection. Based on the obtained isozyme spectra, dendrograms of the phylogenetic relationships between the accessions were constructed which correspond to their botanical and agrobiological descriptions and geographic origin. The average heterozygosity of isozyme forms of esterases in the studied accessions and their variance were established, indicating the reliability of the results obtained.

The work aims to evaluate the polymorphism of esterases in mature radish seeds, its dependence on the origin and agrobiological traits of the accessions, and the suitability of esterases as biochemical markers of the *R. sativus* diversity.

Materials and methods. Mature seeds of eight *Raphanus sativus* varieties of various origins (49 genotypes in total, VIR World Collection) were ground in a porcelain mortar to flour. The100-mg flour specimens were placed into Eppendorf tubes, added with 2 ml of pre-cooled hexane, shaken periodically, and left

overnight in a refrigerator at 4-8 °C. The next day, the tubes were centrifuged (Eppendorf centrifuge 5410, Eppendorf AG, Germany) at 15000 rpm for 10 min, the supernatant was removed, and the fat-free flour precipitate was left under the fume hood to air-dry. Esterase enzymes from defatted and dried plant material were extracted with 0.05 M Tris-HCl buffer, pH 8.3 (flour: buffer ratio 1:4) at 4-8 °C for 14-18 hours. After centrifugation at 15000 rpm for 10 min, the enzyme extracts were poured off the sediment and frozen at –20 °C. Specimens were thawed before electrophoresis and loaded in the pockets of stacking gel.

Esterase isozymes were separated by native polyacrylamide gel electrophoresis [31] (the concentrations of resolving and stacking gel 11 and 5%, respectively, a Mini-PROTEAN Tetra Cell, Bio-Rad Laboratories, Inc., USA). A Prestained Protein Ladder marker (Thermo Scientific, USA) was pipetted into the last pocket of the gel. Protein concentration in enzyme extracts was measured as per Bradford [32]. Depending on the protein concentration, 15-20 μl aliquots were loaded into the gel pockets. Electrophoresis was conducted at 6-10 °C and 10 V/cm for 2.5 h. To visualize isoenzymes, the gel was exposed to a reagent detecting nonspecific esterase [33] for 10-15 min until the zones appeared. The gel was floated in a fresh solution of the dye and substrates, consisting of 100 mg α-naphthyl acetate and 120 mg β-naphthyl acetate (Sigma-Aldrich Chemie GmbH, Switzerland) dissolved in 10 ml of 70% ethanol, 500 mg of Fast Blue RR (Sigma-Aldrich Co., USA), 4 ml of propanol and 60 ml of 0.1 M phosphate buffer (pH 6.0). The excess dye was removed with 7% acetic acid.

The obtained zymograms were scanned (Epson Expression 10000XL, GE Healthcare, USA). The proportion of zones found in the track and the determination of molecular weights according to the corresponding standards based on the Rf value were calculated using the Phoretix 1D Advansed software (Total Lab, Ltd., UK).

Heterozygosity H_l of the population for each locus and the average (total) heterozygosity of $H_{tot.}$ were calculated as per by the formulas [34, 35]:

$$H_l = 2n(1 - \sum_k x_k^2)/2n - 1\ ,$$
$$H_{tot.} = \sum_{l=1}^{r} H_l/r,$$

where l is the ordinal number of the locus, n is the population size, x_k is the frequency of the k-th allele of the l-th locus, and r is the total number of loci.

The variance of heterozygosity $Var\ (H_l)$ for each locus and the variance of the average heterozygosity within the population $Var\ (H_{tot.})$ were calculated as follows [36]:

$$Var(H_l) = H_l(1 - H_l)/n\ ,$$
$$Var(H_{tot.}) = \frac{1}{nr^2}\sum_l H_l(1 - H_l) + \frac{1}{nr^2}\sum_l \sum_{l \neq \hat{l}} (H_{l\hat{l}} - H_l H_{\hat{l}}).$$

The morphological and agrobiological descriptions of the accessions were performed according to Sazonova et al. [37] (Pushkin and Pavlovsk laboratories of VIR, St. Petersburg).

The main morphological and phenological markers for the *R. sativus* intraspecific attributing to varieties and types were the shape and color of the root-crop and the length of the growing season [1, 37]. When constructing a matrix for cluster analysis, the following quantitative and qualitative characteristics were used: the root-crop length and diameter, the root-crop index (the length to diameter ratio), the color of the root-crop bark (1 — white, 3 — green, 5 — pink, 7 — red, 9 — black), the root-crop shape (2 — conical, 3 — cylindrical, 4 — elliptic, 5 — round, 6 — flat round, 7 - cylindrical with a run up), leaf type (1 — solid, 3 — lyre-like), the leaf pubescence (0 — no pubescence, 1 — pubescent), the length of the growing season. Qualitative characteristics were assessed in points according to the descriptor [38].

Cluster analysis based on the profiles of seed esterases only and on a complex of traits (morphological and phenological features and esterase profiles in seeds) was performed by the UPGMA method using the PAST program (http://sonraid.ru/past/), including bootstrap analysis.

Results. Table 1 lists the radish accessions studied in the work.

1. *Raphanus sativus* L. accessions (VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **involved in the study**

VIR-number	Name	Origin	Convariety (convar.)	Variety (var.)	Number on zymogram
		R. sativus subsp. *sativus* (L.) Sazon.			
k-1675	Belaya Adzharskaya	Georgia	*sativus* Sazon.	*sativus* Sazon.	3
k-1833	Odesskaya 5	Belarus	*sativus* Sazon.	*sativus* Sazon.	7
k-2163	Maiskaya belaya	Russia	*sativus* Sazon.	*sativus* Sazon.	44
k-1778	Zimnyaya krukglaya chernaya	Germany	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	4
k-1892	Dazwish ali	Egypt	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	9
k-1971	Round Black Spanish	USA	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	15
k-1764	Local	Russia	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	26
k-2115	Chernaya	Russia	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	35
k-2124		Turkey	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	36
k-1914	Zimnyaya krukglaya belaya	Russia	*hybernus* (Alef.) Sazon.	*hybernus* (Alef.) Sazon.	28
k-2025	Skvirovskaya belaya	Ukraine	*hybernus* (Alef.) Sazon.	*hybernus* (Alef.) Sazon.	32
		R. sativus subsp. *sinensis* Sazon. et Stankev.			
k-698		Asia Minor	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	1
k-1805		Middle Asia	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	5
k-1902	Belaya zelenigolovaya	China	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	11
k-1978	Local	Kyrgyzstan	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	16
k-2101	Chinese White Winter	Chile	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	21
k-2074	Local	Egypt	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	33
k-2151	Altari mu	South Korea	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	39
k-1815	Margilanskaya	Uzbekistan	*lobo* Sazon. et Stankev.	*virens* Sazon.	6
k-1865	Wei-syan	China	*lobo* Sazon. et Stankev.	*virens* Sazon.	8
k-2000	Local	Uzbekistan	*lobo* Sazon. et Stankev.	*virens* Sazon.	17
k-2148	Local	Kazakhstan	*lobo* Sazon. et Stankev.	*virens* Sazon.	38
k-725		Asia Minor	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	2
k-1895	Hun-dyn-lun	China	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	10
k-1903	Krasnaya	China	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	12
k-1935	Nerima Pointed rooted	Japan	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	13
k-1857	Chan-shun-lobo	China	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	27
k-1967	Local	Afghanistan	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	30
k-1983	Nexhnaya	Russia	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	31
		R. sativus subsp. *acanthiformis* (Blanch) Stankev.			
k-1958	Hakata haruwaka	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	14
k-2033	Turnip	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	18
k-2063	Unzen shigatsu	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	20
k-2111	Minotoki No. 2	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	22
k-1946	Unsen-4-gatsu	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	29
k-2134	Eifuku 2	Japan	*minowase* (Kitam.) Sazon.	*minowase*	37
k-2154	Mikura gross F_1	The Netherland	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	40
k-2155	Local	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	41
k-2159	Yasato riso F_1	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	42
k-2161	Horiyou	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	43
k-2184	Cheng sugeng zung	South Korea	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	47
k-2335	April cross	France	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	48
k-2336	Spring Feller	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	49
k-2034	Miyasige Oonaga	Japan	*acanthiformis* (Blanch) Stankev.	–	19
k-2133	Eifuku	Japan	*acanthiformis* (Blanch) Stankev.	–	23
k-2136	Shinuchi Sobutori F_1	Japan	*acanthiformis* (Blanch) Stankev.	–	24
k-2177	Back-ok	South Korea	*acanthiformis* (Blanch) Stankev.	–	25
k-2093	Mijshige long pointed rooted	Japan	*acanthiformis* (Blanch) Stankev.	–	34
k-2175	Sodam	South Korea	*acanthiformis* (Blanch) Stankev.	–	45
k-2178	Shinmyeong	South Korea	*acanthiformis* (Blanch) Stankev.	–	46

N o t e. Dashes mean that varieties are not classified.

Native electrophoresis detected five main esterase isozymes, the B1 (45.3 kDa), B2 (42.9 kDa), B3 (39.7 kDa), B4 (37.1 kDa), and B5 (35.0 kDa) (Fig. 1) in seeds of the 49 studied radish genotypes. All five bands were

characterized by polymorphism among the accessions (Table 2). We did not find monomorphic zones present in all accessions.

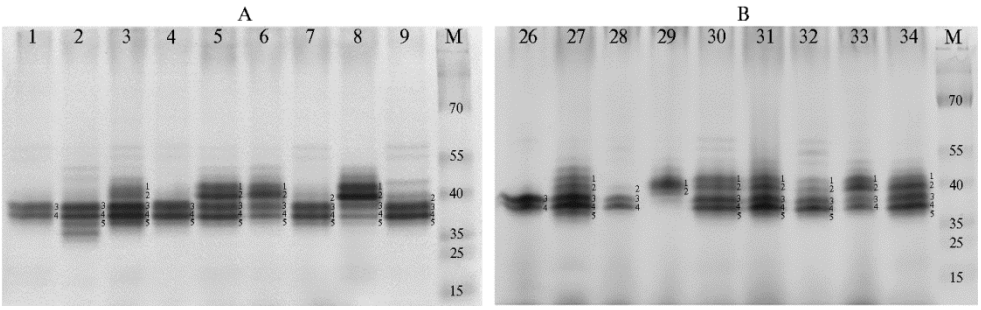


Fig. 1. Zymograms of esterases from mature seeds of *Raphanus sativus* L. (accessions of the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources). Esterase zones are indicated along the tracks on the right, accessions numbers are above the lanes For line numbers, see Table 1. M — molecular weight markers (15-70 kDa; Prestained Protein Ladder, Thermo Scientific, USA).

2. Esterase zymotypes in mature seeds of *Raphanus sativus* L. (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources)

Zymotype	B1 (45,3kDA)	B2 (42,9kDA)	B3 (39,7kDA)	B4 (37,1kDA)	B5 (35,0kDA)	Total number of zones
No.1	+	+	+	+	+	5
No. 2	+	+	+	+	−	4
No. 3	−	+	+	+	−	3
No. 4	−	+	+	+	+	4
No. 5	−	−	+	+	−	2
No.6	−	−	+	+	+	3
No. 7	+	+	−	−	−	2
Total	38	45	48	48	26	205
Frequency of zone occurrence, %	19	22	23	23	13	100

By the esterase profiles, all accessions were attributed to seven winter types differing from each other by the presence or absence of certain zones (Tables 2, 3).

3. Attribution of mature seeds of *Raphanus sativus* L. (accessions of the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **to esterase zymotypes**

Zymotype	Number of zones in zymotype	Number on zymogram	Number of genotypes	
			total	of total number, %
No. 1(B1-B5)	5	3, 5, 6, 8, 10-14, 16, 17, 23, 24, 27,30-32,34,38,40,48	21	43
No. 2(B1-B4)	4	18-22,25,33,36,37,39,41, 42, 44-46,49	16	33
No.3(B2-B4)	3	28,35,43,47	4	8
No.4(B2-B5)	4	7,9,15	3	6
No.5(B3-B4)	2	1,26	2	4
No. No.6(B3-B5)	3	2,4	2	4
№ 7(B1-B2)	2	29	1	2

N o t e. For description of the accessions, see Table 1, for esterase profiles of zymotypes, see Table 2.

4. Esterase is enzyme contents in mature seeds of *Raphanus sativus* L. (accessions of the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources)

Indicator	B1	B2	B3	B4	B5
M_r, kDa	45.3	42.9	39.7	37.1	35.0
Min, %	7.06	7.78	16.77	8.74	4.78
Max, %	67.44	39.91	54.22	52.10	25.96
Mean, %	20.11	25.16	29.28	26.60	11.48

N o t e. M_r— molecular weight, Min — minimum amount, Max — maximum amount.

The amount of esterase within each zone varied greatly (Table 4). The prevalence ranged between zones from 13 to 23%, the minimum was characteristic of B5 zone (4.78%), the maximum of B1 zone (67.44%). The average statistical

value of the content of esterase isozymes varied from 11.48 for zone B5 to 29.28% for zone B3.

Zymotype No. 1 (5 zones) accounted for 43% of the total number of genotypes. The main part of this group was represented by specimens of the Chinese subspecies green (var. *virens* Sazon.), pink-red (var. *rubidus* Sazon.), and white (var. *lobo*) varieties from China and Central Asia. In addition, this group included six accessions of Japanese radish of European and Japanese origin and an accession of summer European radish. Zymotype No. 2 (4 zones) was typical for 33% of the studied accessions. This group consisted mainly of accessions of Japanese subspecies from Japan and South Korea, several genotypes of the white variety from Egypt and Chile, and two accessions of European summer and winter radish. Zymotype No. 3 (3 zones) was characteristic of two accessions of winter European radish from Russia and two accessions of daikon of autumn type and made up 8% of all genotypes. Three accessions (6%) of European radish demonstrated zymotype No. 4 (4 zones), each of the two zymotypes, No. 5 (2 zones) and No. 6 (3 zones) was represented by two accessions (4%) of local lobo radish from Asia Minor and winter black radish. One accession of the daikon Unzen-4-gatsu (k-1946, Japan) of zymotype No. 7 (2 zones) was the least common, that is, having the rarest esterase profile with 2%frequency of occurrence.

The frequency of heterozygotes is one of the most important characteristics of a population, since each heterozygote carries different alleles and provides variability. It should be noted that the smaller the difference between the values of the allele frequencies per locus, the higher the obtained value of heterozygosity for this locus. Our calculation showed the highest heterozygosity (H_l= 0.503) for the isoform B5 (Table 5). For B3 and B4, only one polymorphic allele was identified and, therefore, the heterozygosity was the lowest (H_l= 0.039). Variance is a quantity dependent on heterozygosity, and therefore the patterns identified for heterozygosity are similar to those for variance. The application of the formula for calculating the variance of average heterozygosity [36] is due to covariations between heterozygosities at loci l and $\hat{l}$ determined by the frequencies of double heterozygotes $H_{l\hat{l}}$of these loci.

5. Population heterozygosity and its variance among *Raphanus sativus* L. accessions (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **as calculated based on isozyme analysis data**

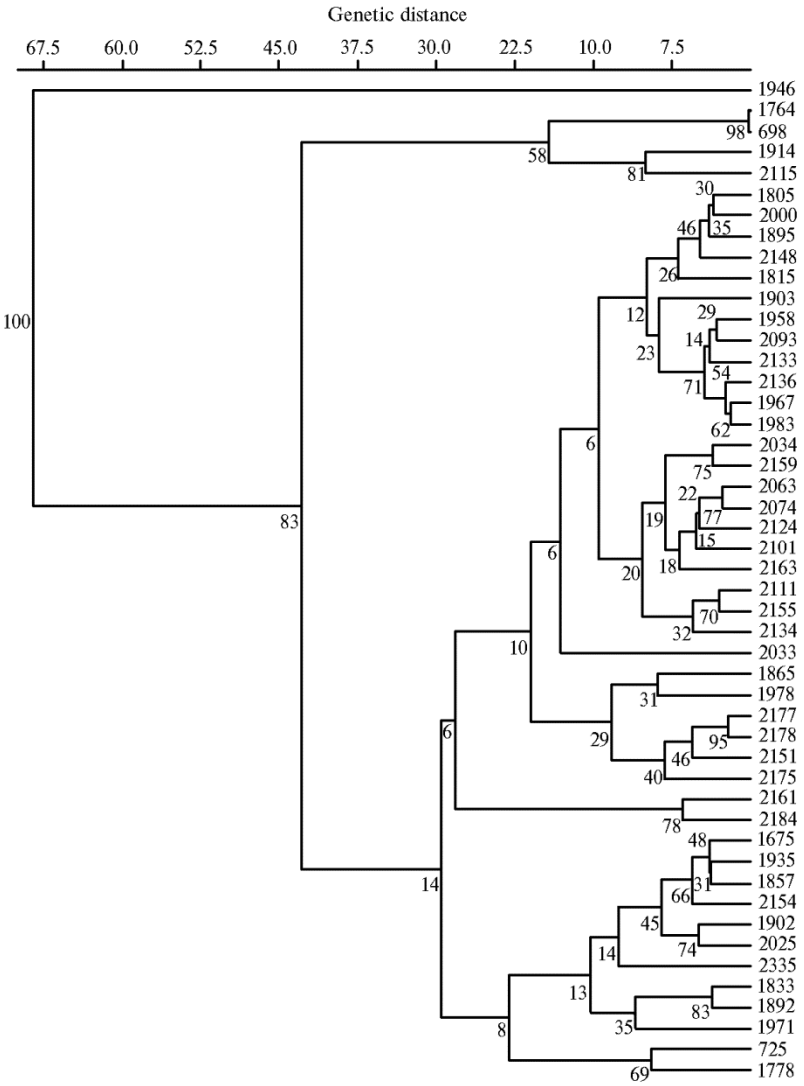
Statistical parameter	Isozyme loci (esterase zones)				
	B1	B2	B3	B4	B5
Heterozygosity H_l	0.328	0.152	0.039	0.039	0.503
Variance $Var(H_l)$	0.004	0.002	0.001	0.001	0.005

N o t e.Average heterozygosity $H_{tot.}$ is 0.212, the variance of the average heterozygosity with in a population $Var(H_{tot.})$ is 0.0007.

The formulas we used in our work allow any polynomial in a set of variables distributed multinomially to be solved, and the calculated heterozygosity is regarded as a measure of polymorphism information which is actively used in genetic research and selection programs.

The isozyme analysis revealed a total of 205 electrophoretic bands of esterase isoforms which were used to construct a dendrogram (Fig. 2). The radish accessions were found to form one large and two small clusters. An accession of the daikon Unzen-4-gatsu (k-1946, Japan) was an out-group. The first small cluster grouped accessions of European winter radishes from Russia (var. *niger* (L.) Sinsk.; var. *hybernus*) and a local white lobo from Asia Minor (k-698). The second large cluster was mainly Chinese and Japanese radish accessions divided into four subclusters. The lobos from Russia, Afghanistan, China (var. *rubidus* Sazon.), Uzbekistan and Kazakhstan (var. *virens* Sazon.), and of spring and autumn daikons

were in the first subcluster. The Japanese and European radishes (var. *sativus*; var. *niger* (L.) Sinsk.), lobos from Egypt and Chile (var. *lobo*) were in the second subcluster. An accession of daikon k-2033 was located outside the first two subclusters. The third subcluster grouped lobo accessions from South Korea, Kyrgyzstan, China (var. *lobo*; var. *virens* Sazon.) and daikons from South Korea and Japan. Note, the genotypes from South Korea and Japan formed two separate groups within the subcluster. Two daikons from South Korea and Japan were the fourth subcluster.



Fir. 2. UPGMA-dendrogram of genetic similarity among *Raphanus sativus* L. accessions (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **based on esterase profiles of mature seeds.** The bootstrap values on the branches indicate the linkage distances. For description of genotypes (numbers on the right), see Table 1.

In the third cluster, mainly accessions of the European subspecies radish, and the Japanese subspecies but of European origin were located. The cluster was divided into two subclusters, the first was the European winter radishes from Canada and Egypt (var. *niger* (L.) Sinsk.) and summer radishes from Belarus (var. *sativus*), the second was daikons from France and the Netherlands, white European radishes from Ukraine and Georgia (var. *hybernus*; var. *sativus*), and Chinese

radishes from China and Japan (var. *rubidus* Sazon.; var. *lobo*). Two accessions remained outside the subclusters, i.e., the winter black radish from Germany and lobo from Asia Minor.

By phenotypic traits, the studied radish accessions were grouped into five clusters (the dendrogram we obtained is not shown, since it corresponded to their botanical and agrobiological attribution). European winter and summer radishes grouped into separate clusters. Asian radishes were represented by two clusters (accessions of the Japanese and Chinese subspecies), and there were no significant differences between the accessions of different ecological and geographical origin. The last cluster was several accessions of lobo from Central Asia and Asia Minor and Chile and a daikon of a local variety population from Japan. That is, the phylogenetic pattern obtained using only phenotypic characters did not fully reflect the peculiarities of origin and evolution of the studied radishes.

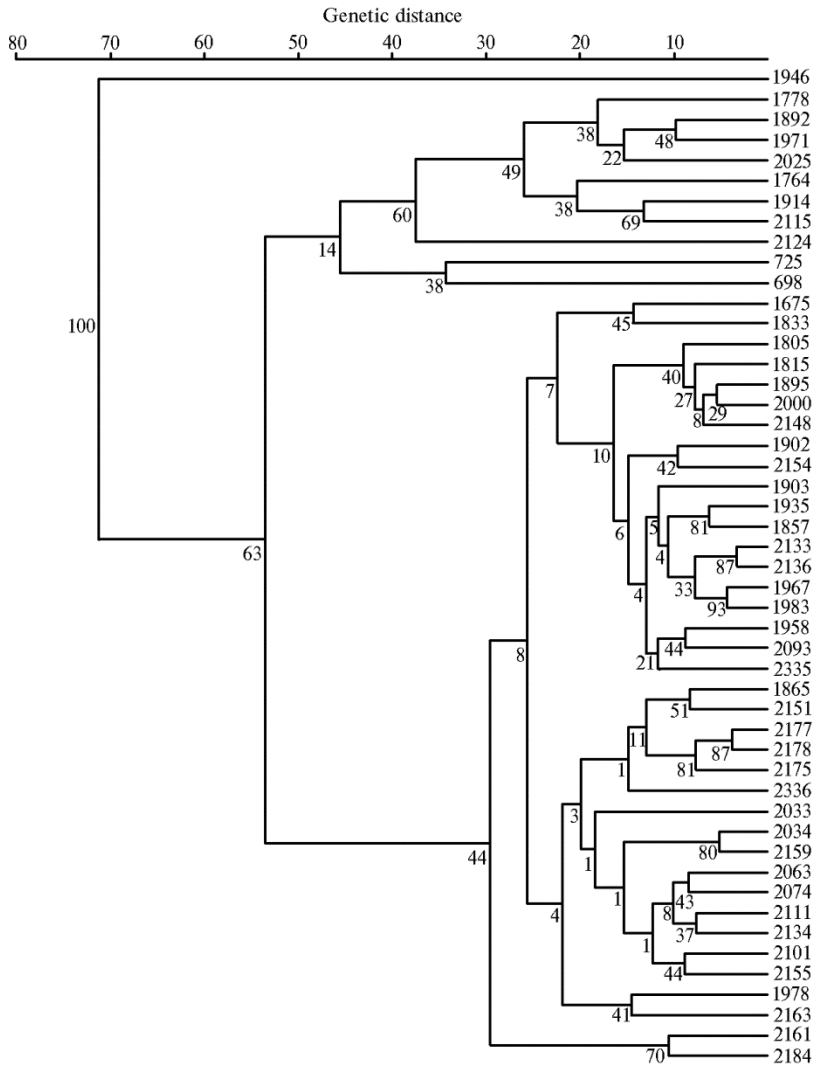


Fig. 3. UPGMA-dendrogram of genetic similarity among *Raphanus sativus* L. accessions (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **based on** morpho-phenological traits and **esterase profiles of mature seeds.** The bootstrap values on the branches indicate the linkage distances. For description of genotypes (numbers on the right), see Table 1.

The dendrogram based on morpho-phenological trait sand esterase profiles

of seeds (Fig. 3) consists of a large and two small clusters. Outside, as in Figure 2, there was a Japanese accession of the Unzen-4-gatsu daikon (k-1946). European radish subspecies were in the first cluster divided into two subclusters. The first subcluster was winter radish of black (var. *niger* (L.) Sinsk.) and white (var. *hybernus*) varieties, the second subcluster was Chinese radish genotypes from Asia Minor (k-725, k-698) which were local variety populations.

The second large cluster comprised the Chinese and Japanese subspecies and the European summer radish. The cluster was divided into four subclusters. Two European summer radishes (var. *sativus*) from Belarus and Georgia were in the first subcluster. The second subcluster consisted of a separate group of lobos from Central Asian, a group of lobos of a pink-red variety (var. *rubidus* Sazon.) from China, Russia, and Afghanistan, and daikons from Japan, France, and the Netherlands. In the third subcluster, there were two large groups of accessions, the first group of the lobo and daikon genotypes from South Korea with a daikon from Japan (k-2336) close to them and the second group of daikons from Japan and two accessions of the white lobos from Egypt (k- 2074) and Chile (k-2101). The fourth subcluster comprised only two accessions, the white lobo from Kyrgyzstan (Local, k-1978) and the summer European radish from Russia (Maiskaya, k-2163). The third small cluster contained two daikon accessions from Japan and South Korea.

A comparative isozyme profiling of the accessions revealed an intraspecific polymorphism and divided the accessions into seven zymotypes different in quantitative ratios of the isozyme zones of esterases. The appearance of all five esterase zones (zymotype No. 1) was more characteristic of the Chinese subspecies, which indicates large intervarietal differences within the subspecies. Four esterase zones (zymotypes Nos. 2 and 4) were found mainly in the Japanese and European subspecies. Three (zymotypes Nos. 3 and 6) and two (zymotypes Nos. 5 and 7) esterase zones were found in European winter radish from Russia, daikons from Japan and South Korea, and lobo from Asia Minor. The zymotypes Nos. 3 and 6 were cultivars presumably resulted from individual selection from populations or through hybridization followed by selection. The accessions of local origin which expressed zymotypes Nos. 5 and 7 are highly homogenous within the cultivar, possibly, their selection was localized in a certain area.

Consequently, the rare esterase zones in these accessions are due to their selection or agrobiological affiliation, which is consistent with other reports [15-17, 39]. We for the first time have shown [15-17] that the assessment of esterase isozyme polymorphism is reliable to evaluate genetic polymorphism not only in radish (*R. sativus*) and lines of *Brassica rapa* L. doubled haploids, but also in hexaploid spring wheat (*Triticum aestivum* L.). Similar results were obtained in studying the polymorphism of various wheat varieties (*Triticum* L.) [39]. In all these studies, polymorphism of the isozyme profile of esterases isolated from mature seeds was revealed in accessions of varietal, linear and collection breeding material. A wide variety of electrophoretic profiles of esterase isozymes of mature seeds has been shown and the possibility of determining the polymorphism of esterases in hybrid generations has been established. Along with the results we obtained in this study, this allows us not only to select promising starting material for breeding, but also to recommend this type of biochemical markers for solving practical problems as a means that can accelerate and simplify selection of breeding material. The fact that similar work on radish, small radish, *B. rapa*, wheat has not been carried out before, once again emphasizes the prospects of using the approach we have proposed.

Cluster analysis of the esterase profiles of seeds showed that the

accessions were grouped mainly by origin and partly due to their botanical affiliation. Accessions of the European subspecies radish were located in two clusters, and the accessions of Russian origin formed a separate group in the first cluster, and the accessions of European origin were in the third cluster which also includes Japanese radishes of European origin. Perhaps this division is due to the peculiarities of the selection of these accessions.

The second large cluster of Asian radishes grouped not only accessions of the Chinese and Japanese subspecies, but also several accessions of the European subspecies of summer and winter varieties, which could be associated with the peculiarities of their origin or with an error in reproduction. Interestingly, South Korean accessions, regardless of their botanical affiliation, formed a separate group within the third subcluster, which made an important addition to intraspecific differentiation.

Thus, European and Asian accessions were distributed in separate clusters, which confirm the origin of the radish diversity from two primary geographic centers, the Mediterranean and Asian [2, 4].

Clustering of accessions by a set of characters (morphological, phenological traits, and seed esterases) showed the results most consistent with botanical and agrobiological division. The first cluster included all accessions of European winter radishes and two variety populations of the lobo. The second large cluster grouped accessions of the Chinese and Japanese subspecies, as well as European summer radish. Summer European radishes are considered the intermediate forms between European winter radishes and small radishes, and lobos are the original forms of Japanese radishes and Chinese small radishes. Perhaps this clusters these two groups of varieties together. The third cluster contained a group of lobo and daikon accessions from South Korea, as in the first dendrogram. This refinement was revealed due to the analysis of esterase enzymes and, probably, indicates a multiple origin of Japanese radishes [3].

The daikon Unzen-4-gatsu (k-1946, Japan) was distinguished by the rare esterase zones (zymotype No. 7) and occupied the out-group position. It belongs to a morphologically sharply different cultivar Ninengo, a characteristic feature of which is a long thin root-crop (50-55 cm in length, 5-6 cm in diameter) and a large rosette of leaves (25-30 cm in height, 35-40 cm in diameter). The cultivars of this group are the most resistant to frost and stemming [40].

So, the performed biochemical analysis of esterases of mature seeds of radish accessions of different origin revealed the isozyme polymorphism. Calculation of heterozygosity H_I for each locus and total heterozygosity of the population $H_{tot.}$ revealed the most (B5) and least (B3 and B4) heterozygous esterase isoforms. The formulas used in this paper make it possible to solve any polynomial in a set of variables distributed multinomially. The calculated heterozygosity $H_{tot.} = 0.212$ and the variance for the same accessions $Var\ (H_{tot.}) = 0.0007$ can be regarded as an effective measure of informational polymorphism to be used in breeding programs. In addition, the cluster analysis based on the seed esterase profiles coupled with phenotypic traits are consistent with botanical and agrobiological classification by origin from two primary geographic centers. Consequently, esterase profiles of mature seeds are convenient biochemical markers in physiological, biochemical, genetic and breeding studies of the crop.

REFERENCES

1. Sinskaya E.N. Redis i red'ka (*Raphanus sativus* L.). *Trudy po prikladnoi botanike, genetike i selektsii*, 1928, 19(3): 448-534 (in Russ.).
2. Sinskaya E.N. *Trudy po prikladnoi botanike, genetike i selektsii*, 1931, 26(2): 3-58 (in Russ.).
3. Sazonova L.V. *Trudy po prikladnoi botanike, genetike i selektsii*, 1971, 45(1): 42-75 (in Russ.).

4. Shebalina M.A., Sazonova L. V. *Kul'turnaya Flora SSSR. T. 18. Korneplodnye rasteniya (semeistvo Kapustnye — repa, turneps, bryukva, red'ka, redis)* /Pod redaktsiei V.T. Krasochkina, V.I. Burenina [Cultural flora of the USSR. Vol. 18. Root plants (*Brassicaceae* family — turnips, turnips, rutabagas, radishes, radishes). T. Krasochkin, V.I. Burenin (eds.)]. Leningrad, 1985 (in Russ.).
5. Tsuro M., Suwabe K., Kubo N., Matsumoto S., Hirai M. Mapping of QTLs controlling root shape and red pigmentation in radish, *Raphanus sativus* L. *Breeding Science*, 2008, 58(1): 55-61 (doi: 10.1270/jsbbs.58.55).
6. Mun J.H., Chung H., Chung W.H., Oh M., Jeong Y.M., Kim N., Ahn B.O., Park B.S., Park S., Lim K.B., Hwang Y.J., Yu H.J. Construction of a reference genetic map of *Raphanus sativus* based on genotyping by whole-genome resequencing. *Theoretical and Applied Genetics*,2015, 128(2): 259-272 (doi: 10.1007/s00122-014-2426-4).
7. Xu L., Wang L., Gong Y., Dai W., Wang Y., Zhu X., Wen T., Liu L. Genetic linkage map construction and QTL mapping of cadmium accumulation in radish (*Raphanus sativus* L.). *Theoretical and Applied Genetics*, 2012, 125(4): 659-670 (doi: 10.1007/s00122-012-1858-y).
8. Hashida T., Nakatsuji R., Budahn H., Schrader O., Peterka H., Fujimura T., Kubo N., Hirai M. Construction of a chromosome-assigned, sequence-tagged linkage map for the radish, *Raphanus sativus* L. and QTL analysis of morphological traits. *Breeding Science*, 2013, 63(2): 218-226 (doi: 10.1270/jsbbs.63.218).
9. Yu X., Choi S.R., Dhandapani V., Rameneni J.J., Li X., Pang W., Lee J.Y., Lim Y.P. Quantitative trait loci for morphological traits and their association with functional genes in *Raphanus sativus. Frontiers in Plant Science*, 2016, 7: 255 (doi: 10.3389/fpls.2016.00255).
10. Kitashiba H., Li F, Hirakawa H., Kawanabe T, Zou Z., Hasegawa Y., Tonosaki K., Shirasawa S., Fukushima A., Yokoi S., Takahata Y., Kakizaki T., Ishida M., Okamoto S., Sakamoto K., Shirasawa K., Tabata S., Nishio T. Draft sequences of the radish (*Raphanus sativus* L.) genome. *DNA Research*, 2014, 21(5): 481-490 (doi: 10.1093/dnares/dsu014).
11. Mitsui Y., Shimomura M., Komatsu K., Namiki N., Shibata-Hatta M., Imai M., Katayose Y., Mukai Y., Kanamori H., Kurita K., Kagami T., Wakatsuki A., Ohyanagi H., Ikawa H., Minaka N., Nakagawa K., Shiwa Y., Sasaki T. The radish genome and comprehensive gene expression profile of tuberous root formation and development. *Scientific Reports*, 2015, 5: 10835 (doi: 10.1038/srep10835).
12. Jeong Y.M., Kim N., Ahn B.O., Oh M., Chung W.H., Chung H., Jeong S., Lim K.B., Hwang Y.J., Kim G.B., Baek S., Choi S.B., Hyung D.J., Lee S.W., Sohn S.H., Kwon S.J., Jin M., Seol Y.J., Chae W.B., Choi K.J., Park B.S., Yu H.J., Mun J.H. Elucidating the triplicated ancestral genome structure of radish based on chromosome-level comparison with the Brassica genomes. *Theoretical and Applied Genetics*, 2016, 129(7): 1357-1372 (doi: 10.1007/s00122-016-2708-0).
13. Ivy N.A., Biswas M.S., Rasul G., Hossain T., Mian M.A.K. Variations of genotypes of radish at molecular level using isozyme analysis for the identification of self-incompatible lines. *Global Journal of Biotechnology & Biochemistry*, 2010, 5(1): 19-26.
14. Cruz S.M., Nery M.C., Pinho E.V., Luiz M. Molecular characterization of radish cultivars. *Revista Ciência Agronômica*, 2014, 45(4): 815-822 (doi: 10.1590/S1806-66902014000400020).
15. Rudakova A.S., Rudakov S.V., Artem'eva A.M., Kurina A.B., Kocherina N.V., Chesnokov Yu.V. *Ovoshchi Rossii*, 2017, 5(38): 3-8 (doi: 10.18619/2072-9146-2017-5-3-8).
16. Rudakova A.S., Rudakov S.V., Davydova N.V., Mirskaya G.V., Zhuravleva E.V., Chesnokov Yu.V. Isozymic analysis of esterases in mature seeds of hexaploid soft wheat (*Triticum aestivum* L.). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2016, 51(3): 327-334 (doi: 10.15389/agrobiology.2016.3.327eng).
17. Rudakova A.S., Rudakov S.V., Artem'eva A.M., Fateev D.A., Kocherina N.V., Chesnokov Yu.V. QTL mapping of esterase isozyme forms in *Brassica rapa* L. mature seeds (review) *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(3): 469-480 (doi: 10.15389/agrobiology.2019.3.469eng).
18. Nakagahra M., Okuno K., Vaughan D. Rice genetic resources: history, conservation, investigative characterization and use in Japan. In: *Oryza: from molecule to plant.* T. Sasaki, G. Moore (eds.). Springer, Dordrecht, 1997: 69-77 (doi: 10.1007/978-94-011-5794-0_7).
19. Alexandre F., Morvan, O., Gaffe J., Mareck A., Jauneau A., Dauchel H., Balange A.P., Morvan C. Pectin methylesterase pattern in flax seedlings during their development. *Plant Physiology and Biochemistry*, 1997, 35(6): 427-436.
20. Timonen S., Sen R. Heterogeneity of fungal and plant enzyme expression in intact Scots pine—*Suillus bovinus* and —*Paxillus involutus* mycorrhizospheres developed in natural forest humus. *New Phytologist*, 1998, 138(2): 355-366 (doi: 10.1046/j.1469-8137.1998.00103.x).
21. Muarlidharan J., John E., Channamma L., Theerthaprasad D. Changes in esterases in response to blast infection in fingermillet seedlings. *Phytochemistry*, 1996, 43(6): 1151-1155 (doi: 10.1016/S0031-9422(96)00478-5).
22. Pappas A.C., Paplomatas E.J. Pyriculria leaf spot: a new disease of ornamental plants of the family Marantaceae. *Plant Disease*, 1998, 82(5): 465-469 (doi: 10.1094/PDIS.1998.82.5.465).
23. Parker D.M., Köller W. Cutinase and other lipolytic esterases protect bean leaves from infection

by *Rhizoctonia solani*. *Molecular Plant-Microbe Interactions*, 1998, 11(6): 514-522 (doi: 10.1094/MPMI.1998.11.6.514).
24. Aung U.T., McDonald M.D. Changes in esterase activity associated with peanut (*Arachis hypogea* L.) seed deterioration. *Seed Science and Technology*, 1995, 23(1): 101-111.
25. Miura G.A., Broomfield C.A., Lawson M.A., Worthley E.G. Widespread occurrence of cholinesterase activity in plants. *Physiologia Plantarum*, 1982, 56(1): 28-32 (doi: 10.1111/j.1399-3054.1982.tb04895.x).
26. Feng P.C.C., Ruff T.G., Rangwala S.H., Rao S.R. Engineering plant resistance to thiazopyr herbicide via expression of a novel esterase deactivation enzyme. *Pesticide Biochemistry and Physiology*, 1997, 59(2): 89-103 (doi: 10.1006/pest.1997.2312).
27. Maier R. Blei und seine Auswirkung auf Aktivitдt und multiple Formen der Alpha-Naphtyl-Easterase in bleichteten und verdunkelten pflanzen. *Berichte der Deutschen Botanischen Gesellschaft*, 1978, 91(1): 339-350.
28. Cachot J., Romaña L.A., Galgani F. In vivo esterase activity in protoplasts as a bioassay of environmental quality. *Aquatic Botany*, 1994, 48(3-4): 297-312 (doi: 10.1016/0304-3770(94)90022-1).
29. Krasnuk M., Witham F.H., Jung G.A. Hydrolytic enzyme differences in cold-tolerant and cold-sensitive alfalfa. *Agronomy Journal*, 1978, 70(4): 597-605 (doi: 10.2134/agronj1978.00021962007000040019x).
30. Taskakorie A., Clerc M., Thi A.T.P., da Silva J.V. Evidence of esterase activity in cotton leaves: effect of drought on this activity. *Comptes rendus del'Academie des Sciences. Serie III. Sciences de la Vie*,2013, 301(6): 343-346.
31. Davis B.J. Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, 1964, 121(2): 404-427 (doi: 10.1111/j.1749-6632.1964.tb14213.x).
32. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, 1976, 72(1-2): 248-254 (doi: 10.1016/0003-2697(76)90527-3).
33. Meon S. Protein, esterase and peroxidase patterns of Phytophtora isolates from cocoa in Malaysia. *Journal of Islamic Academy of Sciences*, 1988, 1(2): 154-158.
34. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 1978, 89(3): 583-590.
35. Lefèvre F., Charrie A. Isozyme diversity within African *Manihot* germplasm. *Euphytica*, 1992, 66(1): 73-80 (doi: 10.1007/BF00023510).
36. Veir B. *Analiz geneticheskikh dannykh* [Analysis of genetic data]. Moscow, 1995 (in Russ.).
37. Sazonova L.V., Vlasova E.A. *Metodicheskie ukazaniya po izucheniyu i podderzhaniyu mirovoi kollektsii korneplodov* [Methodological guidelines for the study and maintenance of the global collection of root crops]. Leningrad, 1989 (in Russ.).
38. IBPGR. *Descriptors for Brassica and Raphanus*. International Board for Plant Genetic Resources, Rome, Italy, 1990.
39. Shayakhmetov I.F., Akhmadieva A.A., Leonova S.A., Nikonov V.I. *Vestnik Bashkirskogo universiteta*, 2012, 17(1): 89-93 (in Russ.).
40. Kurina A.B., Kornyukhin D.L., Artem'eva A.M. *Vestnik Novosibirskogo gosudarstvennogo agrarnogo universiteta*, 2018, 4(49): 81-92 (doi: 10.31677/2072-6724-2018-49-4-81-92) (in Russ.).

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PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF TEA (*Camellia sinensis* L.) MICROSHOOTS in vitro: THE NORM, OSMOTIC STRESS, AND EFFECTS OF CALCIUM

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Abstract

Stress tolerance is an important trait, that determines the productivity of plants under drought, hypothermia, mineral deficiency, and salinity. Numerous studies of various agricultural crops (J.K. Zhu, 2016; E. Fleta-Soriano, S. Munné-Bosch, 2016), including tea crop (*Camellia sinensis* L.), were aimed at solving this problem due to the global aridization of the climate. (T.K. Maritim et al., 2015; L.S. Samarina et al., 2019). Along with the sufficiently detailed physiological, biochemical and molecular studies of tea drought tolerance, the exogenous regulation of tolerance by using of chemical and biological substances is still not investigated. In addition, the important role of calcium ions (Ca^{2+}) in the cell recognition of an external stressor by the triggering signal transduction has been shown in many crops (M.C. Kim, 2009; E.G. Rikhvanov et al., 2014). In these studies, tissue culture media supplemented with the osmotically active substances (R.M. Pérez-Clemente et al., 2012; M.K. Rai et al., 2011) and artificial biosystems (microshoots and tissues in vitro), are often used as "drought models" to reveal cellular adaptation mechanisms. However, just a few studies were conducted aimed at deciphering the biochemical and molecular responses of tea plant to stress using tissue culture tool (L.S. Samarina et al., 2018; M.V. Gvasaliya et al., 2019). In this article, for the first time, we investigated the role of calcium in plant adaptation to long-term osmotic stress based on earlier published protocols of tea tissue culture (M.V. Gvasaliya, 2013) and osmotic stress induction protocols. We also demonstrated the prospect of studying the role of exogenous inducers in increasing plant tolerance using "drought models". This work aimed to identify the effect of different concentrations of calcium (Ca^{2+}) in the culture medium on the functional state of tea microshoots grown under mannitol-induced osmotic stress in vitro comparing with control. The changes in morphophysiological state of the leaves, leaves water content, cells membrane permeability, malondialdehyde, proline, and photosynthetic pigments were analyzed. It was found that increased Ca^{2+} content in the nutrient medium (from 440 to 880 mg/l) resulted the slower leaves development and significant decrease of malondialdehyde and cell membranes permeability of tea microshoots (by 50 %, $p \le 0.05$) during the long-term cultivation of tea microshoots in vitro (4 months), indicating inhibition of lipid peroxidation processes. The addition of mannitol (40 g/l) to the culture medium reduced the water content of the shoots (on average by 2 %, $p \le 0.05$), thereby forming light osmotic stress, which led to the accumulation of proline (an increase of 30-40 %, $p \le 0.05$), as well as to the structural and functional rearrangement of the photosynthetic apparatus (a decrease in the concentration of photosynthetic pigments by an average of 35-40 %). In addition, a significant decrease of malondialdehyde (by 50-70 %, $p \le 0.05$) and the intensity of electrolyte leakage from leaf tissues (on average by 50 %, $p \le 0.05$) were observed, indicating a less pronounced oxidative stress in comparison with control (without mannitol). An increase in the Ca^{2+} concentration in the nutrient medium (from 440 to 880 mg/l) (in the presence of mannitol) did not

significantly affect the water content in the leaves and the photosynthetic apparatus (content and ratio of chlorophylls/carotenoids). An insignificant effect of calcium (in the presence of mannitol) manifested itself in a significant decrease in malondialdehyde by 20 μmol/g dry weight. Consequently, the increased concentration of calcium (660-880 mg/l) in the nutrient medium provides an improvement in the functional state of long-term cultivated tea microshoots in vitro (4 months) by reducing the activity of lipid peroxidation in membranes and increasing their stability. The revealed patterns confirm the positive role of calcium ions in the reduction of combined oxidative stress caused by long-term cultivation of plants *in vitro* in combination with osmotic stress.

Keywords: tea plants, *Camellia sinensis* L., in vitro microshoots, calcium, mannitol, osmotic stress, pigments, proline, malondialdehyde

Stress resistance is essential to determine plant productivity [1, 2]. Osmotic stress caused by drought, hypothermia, and salinity leads to tissue dehydration and even plant death [3, 4]. The effect of stressors is traced by a complex of physiological, biochemical and molecular processes, including growth, water content and water potential of leaves, enzymatic activity, metabolomic profile, and gene expression [1, 5, 6].

Calcium (Ca^{2+}) plays a special role in maintaining plant resistance to adverse environmental factors [7, 8]. A change in its concentration in the cytosol is the first stage in the cell recognition of an external stimulus and the triggering of the signal transduction system for a response [7, 9, 10]. An important group of sensors involved in the cascade of calcium ion signals in higher plant cells are Ca-dependent protein kinases [11, 12]. Under the action of calcium, changes are noted in the growth, photosynthesis and water-air regime of plants, the functions of stomata, and accumulation of stress proteins [7, 13]. The induction of the antioxidant system reduces the oxidative damage [14, 15].

Tea plants (*Camellia sinensis* L.) are cultivated in drought-prone regions of the world [16-18]. According to a number of researches [9, 16, 18], including our own [17], hydrothermal stress leads to the loss of more than 50% of the tea yield under dryland conditions. Progressive climate aridization imposes urgency on the problem of plant drought resistance. Much attention is paid to the mechanisms of tea plant resistance to drought, the search for the most informative markers of drought resistance, and creation of new resistant varieties [4, 9, 15]. However, in-depth understanding of the young tea shoot metabolism under the exogenous effects of biogenic elements and other substances are practically not covered, though being widely studied in other crops where agar nutrient media with the addition of osmotically active substances are used as “drought models” [19-21]. The “drought models” are suitable to control water potential, which is essential to ensure high accuracy, reproducibility and comparative assessment of various experiments [22].

In vitro plant tissues and microshoots are informative model systems for studying metabolic processes and cellular responses to stresses, including physiological and biochemical changes. This technique was used to select resistant genotypes of potato [23], lathyrus culture [24], cucumber [25], and beans [26] under osmotic stress (drought). However, little research has focused on tea plants [27, 28], and these studies have mainly elucidated biochemical and molecular responses to drought for better understanding the changes it causes in plant proteome and metabolome.

In this paper, we have established for the first time the role of calcium in the adaptation of tea microshoots to stress caused by prolonged in vitro growing combined with osmotic stress, and confirmed the prospect of agar culture media added with mannitol as a “drought models” for studying effects of exogenous inducers on plant resistance.

The aim of the work was to assess the reaction functional performance of

in vitro grown tea microshoots under optimal conditions and upon the mannitol-simulated weak osmotic stress as influenced by different concentrations of calcium (Ca^{2+}) in the nutrient medium.

Materials and methods. Microshoots of tea (*Camellia sinensis* L.) local population were grown in vitro on the Murashige-Skoog nutrient medium supplied with 6-benzylaminopurine (BAP, 6 mg/l), 1-naphthaleneacetic acid (NAA, 1 mg/l), gibberellic acid (HA, 2 mg/l) [29]. The treatments were 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. For each treatment, 10-15 microshoots were grown for 4 months in a factorostatic chamber (16/8 h photoperiod, 25±1.0 °C, humidity 70%, illumination 3000 lx, lamps L 36 W/765, OSRAM GmbH, Germany). Leaves of microshoots were collected for analysis.

The water content in leaves was measured by drying samples in a thermostat (BD-115, Binder GmbH, Germany) at 70 °C to constant weight [30].

To assess cell membrane stability, a portion of leaves (50 mg) was placed in deionized water (50 ml). Electrical conductivity was measured in 0, 60, 120 minutes, and 60 minutes after boiling the plant sample (a portable ST300C conductometer, STCON3 sensor with verification, OHAUS Corporation, USA). The relative electrical conductivity of the solution was calculated as $L_1/L_2 \times 100$ %, where L_1 is the electrical conductivity in 0 min, L_2 is the electrical conductivity in the cooled solution after boiling in a water bath for 1 h at 100 °C [31].

Lipid peroxidation (LPO) was assessed the malondialdehyde (MDA) concentration [32]. Leaves of tea microshoots were homogenized in 0.1 M Tris-HCl buffer (pH 7.5) with 0.35 M NaCl followed by adding a 0.5% solution of thiobarbituric acid in a 20% aqueous solution of tri-chloroacetic acid. The reaction mixture was incubated for 30 min in a boiling water bath (WB-4MS, BioSan, Latvia), cooled, and the optical density of the supernatant liquid was measured (SF-46 spectrophotometer, LOMO, Russia) at $\lambda = 532$ nm.

Pigments were extracted from leaves using 96% ethanol [32]. After centrifugation of the homogenate (13000 rpm, 5 min), the chlorophyll a (Chla) ($\lambda = 665$ nm), chlorophyll b (Chlb) ($\lambda = 649$ nm), and carotenoids (Car) ($\lambda = 440$ nm) concentrations were measured in the supernatant. The concentrations of pigments were calculated by the standard method [33].

Proline was extracted from leaves by a standard method and measured with a ninhydrin reagent [27] at $\lambda = 520$ nm (a USF-01 device, VNIIOFI, Russia).

All measurements were conducted in three biological and three analytical replicates. Statistical processing was performed using Microsoft Excel 2010 and SigmaPlot 12.2 (http://www.sigmaplot.co.uk) programs. The table and figures show the arithmetic mean values (*M*) and their standard errors (±SEM). The significance of the differences between the means was assessed by the Student's *t*-test; values marked with different Latin letters differ at $p \leq 0.05$.

Results. Mannitol is known to cause in vitro osmotic stress [27, 34]. In our tests, tea microshoots grown for 4 months in vitro were low, which was also noted earlier [27], and had bright green leaves (Fig. 1). High Ca^{2+} concentration in the basal nutrient medium (880 vs. 440 mg/l $CaCl_2$, that is, 2 times higher than the normal Ca^{2+} level) caused a pronounced decrease in the microshoot height (see Fig. 1, A). Therefore, an increase in Ca^{2+} concentration slowed down the formation and development of leaves in tea microshoots. This tendency continued under osmotic stress, i.e., the higher the Ca^{2+} concentration in the medium, the smaller the leaf size (see Fig. 1, B). Nevertheless, the leaves were well developed, that is, we did not observe apparent stress effects,

which may evidence the Ca^{2+} protectiveness found in other studies, for example, in potatoes [23]. Note that in the available literature, we did not find any reports on the drought modeling in tea plants in vitro, except for our work.

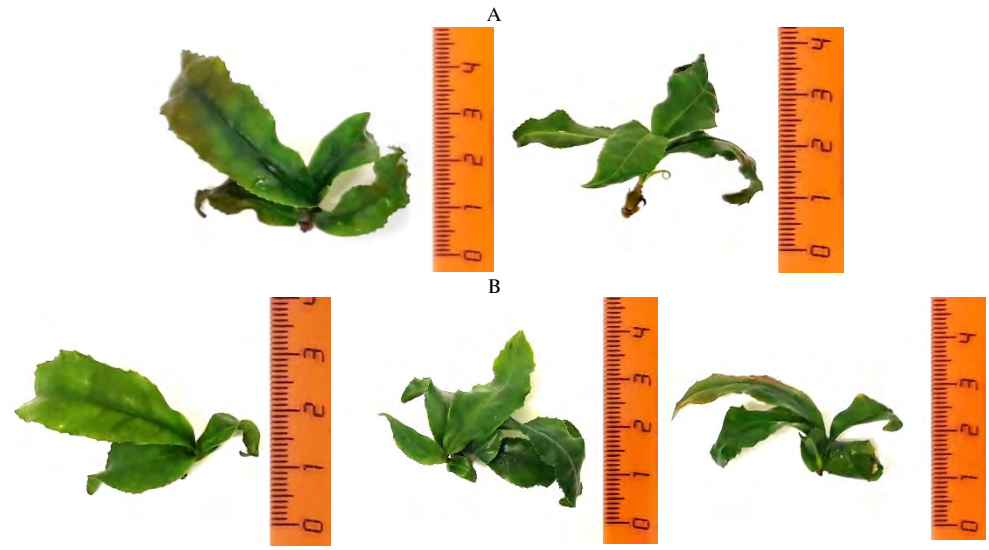

Fig. 1. Microshoots of tea plants (*Camellia sinensis* L.) grown in vitro on the nutrient media with (A) **and without** (B) **osmotic agent mannitol** (40 g/l) **and different concentrations of $CaCl_2$** (top row — 400 and 800 mg/l, bottom row from left to right 400, 600, and 800 mg/l).

The water content in the cells is an important parameter to characterize physiological state of a plant, especially under drought conditions [19]. Our study showed that the water content in leaves of tea microshoots grown on the basal nutrient medium did not depend on the effective concentration of Ca^{2+} and was practically equal for both treatments (Table 1).

Mannitol slightly but reliably decreased water content in leaves (by 2 %, $p \leq 0.05$), and this effect did not depend on the $CaCl_2$ concentration in the medium. Based on this, we assumed that Ca^{2+} contributed to the maintenance of water homeostasis of leaves in vitro in tea microshoots under osmotic stress, and this effect was practically the same at all studied concentrations (from 440 to 880 mg/l). There are reports that Ca^{2+} is involved in the regulation of plant responses to the adverse effects of drought [35]. In seedlings of *Vernicia fordii* Hemsley and *Hordeum vulgare* L. treated with this macronutrient, the leaf water content increased, and this effect depended on the time of exposure, Ca^{2+} concentration, and plant genotype [36, 37].

1. Water content in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the osmotic nutrient media

Mannitol	Treatment	$CaCl_2$ concentration, mg/l	Water content, %
Basal medium (no mannitol)	1	400	66.22±1.92[a]
	2	800	66.73±2.28[a]
Basal medium added with mannitol (40 g/l)	3	400	64.79±0.11[b]
	4	600	64.59±0.76[b]
	5	800	64.40±1.45[b]

[a, b] Differences between values marked with different Latin letters are statistically significant at $p \leq 0.05$.

Stressful conditions, including drought, often promote generation of reactive oxygen species in plant cells and intensify oxidative processes [1, 4, 19, 35]. Thence, LPO parameters in cells are determined, e.g., by the measuring MDA concentration [19, 38]. We noted a higher MDA level in the leaves of microshoots grown on the basal medium with low Ca^{2+} concentration (440 mg/l $CaCl_2$), on

average, approximately 2 times as much as for other treatments (Fig. 2). Perhaps this effect is due to the lack of this compound in the nutrient medium during long-term cultivation of tea microshoots in vitro. With an increase in Ca^{2+} concentration in the medium (up to 880 mg/l $CaCl_2$), the MDA concentration significantly decreased and the values detected under osmotic stress in combination with different Ca^{2+} concentrations. So, in the presence of mannitol in the nutrient medium, the MDA concertation for treatment 3 (440 mg/l $CaCl_2$) was 51.8% lower than for treatment 1 (control), 60.7% lower than for treatment 4 (660 mg/l $CaCl_2$), and almost 70% lower than for treatment 5 (880 mg/l $CaCl_2$) ($p \leq 0.05$). These results draw us to the conclusion that not only mannitol, but also Ca^{2+} (especially in high concentrations) has a regulatory effect on the antioxidant system of tea microshoots, which is confirmed by the data on a decrease in lipid peroxidation level upon treatment 2-5 compared to control treatment 1. As noted, drought conditions contribute to the development of oxidative stress, disrupting the balance between production and detoxification of reactive oxygen species [39]. In this case, Ca^{2+} ions are important secondary messengers in the transduction of intracellular signals in plants and in the regulation of oxidative reactions [7, 8]. The exogenous application of Ca^{2+} was shown to reduce negative impact of drought on *Helianthus annuus* L. seedlings [40]. Our results also indicate a significant decrease in the intensity of oxidative processes in the cells of tea microshoots due to Ca^{2+} application under osmotic stress.

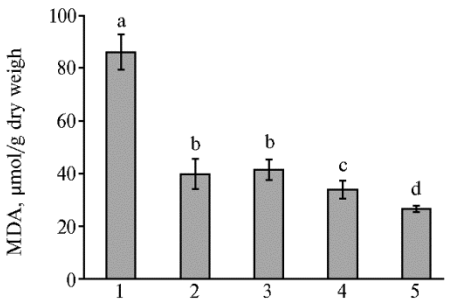

Fig. 2. Malonic dialdehyde (MDA) concentration in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the nutrient media without (1, 2) **or with** (3-5) **mannitol** (40 g/l): 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. Statistically significant differences between mean values ($p \leq 0.05$) are marked by different Latin letters.

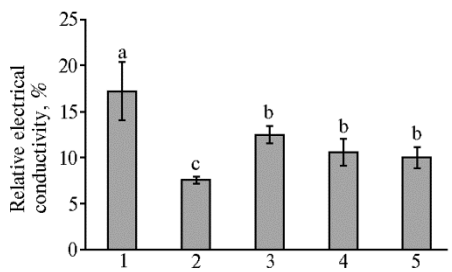

Fig. 3. Relative electrical conductivity which reflects the electrolytes leakage form leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the nutrient media without (1, 2) **or with** (3-5) **mannitol** (40 g/l): 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. Statistically significant differences between mean values ($p \leq 0.05$) are marked by different Latin letters.

An altered state of plant cell membranes resulted in a change in relative electrical conductivity is one of the initial stages of plant response to stressors [7, 10]. Cell membrane regulation, being a part of the entire regulation systems in the plant, is the most important adaptation mechanism that determines the preservation of viability, and the permeability of plant cell membranes (the rate of release of electrolytes from tissues) is an indicator of plant resistance to stresses, including the osmotic stress [41]. By analogy with the MDA level, the highest electrolyte efflux from leaf tissues occurred in tea microshoots grown on the basal medium with Ca^{2+} (Fig. 3). With an increase in the Ca^{2+} concentration (880 mg/l $CaCl_2$) in the presence of mannitol, the leakage of electrolytes decreased 1.5-2.0 times on average ($p \leq 0.05$), which indicates an increase in the stability of cell membranes.

Under osmotic stress, the effect was more apparent at higher concentrations of Ca^{2+} (660 and 880 mg/l $CaCl_2$) when we noted a decrease in relative electrical conductibility (see Fig. 3). Such a decrease indicated a less pronounced lipid peroxidation in cell membranes of these in vitro grown microshoots upon application of higher concentrations of Ca^{2+}, which is in line with literature data [14, 15]. The changes in the analyzed parameter directly correlate with the MDA levels in leaves, demonstrating a more pronounced development of lipid peroxidation processes in cells when Ca^{2+} concentrations in the medium during long-term culture is insufficient (up to 440 mg/l $CaCl_2$).

It is known that stress enhances hydrolytic processes, which leads to the accumulation of so-called stress metabolites, for example, proline, a low-molecular-weight osmotically active compound capable of forming hydrophilic colloids which protects proteins from denaturation under various stresses [9]. Our studies have shown an increase in the free proline in tea microshoots under osmotic stress (Fig. 4). An increase in the Ca^{2+} concentration to 880 mg/l significantly decreased proline concentration in the leaves of tea microshoots. The ratio of the absolute content of proline in microshoots for +mannitol/–mannitol treatments (after stress/before stress) which characterizes the rate of proline production was the highest for 400 mg/l $CaCl_2$ and amounted to 1.45 vs. 1.36 for 880 mg/l $CaCl_2$.

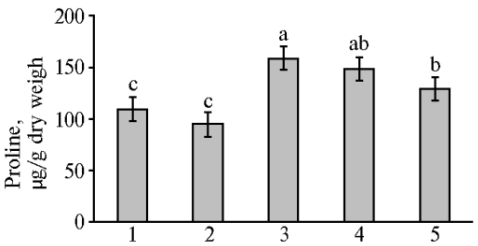

Fig. 4. Proline concentration in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the nutrient media without (1, 2) **or with** (3-5) **mannitol** (40 g/l): 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. Statistically significant differences between mean values ($p \leq 0.05$) are marked by different Latin letters.

2. Concentration (mg/g dry weigh) **of chlorophyl a** (Chla)**, chlorophyl b** (Chlb), **and carotenoids** (Car) **in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the osmotic nutrient media**

Treatment	Chla	Chlb	Car	Chla + Chlb	(Chla + Chlb)/Car
Basal medium without mannitol:					
440 mg/l $CaCl_2$	3.68±0.84[a]	0.90 ±0.30[a]	1.74±0.34[a]	4.58	2.63
880 mg/l $CaCl_2$	3.91±0.46[a]	0.95 ±0.17[a]	1.82±0.20[a]	4.86	2.67
Basal medium with mannitol (40 g/l):					
440 mg/l $CaCl_2$	2.39±0.32[b]	0.73±0.05[a]	1.26±0.12[a]	3.12	2.48
660 mg/l $CaCl_2$	1.95±0.11[b]	0.54±0.04[b]	0.98±0.04[b]	2.49	2.54
880 mg/l $CaCl_2$	2.07±0.16[b]	0.55±0.04[b]	1.07±0.05[b]	2.62	2.45

[a, b] Differences between values marked with different Latin letters are statistically significant at $p \leq 0.05$.

Osmotic compounds can cause a stress response in plants, including that leading to structural and functional rearrangement of the photosynthetic apparatus and inhibition of photosynthesis [42, 43]. The leaves of photosynthetic pigments in tea microshoots grown on the basal nutrient medium with Ca^{2+} were the highest and almost equal at both Ca^{2+} concentrations (Table 2). This may be a consequence of the structural and functional rearrangement of the photosynthetic apparatus, as well as a change in the concertation of reactive oxygen species, which explains the high level of LPO and the release of electrolytes (see Fig. 2 and 3) in long-term culture of microshoots on a nutrient medium with low concentrations of Ca^{2+} (440 mg/l $CaCl_2$). This aspect seems to us interesting and will be further studied.

When microshoots were grown on the basal medium with various Ca^{2+}

concentrations, the Chla levels in their leaves were the highest, while when mannitol was added, the Chla decreased upon all treatments by 35-40% ($p \leq 0.05$), that is, almost equally (see Table 2). A similar trend is characteristic of the Chlb levels. The level of chlorophyll b was high in the control (440 mg/l $CaCl_2$), mannitol added to this medium, decreased Chlb concentration by 20% ($p \leq 0.05$). For combination of mannitol and higher concentrations of Ca^{2+} (660 and 880 mg/l $CaCl_2$), the decrease was 40% compared to the control ($p \leq 0.05$). A decrease in the concentrations of chlorophylls in plant tissues is considered a manifestation of oxidative stress and can be the result of both degradation of pigments and structural reorganization of chloroplasts [43, 44].

The pigment system of plants is not only chlorophylls but also carotenoids that absorb the blue spectrum light, protect photosynthetic apparatus from photodegradation, and perform other protective functions [45]. As follows from our data, the trends in Car concentration changes were largely similar to those for chlorophylls, especially for Chlb (see Table 2). A higher content of carotenoids was characteristic of the leaves of tea microshoots grown on the basal medium with Ca^{2+}; the mannitol added to the medium, decreased carotenoids, but only for 660 and 880 mg/l $CaCl_2$ (treatments 4 and 5).

The revealed changes in the levels of pigments reflect the structural and functional reorganization of the photosynthetic apparatus of tea microshoots under low osmotic stress caused by mannitol. It is also possible that the observed changes occur under in vitro conditions and are largely due to in vitro differentiation of plant tissue structures, which was repeatedly reported [21, 23, 29].

Thus, a Murashige-Skoog nutrient medium added with mannitol (40 g/l) and 440-880 mg/l $CaCl_2$ causes changes in physiological and biochemical properties of tea microshoots in in vitro culture. This is manifested in a decrease in leaf water content (by 2%, $p \leq 0.05$), which is indicative of weak osmotic stress, in the accumulation of free proline in leaves, and in a decrease in the levels of photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids). When mannitol is added and the concentration of Ca^{2+} increases, the relative electrical conductivity and the concentration of malondialdehyde decrease, which indicates a decrease in cell membrane lipid peroxidation. Our study discloses some cellular mechanisms of action and the role of exogenous Ca^{2+} in tea microshoots during long-term culture and under osmotic stress. These data are of great importance with regard to depositing tea plants in biotechnological collections. In addition, they testify to the significant role of osmolytes in the preservation and maintenance of plant viability, especially in vitro.

REFERENCES

1. Zhu J.K. Abiotic stress signaling and responses in plants. *Cell*, 2016, 167(2): 313-324 (doi: 10.1016/j.cell.2016.08.029).
2. Fleta-Soriano E., Munné-Bosch S. Stress memory and the inevitable effects of drought: a physiological perspective. *Frontiers in Plant Science*, 2016, 7: 143 (doi: 10.3389/fpls.2016.00143).
3. Marcińska I., Czyczyło-Mysza I., Skrzypek E., Filek M., Grzesiak S., Grzesiak M.T., Janowiak F., Hura T., Dziurka M., Dziurka K., Nowakowska A., Quarrie S.A. Impact of osmotic stress on physiological and biochemical characteristics in drought-susceptible and drought-resistant wheat genotypes. *Acta Physiologiae Plantarum*, 2013, 35(2): 451-461 (doi: 10.1007/s11738-012-1088-6).
4. Samarina L.S., Ryndin A.V., Malyukova L.S., Gvasaliya M.V., Malyarovskaya V.I**.** Physiological mechanisms and genetic factors of the tea plant *Camellia sinensis* (L.) Kuntze response to drought (review**)**. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(3): 458-468 (doi: 10.15389/agrobiology.2019.3.458eng).
5. Fayez K.A., Bazaid S.A. Improving drought and salinity tolerance in barley by application of

salicylic acid and potassium nitrate. *Journal of the Saudi Society of Agricultural Sciences*, 2014, 13(1): 45-55 (doi: 10.1016/j.jssas.2013.01.001).
6. Yang Y., Guo Y. Unraveling salt stress signaling in plants. *Journal of Integrative Plant Biology*, 2018, 60(9): 796-804 (doi: 10.1111/jipb.12689).
7. Medvedev S.S. *Fiziologiya rastenii*, 2005, 52(2): 282-305 (in Russ.).
8. Song W.Y., Zhang Z.B., Shao H.B., Guo X.L., Cao H.X., Zhao H.B., Fu Z.Y., Hu X.J. Relationship between calcium decoding elements and plant abiotic-stress resistance. *International Journal of Biological* Sciences, 2008, 4(2): 116-125 (doi: 10.7150/ijbs.4.116).
9. Maritim T.K., Kamunya S.M., Mireji P., Wendia C.M., Muoki R.C., Cheruiyot E.K., Wachira F.N. Physiological and biochemical response of tea (*Camellia sinensis* (L.) O. Kuntze) to water-deficit stress. *The Journal of Horticultural Science and Biotechnology*, 2015, 90(4): 395-400 (doi: 10.1080/14620316.2015.11513200. 0.4236/ajps.2012.34054).
10. Hetherington A.M., Brownlee C. The generation of Ca^{2+} signals in plants. *Annual Review of Plant Biology*, 2004, 55: 401-427 (doi: 10.1146/annurev.arplant.55.031903.141624).
11. Kim M.C. Calcium and calmodulin-mediated regulation of gene expression in plant. *Molecular Plant*, 2009, 2(1): 13-21 (doi: 10.1093/mp/ssn091).
12. Saidi Y., Finka A., Muriset M., Bromberg Z., Weiss Y. G., Maathuis F.J., Goloubinoff P. The heat shock response in moss plants is regulated by specific calcium-permeable channels in the plasma membrane. *Plant Cell*, 2009, 21: 2829-2843 (doi: 10.1105/tpc.108.065318).
13. Rikhvanov E.G., Fedoseeva I.V., Pyatrikas D.V., Borovskii G.B., Voinikov V.K. *Fiziologiya rastenii*, 2014, 61(2): 155-169 (doi: 10.7868/S0015330314020134) (in Russ.).
14. Shu M.Y., Fan M.Q. Effect of osmotic stress and calcium on membrane-lipid peroxidation and the activity of defense enzymes in fir seedling. *Forest Research*, 2000, 4: 391-396.
15. Upadhyaya H., Panda S.K., Dutta B.K. $CaCl_2$ improves post-drought recovery potential in *Camellia sinensis* (L) O. Kuntze. *Plant Cell Reports*, 2011, 30(4): 495-503 (doi: 10.1007/s00299-010-0958-x).
16. Bhagat R.M., Baruah R.D., Cacigue S. Climate and tea [*Camellia sinensis* (L.) O. Kuntze] production with special reference to north eastern India: a review. *Journal of Environmental Research and Development*, 2010, 4(4): 1017-1028.
17. Malyukova L.S., Kozlova N.V., Rogozhina E.V., Strukova D.V., Kerimzade V.V., Velikii A.V. Cultivating subtropical crops on the Black Sea coast of Russia: ecological and agrochemical aspects. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2014, 3: 24-31 (doi: 10.15389/agrobiology.2014.3.24eng) (in Russ.).
18. Baruah R.D., Bhagat R.M. Climate trends of Northeastern India: a longterm pragmatic analysis for tea production. *Two and a Bud*, 2012, 59(2): 46-49.
19. Osmolovskaya N., Shumilina J., Kim A., Didio A., Grishina T., Bilova T., Frolov A. Methodology of drought stress research: experimental setup and physiological characterization. *International Journal of Molecular Sciences*, 2018, 19(12): 4089-4114 (doi: 10.3390/ijms19124089).
20. Rai M.K., Kalia R.K., Singh R., Gangola M.P., Dhawan A.K. Developing stress tolerant plants through in vitro selection — an overview of the recent progress. *Environmental and Experimental Botany*, 2011, 71(1): 89-98 (doi: 10.1016/j.envexpbot.2010.10.021).
21. Pérez-Clemente R.M., Gómez-Cadenas A. In vitro tissue culture, a tool for the study and breeding of plants subjected to abiotic stress conditions. In: *Recent advances in plant in vitro culture*. A. Leva, L.M.R. Rinaldi (eds.). IntechOpen Limited, London, 2012: 91-108 (doi: 10.5772/50671).
22. Sunaina N.A., Singh N.B. PEG imposed water deficit and physiological alterations in hydroponic cabbage. *Iranian Journal of Plant Physiology*, 2016, 6(2): 1651-1658.
23. Gelmesa D., Dechassa N., Mohammed W., Gebre E., Monneveux P., Bьndig C., Winkelmann T. In vitro screening of potato genotypes for osmotic stress tolerance. *Open Agriculture*, 2017, 2(1): 308-316 (doi: 10.1515/opag-2017-0035).
24. Piwowarczyk B., Kami ska I., Rybi ski W. Influence of PEG generated osmotic stress on shoot regeneration and some biochemical parameters in Lathyrus culture. *Czech Journal of Genetics and Plant Breeding*, 2014, 50(2): 77-83 (doi: 10.17221/110/2013-cjgpb).
25. Abu-Romman S., Suwwan M., Al-Shadiadeh A., Hasan H. Effects of osmotic stress on cucumber (*Cucumis sativus* l.) microshoots cultured on proliferation medium. *World Applied Sciences Journal*, 2012, 20(2): 177-181 (doi: 10.5829/idosi.wasj.2012.20.02.2817).
26. Tejavathi D.H., Devaraj V.R., Murthy S.M., Nijagunaiah R., Shobha K. Effect of PEG induced osmotic stress on proline, protein and relative water content in vitro plants of *Macrotyloma uniflorum* (Lam.) Verdc. *Acta Hortic*., 2010, 865: 87-93 (doi: 10.17660/ActaHortic.2010.865.10).
27. Gvasaliya M.V., Samarina L.S., Malyukova L.S., Malyarovskaya V.I., Rakhmangulov R.S., Koninskaya N.G., Platonova N.B., Pashchenko O.I. *Vestnik Michurinskogo gosudarstvennogo agrarnogo universiteta*, 2019, 4(59): 49-53 (in Russ.).
28. Gvasaliya M.V. *Novye tekhnologii,* 2020, 3: 117-124 (doi: 10.24411/2072-0920-2020-10313) (in Russ.).
29. Gvasaliya M.V. *Sadovodstvo i vinogradarstvo*, 2013, 4: 20-22 (in Russ.).

30. Zubova M.Yu., Nikolaeva T.N., Nechaeva T.L., Malyukova L.S., Zagoskina N.V. *Khimiya rastitel'nogo syr'ya*, 2019, 4: 249-257 (doi: 10.14258/jcprm2019046065) (in Russ.).
31. Yoshida K., Matsuo K. A simple method of evaluating the freezing resistance of tea plants (*Camellia sinensis* (L.) Kuntze) by measuring electrolyte leakage from low-temperature-treated overwintering buds and leaves. *Chagyo Kenkyu Hokoku (Tea Research Journal)*, 2012, 113: 63-69 (doi: 10.5979/cha.2012.113-63).
32. Tsypurskaya E.V., Kazantseva V.V., Fesenko A.N., Zagoskina N.V. Growth of buckwheat (*Fagopyrum esculentum* Moench) seed-lings and the accumulation of primary and secondary metabolites under various mineral nutrition conditions. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(5): 946-957 (doi: 10.15389/agrobiology.2019.5.946eng).
33. Shlyk A.A. V sbornike: *Biokhimicheskie metody v fiziologii rastenii* [In: Biochemical methods in plant physiology]. Moscow, 1971: 154-170 (in Russ.).
34. Tholakalabavi A., Zwiazek J.J., Thorpe T.A. Effect of mannitol and glucose-induced osmotic stress on growth, water relations, and solute composition of cell suspension cultures of poplar (*Populus deltoides* var. *occidentalis*) in relation to anthocyanin accumulation. *In Vitro Cellular & Developmental Biology-Plant*, 1994, 30(3): 164-170 (doi: 10.1007/bf02632208).
35. Xu C., Li X., Zhang L. The effect of calcium chloride on growth, photosynthesis, and antioxidant responses of *Zoysia japonica* under drought conditions. *PloS ONE*, 2013, 8(7): e68214 (doi: 10.1371/journal.pone.0068214).
36. Li Z., Tan X.F., Lu K., Liu Z.M., Wu L.L. The effect of $CaCl_2$ on calcium content, photosynthesis, and chlorophyll fluorescence of tung tree seedlings under drought conditions. *Photosynthetica*, 2017, 55(3): 553-560 (doi: 10.1007/s11099-016-0676-x).
37. Kaczmarek M., Fedorowicz-Stronska O., Głowacka K., Waskiewicz A., Sadowski J. $CaCl_2$ treatment im-proves drought stress tolerance in barley (*Hordeum vulgare L.*). *Acta Physiologiae Plantarum*, 2017, 39(1): 41-52 (doi: 10.1007/s11738-016-2336-y).
38. Farmer E.E., Mueller M.J. ROS-mediated lipid peroxidation and RES-activated signaling. *Annual Review of Plant Biology*, 2013, 64: 429-450 (doi: 10.1146/annurev-arplant-050312-120132).
39. Noctor G., Mhamdi A., Foyer C.H. The roles of reactive oxygen metabolism in drought: not so cut and dried. *Plant Physiology*, 2014, 164(4): 1636-1648 (doi: 10.1104/pp.113.233478).
40. Madany M., Khalil R. Seed priming with ascorbic acid or calcium chloride mitigates the adverse effects of drought stress in sunflower (*Helianthus annuus* L.) seedlings. *The Egyptian Journal of Experimental Biology (Botany)*, 2017, 13(1): 119-133 (doi: 10.5455/egyjebb.20170409090612).
41. Demidchik V., Straltsova D., Medvedev S.S., Pozhvanov G.A., Sokolik A., Yurin V. Stress-induced electrolyte leakage: the role of K+-permeable channels and involvement in programmed cell death and metabolic adjustment. *Journal of Experimental Botany*, 2014, 65(5): 1259-1270 (doi: 10.1093/jxb/eru004).
42. Hu W., Tian S.B., Di Q., Duan S.H., Dai K. Effects of exogenous calcium on mesophyll cell ultrastructure, gas exchange, and photosystem II in tobacco (*Nicotiana tabacum* Linn.) under drought stress. *Photosynthetica*, 2018, 56(4): 1204-1211 (doi: 10.1007/s11099-018-0822-8).
43. Sofronova V.E., Chepalov V.A., Dymova O.V., Golovko T.K. *Fiziologiya rastenii*, 2014, 61(2): 266-274 (doi: 10.7868/s001533031401014x) (in Russ.).
44. Fathi A., Tari D.B. Effect of drought stress and its mechanism in plant. *International Journal of Life Sciences*, 2016, 10(1): 1-6 (doi: 10.3126/ijls.v10i1.14509).
45. Sun T., Yuan H., Cao H., Yazdani M., Tadmor Y., Li L. Carotenoid metabolism in plants: the role of plastids. *Molecular Plant*, 2018, 11(1): 58-74 (doi: 10.1016/j.molp.2017.09.010).

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ON USING DATA FROM MARKER-ASSISTED SELECTION OF SOURCE MATERIAL AND INTERVARIETAL HYBRIDS IN PRACTICAL POTATO BREEDING

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Abstract

The success of breeding research is in many ways determined by the successful selection of parental forms for hybridization. In recent years, along with traditional approaches, the results of marker-assisted selection (MAS) are actively used for the selection of parental lines, in order to combine valuable alleles of parental genotypes. Such programs are widely used for different crops in many countries, including Russia. The use of MAS is promising both at the initial stage in the selection of parental samples for crosses and at the stages of analysis of segregating hybrid populations. In this work, the selection of parental potato varieties for crosses was carried out based on the results of MAS of initial varieties with markers of *R*-genes conferring resistance to various harmful organisms as well as based on their economically valuable characters. To increase the efficiency of the selection of promising hybrid genotypes obtained in intervarietal crosses, we used an integrated approach that combines MAS with markers of *R*-genes for resistance to various diseases and pests with traditional methods for assessing economically valuable traits of hybrid populations. The resulting hybrids of three combinations (Gusar × Charoit), (Gusar × Aliy Parus), (Gusar × Sireneviy tuman) also participated in MAS with 8 markers of 6 *R*-genes conferring resistance to potato virus Y (*Ry$_{sto}$*) and potato virus X (*Rx1*), to golden (*H1*) and pale (*Gpa2*) potato cyst nematodes, and race-specific resistance to late blight (*R1*, *R3A*). Almost all the hybrids had different combinations of *R*-gene markers. To identify the allelic composition of the *R*-genes in the parental varieties, the segregation of DNA markers in each combination was analyzed, which allowed us to determine the level of heterozygosity of the marked loci in the parental varieties. Main economically valuable characters of the hybrids were also evaluated in the field trials. As a result, out of 144 hybrids, 31 genotypes were identified that have one or the other economically valuable traits (yield, marketability, shape of tubers, starch content, field resistance to late blight), and 113 hybrids were rejected. In 23 of the 31 selected hybrids, productivity varied from 600 to 1525 grams per plant. Twelve of these 23 hybrid genotypes combined relatively high productivity and marketability of tubers with various combinations of *Ry$_{sto}$*, *Rx1*, *H1*, *Gpa2*, *R1*, and *R3A* gene markers. Thus, the use of an integrated approach that combines traditional breeding methods and MAS increases the efficiency of the selection of promising genotypes with a given set of traits. The selected hybrid genotypes are of interest for further breeding aimed at creating competitive varieties with complex resistance to various pathogens and pests, including viral and nematode resistance, that will need fewer chemical treatments to protect the crop.

Keywords: potato, varieties, hybrids, valuable traits, disease resistance, *R*-genes, DNA

markers, MAS, PVY, PVX, potato viruses, *Globodera rostochiensis*, the golden potato cyst nematode, *Globodera pallida*, the pale potato cyst nematode, late blight, *Phytophthora infestans*

Plant breeders use various approaches and methods to choose pairs for hybridization which are based on ecological and geographical features, differences in productivity parameters, duration of growing phases or unequal resistance to diseases and pests, and the combining ability of parents [1, 2]. However, despite the long history of investigations aimed to improve these methods, successful hybrid combinations remain largely unpredictable [3]. In recent years, along with conventional approaches, marker assisted selection (MAS) are used to combine valuable alleles of parental genotypes. In potato breeding, their complementarity is mostly used in combination with MAS for DNA markers of different genes of plant resistance to diseases and pests [4, 5]. The combination of MAS with markers of resistance *R*-genes with common selection of hybrid genotypes by yield and other agronomical traits increases the breeding efficiency, which was shown by programs of creating nematode-resistant [6] and virus-resistant [7, 8] breeding material and selection of new promising potato clones with complex resistance to various pathogens [9-13]. Similar programs are being implemented in Russia [14-16].

Since potato cultivars are highly heterozygous tetraploid genotypes, intervarietal hybrids segregate, including by the presence/absence of DNA markers of a certain locus. Data on the inheritance of DNA markers of the dominant alleles of the resistance *R*-genes facilitate selection of promising intervarietal hybrid genotypes in the offspring. In addition, characterization of the allelic composition and heterozygosity of the marked loci in parental varieties makes it possible to predict appearance of the offspring resistant to a specific pathogen and the siaze of analyzed segregating populations [17-21]. In hybrid populations, depending on the degree of heterozygosity of the marked *R*-locus in the initial cultivar, the frequency of resistant genotypes carrying at least one dominant allele will be 100% for quadriplex (*RRRR*) or triplex (*RRRr*), 83.4% for duplex (*RRrr*), and 50% for simplex (*Rrrr*) [17].

Potato varieties with economically valuable traits registered for use in the North-West region of Russia have derived from the original multispecies hybrids [22-24]. Our earlier MAS results for these varieties [25, 26] detected different DNA markers associated with genes for resistance to the causative agent of potato wart, the most common and harmful Y and X potato viruses (PVY and PVX), markers of genes for race-specific resistance to the late blight (*Phytophthora infestans* Mont. de Bary), and markers of genes for resistance to various types of cyst nematodes, i.e., the golden potato nematode (GPN) *Globodera rostochiensis* (Wollenweber) Behrens and pale potato nematode (PPN) *G. pallida* (Stone) Behrens. The selection of genotypes resistant to these nematodes using methods of plant pathology is very difficult because of internal and external quarantine for GPN and PPN, respectively. Markers of genes for resistance to *G. pallida* revealed in breeding material is of particular value, since this nematode species has not yet been found in the Russian Federation, although there is a great danger of its introduction from the outside [27].

In this work, based on the MAS data [26], we selected parental pairs and performed a series of intervrietal crosses in order to combine SCAR markers of *R*-genes conferring resistance to various diseases and pests in one genotype. The maternal form was the cv. Gusar possessing markers of genes for PVY and GPN resistance. This variety is unsuitable as a pollinator due to the inherent cytoplasmic male sterility [26, 28]. The paternal forms in three combinations were cv. Charoit, Alyi Parus, and Sirenevyi tuman in which DNA markers of genes for resistance to PVX and to PPN were identified. In this study, segregating hybrid populations derived from these

combinations were involved in MAS to revealed promising genotypes with various combinations of markers of dominant alleles of *R*-genes conferring resistance to various diseases and pests and to search among them for clones with a complex agronomically valuable traits (productivity, marketability, flattened surface of tubers, and starchiness).

The aim of the work was to increase the efficiency of breeding with the use of an integrated approach that combines MAS and common breeding methodology to select promising intervarietal hybrids.

Materials and methods. Experiments were performed in 2017-2019 (Belogorka Leningrad Research Institute of Agriculture, Gatchinsky District, Leningrad Province).

Parental pairs used in intervarietal crosses possessed economically valuable traits (productivity, quality of tubers, and resistance to the most common diseases). The varieties were created in different years and adapted to the conditions of the North-West region of Russia (the originators are Belogorka Leningrad Research Institute of Agriculture and LLC Breeding firm LiGa, Leningrad Province) [29-31]. Data on molecular screening of potato varieties [26] performed by us earlier with DNA markers associated with *R*-genes for resistance to PVY, PVX, GPN, BKN and genes for race-specific resistance to late blight were also used to involve varieties with markers of different *R* genes in hybridization. Crossings were performed in 2017 in three combinations: Gusar × Charoit, Gusar × Alyi Parus, and Gusar × Sirenevyi tuman.

In 2018, seeds of hybrid offsprings were sown in a greenhouse followed by open-field planting of the seedlings. In July 2018, leaves of each hybrid genotype plants were collected to extract DNA, and in September 2018, tubers were collected individually from each hybrid. In the spring of 2019, based on the results of storing tubers, 144 hybrids from three combinations were selected and planted (a 2.3 m^2 8-tuber plot for each hybrid genotype). The best hybrid genotypes were selected in 2019 (an experimental field, Belogorka Leningrad Research Institute of Agriculture, Leningrad Provinceregion) by late blight resistance and resistance to viral diseases during the growing season and by a complex of economically valuable traits (productivity, presentation of tubers, uniformity of nests and tubers in the nest, content of dry matter and starch in tubers) in lab test and after harvesting.

MAS was performed for 112 out of 144 hybrid genotypes, including 27 hybrids for Gusar × Charoite combination, 30 hybrids for Gusar × Alyi Parus, and 55 hybrids for Gusar × Sirenevyi tuman hybrids.

The leaves collected from individual hybrid plants in the summer of 2018 (an experimental field, Belogorka Leningrad Research Institute of Agriculture, Leningrad Province), were fixed in liquid nitrogen and delivered to the laboratory in dry ice. Total DNA was CTAB-extracted in our proposed modification [32].

MAS of intervarietal hybrids was performed with 8 SCAR (sequence characterized amplyfied region) markers associated with six *R*-genes for resistance to viruses PVY (Ry_{sto}), PVX (*Rx1*), to cyst potato nematodes *G. rostochiensis* of pathotype Ro1 (gene *H1*), *G. pallida* of pathotypes Pa2/Pa3 (gene *Gpa2*), and with genes for race-specific resistance to late blight (*R1*, *R3a*).

PCR was run in a 20 μl reaction mixture containing 40 ng of total DNA, 1× reaction buffer (Dialat Ltd, Moscow), 2.5 mM $MgCl_2$, 0.6 mM each dNTPs, 0.25 μM forward and reverse primers and 1 unit of BioTaq-DNA polymerase (Dialat Ltd, Moscow) (a Mastercycler® nexus gradient DNA amplifier, Eppendorf, Germany). The PCR protocols and annealing temperatures generally corresponded to those given in the literature for each of the markers

used [10, 33-38]; the TouchDown function was added to the protocols as indicated below to increase the specificity.

The PCR protocols for the markers were as follows. For YES3-3A: 3 min 30 s at 94 °C; 45 s at 94 °C, 1 min at 60 °C with a decrease in the annealing temperature by 1 °C per cycle, 1 min at 72 °C (5 cycles); 40 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C (35 cycles); 10 min at 72 °C (TouchDown) [34]. For YES3-3B: 3 min 30 s at 94 °C; 45 s at 94 °C, 45 s at 58 °C with a decrease in the annealing temperature by 1 °C per cycle, 1 min at 72 °C (5 cycles); 40 s at 94 °C, 40 s at 53 °C, 1 min at 72 °C (35 cycles); 10 min at 72 °C (TouchDown) [34]. For 5Rx1: 3 min 30 s at 94 °C; 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C (35 cycles); 10 min at 72 °C (with an increased time for denaturation and annealing) [33]. For 57R: 3 min 30 s at 94 °C; 45 s at 94 °C, 1 min at 65 °C with a decrease in the annealing temperature by 1 °C per cycle, 1 min at 72 °C (5 cycles); 45 s at 94 °C, 45 s at 60 °C, 45 s at 72 °C (35 cycles); 10 min at 72 °C (TouchDown) [35]. For N195: 3 min 30 s at 94 °C; 45 s at 94 °C, 45 s at 66 °C with a decrease in the annealing temperature by 1 °C per cycle, 1 min 30 s at 72 °C (8 cycles); 30 s at 94 °C, 30 s at 58 °C, 1 min 30 s at 72 °C (35 cycles); and then 10 min at 72 °C (TouchDown) [10]. For Gpa2-2: 4 min 30 s at 94 °C; 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C (35 cycles); 10 min at 72 °C [36]. For R1: 3 min 30 s at 94 °C; 45 s at °C, 1 min at 65 °C with a decrease in the annealing temperature by 1 °C per cycle, 1 min 30 s at 72 °C (10 cycles); 45 s at 94 °C, 45 s at 55 °C, 1 min 30 s at 72 °C (30 cycles); 10 min at 72 °C (TouchDown) [37]. For R3a: 3 min 30 s at 94 °C; 45 s 94 °C, 45 s at 68 °C with a decrease in the annealing temperature by 1 °C per cycle, 1 min 30 s at 72 °C (10 cycles); 45 s at 94 °C, 45 s at 58 °C, 1 min 30 s at 72 °C (35 cycles); 10 min at 72 °C (TouchDown) [38]. DNA of parental cultivars were positive controls and distilled water was a negative control.

Amplified DNA fragments were separated in 2% agarose gels in TBE buffer. The gels were stained with ethidium bromide, followed by visualization in transmitted UV light.

Assessed economically valuable traits of intervarietal hybrids were their productivity, marketable type of tubers, and content of dry substances and starch in tubers. The study involved 144 hybrid genotypes, for each of them the indicators for 8 plants were determined. Productivity was assessed gravimetrically, the average tuber weight, the number of tubers per plant, and the average tuber weight per nest were calculated. The number of commercial and non-commercial tubers, their weight per plant, the evenness of the nests and the number of tubers per nest were recorded. The marketability was assessed by Banadisev wet al. [39]. The contents of dry matter and starch were determined by the specific weight of tubers converted into the percentage of dry matter and starch using a special table [40]. The taste of tubers was scored according to a 9-point scale where 9 points are excellent, 1 point is bad (bitter, unpleasant) [41]. The culinary type was assigned to A (table potatoes, not boiled soft), B (poorly boiled soft), C (boiled soft), and D (highly boiled soft) as per the international classification of potato tuber table qualities [41]. The plant resistance to late blight was assessed under strong natural infection in 2019 (an experimental field, Belogorka Leningrad Research Institute of Agriculture, Leningrad Province) using a scale from 1 (whole plant affected) to 9 points (no disease symptoms).

Statistical processing of agronomic trait characteristics was performed by conventional methods [42]. Chi-squared (χ^2) test was used to estimate deviations from the theoretically expected segregation in hybrid populations (1:1 or 5:1) at different levels of heterozygosity of marked *R*-loci in the carriers of dominant alleles.

1. Characterization of parental potato varieties involved in intervarietal hybridization in 2017 (the originators are Belogorka Leningrad Research Institute of Agriculture and LLC Breeding firm LiGa, Leningrad Province)

Trait	Gusar	Alyi Parus	Sirenevyi tuman	Charoit
Markers of *R*-genes [26] for resistance:				
to viruses				
YES3-3A/YES3-3B (PVY, Ry_{sto})	+/+	–/–	–/–	–/–
5Rx1 (PVX, *Rx1*)	–	+	+	+
to late blight				
R1 (*R1*)	+	-	–	–
RT-R3a (*R3a*)	–	+	+	+
to potato cyst nematodes				
57R/N195 (golden, *H1*)	+/+	+/+	–/–	–/–
Gpa2-2 (pale, *Gpa2*)	–	+	+	+
Commercially valuable traits and diseases resistance ([lant disease estimates) [29-31]	Mid-season, the yield up to 60 t/ha, 15-19% starch, long period of tuber dormancy; resistant to golden potato nematode, potato wart, late blight, common scab, and rhizoctonia	Mid-season, the yield up to 50 t/ha, 18-23% starch, resistant to golden potato nematode, viruses, and potato wart	Mid-season, the yield up to 60 t/ha, 14-17% starch, resistant to potato wart, late blight, and viruses	Super-early potato, the yield up to 55 t/ha, resistant to potato wart, rhizoctonia, and common scab

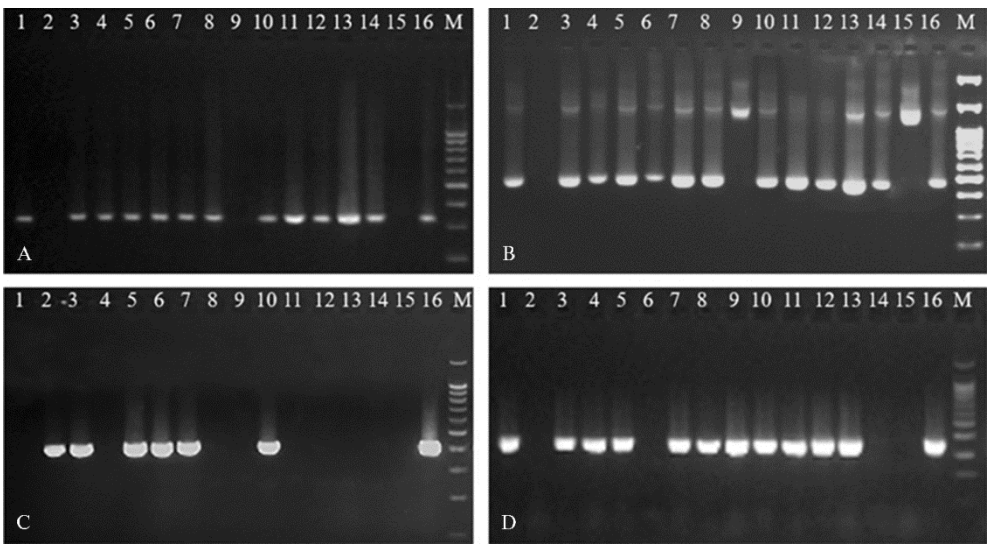
N o t e. Marker RT-R3a was detected in cv. Alyi Parus in biomaterial received directly from the originators (N.M. Gadzhiev, V.A. Lebedeva). In a plant of cv. Alyi Parus cultivar that we obtained from other sources, this marker was not previously detected [26].

Results. Table 1 shows varieties with SCAR markers of different *R*-genes involved in hybridization.

Molecular screening of intervarietal hybrids for markers of *R*-genes conferring plant resistance to diseases and pests. Three combinations of crosses produced 144 intervarietal hybrids of which 112were screened 8 SCAR markers associated with six *R*-genes, the Ry_{sto} and *Rx1* (PVY and PVX resistance), *H1* and *Gpa2* (resistance to golden and pale potato nematodes), and *R1* and *R3a* (race-specific resistance to late blight).

One or another *R*-resistance gene marker combination was characteristic of almost all 112 studied hybrid genotypes (Fig.). The only exception was 7.18-7 (Hussar × Sirenevyi tuman) in which we did not find any of the 8 markers used in MAS. Of the 112 hybrid genotypes, there were 58 hybrids with markers of two genes, the *H1* and *Gpa2*, and 42 hybrids with markers of Ry_{sto} и *Rx1*. In four of the 112 studied hybrid seedlings (5.18-9 and 5.18-17 from Gusar × Charoite and 6.18-12 and 6.18-37 from Gusar × Alyi Parus), we detected all eight markers of six resistance genes used in MAS.

Intervarietal hybrids derived from all three combinations segregated by the presence or absence of DNA markers of the six *R*-loci (Table 2), therefore, no quadriplexes or triplexes for the marked *R*-genes were identified among the initial varieties. Segregation could be attributed to two variants of crosses with different levels of heterozygosity of dominant alleles, the first one is a simplex *Rrrr* × nulliplex *rrrr* with theoretical segregation of 1 *R–*:1 *rrrr*, the second one is a duplex *RRrr* × a nulliplex with theoretical segregation of 5 *R–*:1 *rrrr*.



Screening of the intervarietal potato hybrid Gusar × Syrenevyi tuman for ДНК markers of *R*-genes: N195 (A) and 57R (B) for resistance to golden cyst potato nematode, Gpa2-2 (C) to pale potato nematode, and YES3-3a (D) to PVY; 1 — Gusar, 2 — Sirenevyi tumanман, 3 — hybryd 7.18-1, 4 — hybryd 7.18-8, 5 — hybryd 7.18-2, 6 — hybryd 7.18-9, 7 — hybryd 7.18-3, 8 — hybryd 7.18-10, 9 — hybryd 7.18-4, 10 — hybryd 7.18-11, 11 — hybryd 7.18-5, 12 — hybryd 7.18-12, 13 — hybryd 7.18-6, 14 — hybryd 7.18-13, 15 — hybryd 7.18-7, 16 — hybryd 7.18-14; M — molecular weight marker 100 bp + 1500 + 3000 (NPO SibEnzyme, Russia).

Analysis of the inheritance pattern of DNA markers in each combination (see Table 2) revealed the allelic composition of *R*-genes and heterozygosity of the marked loci in parental forms. For example, Gusar has one dominant allele (simplex) of Ry_{sto} and *R1* genes and two dominant alleles (duplex) of *H1* gene, Charoite was a simplex for *R3a* gene and duplexes for *Rx1* and *Gpa2-2* genes, Alyi

Parus had *R3a* and *H1* simplexes and duplexes for of *Gpa2* and *Rx1* genes, and Syrenevyi tuman had one dominant allele (simplixes) of *R3a*, *Rx1* and *Gpa2-2*.

2. Segregation of potato inrervarietal hybrid offspring derived from three combination of cv. Gusar crossing by DNA marker of *R*-genes conferring resistance to diseases and pests (ratio of hybrids with and without marker of a corresponding gene) (Belogorka Leningrad Research Institute of Agriculture, Leningrad Province, 2018)

Crossing	YES3-3A/YES3-3B (gene *Ry*$_{sto}$)	5Rx1 (gene *Rx1*)	57R/N195 (gene *H1*)	Gpa2-2 (gene *Gpa2*)	R1 (gene *R1*)	RT-R3a (gene *R3a*)
Gusar × Charoit	16:11[a]	22:5[b]	23:4[b]	22:5[b]	11:16[a]	10:17[a]
Gusar × Alyi Parus	15:15[a]	22:8[b]	27:3[c]	21:9[b]	15:15[a]	14:16[a]
Gusar × Syrenevyi tuman	33:22[a]	28:27[a]	46:9[b]	26:29[a]	18:37	22:33[a]

N o t e. DNA markers YES3-3A/YES3-3B (*Ry*$_{sto}$, PVY resistance), 5Rx1 (*Rx1*, PVX resistance), 57R (*H1*, resistance to pathotype Ro1 of golden potato nematode), N195 (*H1*, resistance to pathotype Ro1 of golden potato nematode), R1 (*R1*, resistance to *Phytophthora infestans*), and RT-R3a (*R3a*, resistance to *Ph. infestans*). Superscripts: a — segregation for the specified *R*-locus in the combination at p_{05} corresponds to the theoretical 1:1 for simplex × nulliplex (*Rrrr* × *rrrr*); b — segregation at p_{05} corresponds to the theoretical 5:1 for duplex × nulliplex (*RRrr* × *rrrr*); c — segregation at p_{05} corresponds to the theoretical 11:1 for duplex × simplex (*RRrr* × *Rrrr*).

Agronimical traits of the intervarietal hybrids. The best hybrid genotypes were selected in 2019 by tuber productivity, shape and marketability, field resistance to late blight and viruses, and MAS. Economically valuable traits were measured for 8 plants for each of 144 hybrid genotypes. As a result, 31 promising hybrids were detected which possess certain economically valuable traits and various combinations of *R*-gene markers. Of these, 23 hybrid genotypes showed relatively high productivity (Table 3), in the remaining 8 hybrids, productivity was low but they had high field resistance to late blight and should be involved in further crosses. The reason for the rejection of the remaining 113 hybrids was excessively long stolons or damage to plants by late blight and viruses.

In 23 selected hybrids, total tuber weigh per plant varied from 600 to 1525 g which corresponds to a yield of 24.5-62.2 t/ha; some of these hybrids were distinguished by other economically valuable traits (high marketability of tubers, good taste, starchiness) (see Table 3). For example, we note the hybrid 7.18-3 (Gusar × Syrenevyi tuman) with gene markers for resistance to PVY, PVX, and both nematodes, with high marketability of tubers and a productivity of 1350 g per plant which corresponds to a 55.1 t/ha yield (see Table 3).

The highest productivity (1525 g per plant or 62.2 t/ha) was characteristic of the hybrid 5.18-7 which also carried markers YES3-3A/3B, 5Rx1, N195, 57R, and Gpa2-2. That is, the 5.18-7 potentially has complex resistance to PVY and PVX viruses and to two types of nematodes, GPN and PPN (see Table 3).

Approximately half of hybrid genotypes with high productivity, 12 of 23, show evenness of nests and tubers in the nest, even and regular shape of tubers (Table 4).

DNA markers facilitate selection of target genotypes with dominant alleles of various *R*-genes in breeding, which is especially important when identifying clones with markers of resistance genes to quarantine objects, since assessments of plant resistance to such agents using methods of plant pathology is very complicated and laborious [27, 43]. In this work, we identified more than 40% of hybrids with markers of two genes (*H1* and *Gpa2-2*) for resistance to Ro1 pathotype of golden potato nematode and Pa2/Pa3 pathotypes of pale potato nematode. The high frequency of such hybrids is explained by the presence of duplexes of these genes in the parental varieties, the *H1* gene in the cv. Gusar and the *Gpa2-2* gene in the Alyi Parus and Charoit cultivars. Note that Ermishin et al. [20], when analyzed segregation for DNA markers in 11 intervarietal potato populations, came

3. Selection of 23 potato hybrid genotypes derived from three intervarietal for DNA markers of *R*-genes for resistance to diseases, pests and by rconomically valuable traits устойчивости к болезням и вредителям и по хозяйственно ценным признакам (an experimental field of Belogorka Leningrad Research Institute of Agriculture, Leningrad Province, 2019, 8 plants of each genotype tested)

Variety, hybrid	Productivity, g/plant (min-max)	Marketability, %	Content in tubers, %		Taste, points	Cooking type	DNA markers of genes for resistance to PVY, PVX, late blight, golden and pale potato nematodes
			starch	dry matter			
Невский (st)	700 (560-810)	92	14.2	20.0	5.5	A	RT-R3a
Ten hybrids from crossing Gusar × Charoit							
5.18-3	787 (690-855)	97	15.4	21.1	7.5	B	5Rx1, N195, 57R, Gpa2-2, RT-R3a
5.18-7^{hp}	1525 (1300-1740)	97	10.0	15.0	7.0	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2
5.18-15	643 (590-735)	98	12.9	18.6	8.0	AB	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, R1
5.18-17	600 (430-810)	95	14.2	20.0	7.0	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, R1, RT-R3a
5.18-19^{hp}	925 (780-1060)	97	11.7	17.4	5.0	A	YES3-3A/3B, 5Rx1, Gpa2-2, R1
5.18-24^{hp}	1253 (1010-1470)	94	14.7	20.4	6.0	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, RT-R3a
5.18-25	860 (620-910)	99	11.7	17.4	5.5	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, R1
5.18-27	800 (680-865)	91	13.4	19.1	6.7	A	Нет данных
5.18-32	630 (520-700)	86	14.4	20.1	6.0	A	YES3-3A/3B, 5Rx1, Gpa2-2, R1
5.18-34^{hp}	1340 (1100-1480)	94	15.7	21.5	5.5	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2,
Eight hybrids from crossing Gusar × Alyi Parus							
6.18-2	685 (583-770)	99	14.2	20.0	7.5	A	5Rx1, N195, 57R, Gpa2-2, R1, RT-R3a
6.18-9	735 (615-842)	95	15.2	21.0	6.5	A	YES3-3A/3B, N195, 57R
6.18-13^{hp}	1340 (1200-1420)	94	12.9	18.6	5.5	A	5Rx1, Gpa2-2
6.18-17	770 (630-865)	97	11.0	16.7	6.5	A	5Rx1, N195, 57R, Gpa2-2
6.18-24^{hp}	1015 (810-1360)	94	11.0	16.7	6.0	A	YES3-3A/3B, N195, 57R, RT-R3a
6.18-26^{hp}	1155 (900-1320)	88	14.2	20.0	6.0	A	YES3-3A/3B, N195, 57R, R1
6.18-36^{hp}	870 (820-945)	93	13.4	19.1	7.5	AB	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2,
6.18-42	765 (630-880)	96	14.7	20.5	6.0	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, RT-R3a
Five hybrids from crossing Gusar × Sirenevyi tuman							
7.18-3^{hp}	1350 (1210-1545)	96	11.0	16.8	5.5	A	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, RT-R3a
7.18-4	780 (660-863)	97	12.9	18.6	6.5	AB	YES3-3A/3B, R1, RT-R3a
7.18-42^{hp}	1010 (820-1220)	92	12.2	18.0	6.0	A	5Rx1, N195, 57R
7.18-55^{hp}	1255 (960-1320)	96	13.4	19.1	8.0	B	N195, 57R, R1, RT-R3a
7.18-56^{hp}	1280 (1000-1380)	91	12.2	18.0	7.0	AB	YES3-3A/3B, 5Rx1, Gpa2-2, R1, RT-R3a

Note. Cv. Nevskii (st) is a standard in assessing agronomic characteristics in the Belogorka Leningrad Research Institute of Agriculture. All markers of resistance genes were detected in hybrid 5.18-17; hp — 12 high-productive hybrid genotypes (for their additional characteristics, see Table 4).

4. Economically valuable traits of 12 high-productive hybrid genotypes (n = 8, (an experimental field of Belogorka Leningrad Research Institute of Agriculture, Leningrad Province, 2019)

Variety, hybrid	DNA markers of *R*-genes	Tuber skin color	Tuber shape	Tuber eye depth and color	Evenness, points from 9 to 1)	
					nests	tubers in nest
Невский (st)	RT-R3a	White	Ovate	Small, малиновые	8	8
5.18-7	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2	White	Ovate	Small	7	8
5.18-19	YES3-3A/3B, 5Rx1, Gpa2-2, R1	White	Elongated	Small	7	7
5.18-24	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, RT-R3a	Yellow	Ovate	Small	8	8
5.18-34	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2,	Yellow	Ovate	Small	8	8
6.18-13	5Rx1, Gpa2-2	Pink	Elongated oval	Small, crimson	8	8
6.18-24	YES3-3A/3B, N195, 57R, RT-R3a	White	Elongated oval	Small	8	8
6.18-26	YES3-3A/3B, N195, 57R, R1	Pink	Ovate	Small, crimson	8	8
6.18-36	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2,	White	Ovate	Small	7	8
7.18-3	YES3-3A/3B, 5Rx1, N195, 57R, Gpa2-2, RT-R3a	White	Rounded oval	Small	8	8
7.18-42	5Rx1, N195, 57R	Pink	Ovate	Small, crimson	8	8
7.18-55	N195, 57R, R1, RT-R3a	Pink	Ovate	Small	8	8
7.18-56	YES3-3A/3B, 5Rx1, Gpa2-2, R1, RT-R3a	Yellow	Elongated	Small	8	7

N o t e. Cv. Nevskii (st) is a standard in assessing agronomic characteristics in the Belogorka Leningrad Research Institute of Agriculture.

to the conclusion that duplexes of genes for resistance to quarantine objects are most often detected. In addition, in the cultivars Alyi Parus and Charoit, duplexes were found for the *Rx1* gene which is closely linked to the *Gpa2-2* gene [44]. Therefore, in the hybrid combinations with the participation of these parental cultivars, there was a joint inheritance of the DNA markers of genes for resistance to PPN and to PVX.

Thus, traditional methods of breeding hybrids for economically valuable traits coupled with DNA marking allow us to reveal 12 high-productive hybrid genotypes with tubers even and regular in shape and markers of genes for resistance to cyst forming nematodes. Seven of these hybrids additionally have SCAR markers of genes for resistance to potato viruses Y and X (Ry_{sto} and *Rx1*). These hybrids are of interest for creating competitive varieties resistant to different groups of pests and pathogens, including group resistance to cyst nematodes and group resistance to potato viruses.

REFERENCES

1. Zykin V.A. Sistemnyi analiz problemy podbora par dlya gibridizatsii. In: *Selektsiya i semenovodstvo sel'skokhozyaistvennykh kul'tur v Zapadnoi Sibiri* [Selection and seed production of agricultural crops in Western Siberia]. Novosibirsk, 1984: 3-12 (in Russ.).
2. Syukov V.V. *Metody podbora roditel'skikh par dlya gibridizatsii u samoopylyayushchikhsya rastenii* [Methods for the selection of parental pairs for hybridization in self-pollinating plants]. Samara, 2014 (in Russ.).
3. Lepekhov S.B. *Trudy po prikladnoi botanike, genetike i selektsii*, 2017, 178(4): 76-89 (doi: 10.30901/2227-8834-2017-4-76-89) (in Russ.).
4. Simko I., Jansky S., Stephenson S., Spooner D. Genetics of resistance to pests and disease. In: *Potato biology and biotechnology: advances and perspectives.* D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, M. Taylor, D. MacKerron, H. Ross (eds.). Elsevier, St. Louis, MO, 2007: 117-155.
5. Gebhardt C. Bridging the gap between genome analysis and precision breeding in potato. *Trends Genet.,* 2013, 29(4): 248-256 (doi: 10.1016/j.tig.2012.11.006).
6. Milczarek D., Przetakiewicz A., Kamiński P., Flis B. Early selection of potato clones with the *H1* resistance gene — the relation of nematode resistance to quality characteristics. *Czech J. Genet. Plant Breed.*, 2014, 50(4): 278-284 (doi: 10.17221/114/2014-CJGPB).
7. Ottoman R.J., Hane D.C., Brown C.R., Yilma S., James S.R., Mosley A.R., James M.C., Vales M.I. Validation and implementation of marker-assisted selection (MAS) for PVY resistance (Ry_{adg} gene) in a tetraploid potato breeding program. *American Journal of Potato Research*, 2009, 86: 304-314 (doi: 10.1007/s12230-009-9084-0).
8. Nie X., Chen H., Zhang J., Zhang Y., Yang J., Pan H., Song W.X., Murad F., He Y.Q. Bian K. Rutaecarpine ameliorates hyperlipidemia and hyperglycemia in fat-fed, streptozotocin-treated rats via regulating the IRS-1/PI3K/Akt and AMPK/ACC2 signaling pathways. *Acta Pharmacologica Sinica*, 2016, 37(4): 483-496 (doi: 10.1038/aps.2015.167).
9. Gebhardt C., Bellin D., Henselewski H., Lehmann W., Schwarzfischer J., Valkonen J.P.T. Marker-assisted combination of major genes for pathogen resistance in potato. *Theoretical and Applied Genetics*, 2006, 112: 1458-1464 (doi: 10.1007/s00122-006-0248-8).
10. Mori K., Sakamoto Y., Mukojima N., Tamiya S., Nakao T., Ishii T., Hosaka K. Development of a multiplex PCR method for simultaneous detection of diagnostic DNA markers of five disease and pest resistance genes in potato. *Euphytica*, 2011, 180: 347-355 (doi: 10.1007/s10681-011-0381-6).
11. Mori K., Asano K., Tamiya S., Nakao T., Mori M. Challenges of breeding potato cultivars to grow in various environments and to meet different demands. *Breeding Science*, 2015, 65: 3-16 (doi: 10.1270/jsbbs.65.3).
12. Asano K., Tamiya S. Breeding of pest and disease resistant potato cultivars in Japan by using classical and molecular approaches. *The Japan Agricultural Research Quarterly*, 2016, 50(1): 1-6 (doi: 10.6090/jarq.50.1).
13. Milczarek D., Plich J., Tatarowska B., Flis B. Early selection of potato clones with resistance genes: the relationship between combined resistance and agronomical characteristics. *Breeding Science,* 2017, 67: 416-420 (doi: 10.1270/jsbbs.17035).
14. Zoteeva N.M., Antonova O.Yu., Klimenko N.S., Apalikova O.V., Carlson-Nilsson U., Karabitsina Yu.I., Ukhatova Yu.V., Gavrilenko T.A. Facilitation of introgressive hybridization of wild polyploid mexican potato species using DNA markers of *r* genes and of different cytoplasmic

types. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(5): 964-975 (doi: 10.15389/agrobiology.2017.5.964eng).
15. Sainakova A.B., Romanova M.S., Krasnikov S.N., Litvinchuk O.V., Alekseev Ya.I., Nikulin A.V., Terent'eva E.V. *Vavilovskii zhurnal genetiki i selektsii*, 2018, 22(1): 18-24 (doi: 10.18699/VJ18.326) (in Russ.).
16. Rogozina E.V., Terent'eva E.V., Potokina E.K., Yurkina E.N., Nikulin A.V., Alekseev Ya.I. Multiplex PCR-based identification of potato genotypes as donors in breeding for resistance to diseases and pests. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(1): 19-30 (doi: 10.15389/agrobiology.2019.1.19eng).
17. Bradshaw J.E., Mackay G.R. Breeding strategies for clonally propagated potatoes. In: *Potato genetics*. J.E. Bradshaw, G.R. Mackay (eds.). CABI, Wallingford (UK), 1994: 467-497.
18. Wu R., Gallo-Meagher M., Littell R.C., Zeng Z.-B. A general polyploid model for analyzing gene segregation in outcrossing tetraploid species. *Genetics*, 2001, 159(2): 869-882.
19. Skupinova S., Vejl P., Sedlak P., Domkarova J. Segregation of DNA markers of potato (*Solanum tuberosum* ssp. *tuberosum* L.) resistance against Ro1 pathotype *Globodera rostochiensis* in selected F1 progeny. *Rostlinna vyroba*, 2002, 48(11): 480-485.
20. Ermishin A.P., Svitoch O.V., Voronkova E.V., Gukasyan O.N., Luksha V.I. Opredelenie sostava i allel'nogo sostoyaniya genov ustoichivosti k boleznyam i vreditelyam u roditel'skikh linii kartofelya s pomoshch'yu DNK markerov. *Genetika*, 2016, 52(5): 569-578 (doi: 10.7868/S0016675816050052).
21. Kneib R., Kneib R., da Silva Pereira A., Castro C.M. Allele dosage of PVY resistance genes in potato clones using molecular markers. *Crop Breeding and Applied Biotechnology*, 2017, 17: 306-312 (doi: 10.1590/1984-70332017v17n4a47).
22. Lebedeva N.A. *Trudy MOIP, otd. biol.*, 1962, 5: 215-221 (in Russ.).
23. Lebedeva N.A. *Tezisy dokladov 2-go soveshchaniya po poliploidii* [Theses of reports of the 2nd Meeting on polyploidy]. Leningrad, 1963: 28-29 (in Russ.).
24. Lebedeva N.A. *Kartofel' i ovoshchi*, 1965, 4: 20-24 (in Russ.).
25. Antonova O.Yu., Shvachko N.A., Novikova L.Yu., Shuvalov O.Yu., Kostina L.I., Klimenko N.S., Shuvalova A.R., Gavrilenko T.A. *Vavilovskii zhurnal genetiki i selektsii*, 2016, 20(5): 596-606 (doi: 10.18699/VJ16.181) (in Russ.).
26. Gavrilenko T.A., Klimenko N.S., Antonova O.Yu., Lebedeva V.A., Evdokimova Z.Z., Gadzhiev N.M., Apalikova O.V., Alpat'eva N.V., Kostina L.I., Zoteeva N.M., Mamadbokirova F.T., Egorova K.V. *Vavilovskii zhurnal genetiki i selektsii*, 2018, 22(1): 35-45 (doi: 10.18699/VJ18.329) (in Russ.).
27. Khyutti A.V., Antonova O.Yu., Mironenko N.V., Gavrilenko T.A., Afanasenko O.S. *Vavilovskii zhurnal genetiki i selektsii*, 2017, 21(1): 51-61 (doi: 10.18699/VJ17.223) (in Russ.).
28. Gavrilenko T.A., Klimenko N.S., Alpat'eva N.V., Kostina L.I., Lebedeva V.A., Evdokimova Z.Z., Apalikova O.V., Novikova L.Yu., Antonova O.Yu. *Vavilovskii zhurnal genetiki i selektsii*, 2019, 23(6): 753-764 (doi: 10.18699/VJ19.534) (in Russ.).
29. Lebedeva V.A. *Selektsiya kartofelya na osnove mezhvidovoi gibridizatsii* [Potato breeding based on interspecific hybridization]. St. Petersburg, 2010 (in Russ.).
30. Lebedeva V.A., Gadzhiev N.M. *Materialy Mezhdunarodnogo kongressa «Agrorus»* [Proc. Int. Congress «Agrorus»]. St. Petersburg, 2014: 19-20 (in Russ.).
31. Gadzhiev N.M., Lebedeva V.A. *Zashchita kartofelya*, 2015, 2: 16 (in Russ.).
32. Gavrilenko T., Antonova O., Shuvalova A., Krylova E., Alpatyeva N., Spooner D.M., Novikova L. Genetic diversity and origin of cultivated potatoes based on plastid microsatellite polymorphism. *Genetic Resources and Crop Evolution*, 2013, 60(7): 1997-2015 (doi: 10.1007/s10722-013-9968-1).
33. Ahmadvand R., Wolf I., Gorji A.M., Polgár Z., Taller J. Development of molecular tools for distinguishing between the highly similar *Rx1* and *Rx2* PVX extreme resistance genes in tetraploid potato. *Potato Research*, 2013, 56(4): 277-291 (doi: 10.1007/s11540-013-9244-y).
34. Song Y.-S., Schwarzfischer A. Development of STS markers for selection of extreme resistance (*Ry*$_{sto}$) to PVY and maternal pedigree analysis of extremely resistant cultivars. *American Journal of Potato Research*, 2008, 85(2): 159-170 (doi: 10.1007/s12230-008-9012-8).
35. Schultz L., Cogan N.O.I., Mclean K., Dale M.F.B., Bryan G.J., Forster J.N.W., Slater A.T. Evaluation and implementation of a potential diagnostic molecular marker for *H1*-conferred potato cyst nematode resistance in potato. *Plant Breeding*, 2012, 131: 315-321 (doi: 10.1111/j.1439-0523.2012.01949.x).
36. Asano K., Kobayashi A., Tsuda S., Nishinaka M., Tamiya S. DNA marker-assisted evaluation of potato genotypes for potential resistance to potato cyst nematode pathotypes not yet invading into Japan. *Breeding Science*, 2012, 62(2): 142-150 (doi: 10.1270/jsbbs.62.142).
37. Ballvora A., Ercolano M.R., Weiss J., Meksem K., Bormann C.A., Oberhagemann P., Salamini F., Gebhardt C. The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *The Plant Journal*, 2002, 30(3): 361-371 (doi: 10.1046/j.1365-313X.2001.01292.x).

38. Huang S., van der Vossen E.A.G., Kuang H., Vleeshouwers V.G., Zhang N., Borm T.J.A., van Eck H.J., Baker B., Jacobsen E., Visser R.G.F. Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. *The Plant Journal*, 2005, 42(2): 251-261 (doi: 10.1111/j.1365-313X.2005.02365.x).
39. Banadysev S.A., Starovoitov A.M., Kolyadko I.I., Makhan'ko V.L., Fando V.V., Kozlova L.I., Kolyadko O.M., Nezakonova L.V., Goncharova N.N., Vologdina L.N., Stadnikov I.A., Gribko A.P. *Metodicheskie rekomendatsii po spetsializirovannoi otsenke sortov kartofelya* [Methodical recommendations for specialized assessment of potato varieties]. Minsk, 2003 (in Russ.).
40. Simakov E.A., Sklyarova N.P., Yashina I.M. *Metodicheskie ukazaniya po tekhnologii selektsionnogo protsessa kartofelya* [Methodical instructions on the technology of the potato breeding]. Moscow, 2006 (in Russ.).
41. Simakov E.A. Anisimov B.V., Shabanov A.E., Zebrin S.N., Yurlova S.M., Oves E.V., Zeiruk V.N., Uskov A.I., Fedotova L.S., Filippova G.I. *Metodicheskie polozheniya po provedeniyu otsenki sortov kartofelya na ispytatel'nykh (testovykh) uchastkakh* [Methodological provisions for the assessment of potato varieties at test sites]. Moscow, 2013 (in Russ.).
42. Rokitskii P.F. *Biologicheskaya statistika* [Biostatistics]. Moscow, 1973 (in Russ.).
43. Dalamu V.B., Umamaheshwari R., Sharma R., Kaushik S., Joseph T., Singh B., Gebhardt C. Potato cyst nematode (PCN) resistance: genes, genotypes and markers. *SABRAO Journal of Breeding and Genetics*, 2012, 44(2): 202-228.
44. Van der Vossen E.A.G., van der Voort J.R., Kanyuka K., Bendahmane A., Sandbrink H., Baulcombe D.C., Bakker J., Stiekema W.J., Klein-Lankhorst R.M. Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. *The Plant Juornal*, 2000, 23(5): 567-576 (doi: 10.1046/j.1365-313x.2000.00814.x).

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COMPOSITION AND QUANTIFICATION OF ANTHOCYANINS IN HEALTHY-DIET POTATO (*Solanum tuberosum* L.) VARIETIES FOR BREEDING AND GROWING IN THE RUSSIAN FAR EAST

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Abstract

Potato (*Solanum tuberosum* L.) has been the focus of dietary research in recent decades due to its ability to accumulate phenolic substances (anthocyanins) in tubers. In Russia, such varieties have been created for a number of regions, but in the Far East, the program to increase the content of anthocyanins in potato tubers has recently begun. In the presented study using liquid chromatography and second-order mass spectrometry methods, we profiled anthocyanins and quantified their accumulation for potato varieties of different origin (Russia, Ukraine, Belarus, Kazakhstan, Germany, the Netherlands), which had not previously been characterized by this feature. For the first time, four dietary Russian varieties with an increased content of anthocyanins have been identified. This work aimed to determine the profiles of anthocyanins and their content in tubers in the conditions of the Russian Far East. We used 22 varieties selected for early maturity, productivity, low starch content and different colors of tubers and flowers. The anthocyanin profile was double-identified, by the retention time on a C18 reverse phase column with detection of absorbance at wavelength range 400-700 nm and ESI-MS/MS in positive ionization mode. Anthocyanins were quantified spectrophotometrically. The anthocyanins identified in the tubers were delphinidin, petunidin, malvidin, cyanidin, and pelargonidin. Delphinidin and cyanidin were found in mono- and diglycosylated forms. It was revealed that petunidin-3-glucoside is the most common anthocyanin which is present in almost all of the studied varieties. Depending on varietal specificity, there were from 1 to 5 anthocyanins of those found. Varieties with yellow skin and pink spots on the yellow tuber skin were characterized by a low content of anthocyanins. The pink and dark pink color of the skin positively correlates with the content of pelargonidin-3-glucoside, while petunidin-3-glucoside and cyanidin-3-glucoside give purple and blue-violet color to the skin of the tuber. The anthocyanin level was the highest in Phioletovii variety (310 mg/kg cyanidin-3-glucoside, 50 mg/kg malvidin, 30 mg/kg delphinidin), Vasilyok variety (150 mg/kg petunidin), and in Mayak (95 mg/kg pelargonidin) and Kuznechanka (78 mg/kg pelargonidin) varieties. In the Phioletovi variety, the cyanidin-3-glucoside prevailed. We recommend to involve varieties with pink, dark pink, purple and blue-purple tuber skin in selection for an increased content of anthocyanins.

Keywords: *Solanum tuberosum* L., potatoes, dietary varieties, anthocyanins, delphinidin, petunidin, malvidin, cyanidin, pelargonidin, mass spectrometry, HPLC

Potato (*Solanum tuberosum* L., *Solanaceae*) is the fourth most important food crop after wheat, rice, and corn, and the first among tuber and root crops in terms of grown areas [1]. The nutritional value of potatoes is largely due to the high content of carbohydrates, a significant amount of ascorbic acid and anthocyanins, highly digestible proteins, as well as potassium, calcium, and magnesium

salts [2].

Anthocyanin pigments are synthesized in the skin and flesh of potato tubers. In addition, flowers, leaves, stems, and eyes can be colored. In the potato as a food crop, the content of anthocyanins in the edible parts, i.e., in the flesh of tubers, should be increased [3].

Anthocyanin pigments are used in the food and pharmaceutical industries. These antioxidants are water-soluble natural dyes that color food in different shades of red. They are approved by Russian sanitary rules and regulations for coloring cheeses, wines, soft drinks, canned vegetables, breakfast cereals (up to 200 mg/kg), jams, jellies, and marmalades, and serve as an alternative to carcinogenic azo dye carmoisine [4, 5].

The anthocyanins which possess capillary-strengthening, anti-oxidant, antibacterial and anticarcinogenic properties are used for treating and preventing various diseases, for example, type II diabetes and some types of tumors [6-9].

Development and application of DNA markers for target genes involved in anthocyanin biosynthesis facilitate breeding for red and purple potato varieties. It is known that the chalcone synthase (CHS), chalconflavanone isomerase (CHI), dihydroflavonol-4-reductase (DFR), flavonone-3-hydroxylase (F3H), flavonoid-3′-hydroxylase (F3′H), flavonoid-3′,5′-hydroxylase (F3′5′H), and anthocyanidin synthase (ANS) genes are involved in biosynthesis of anthocyanins [10, 11]. *S. tuberosum* also has gene loci with regulatory properties. Locus *D* (developer, designated in the diploid potato *S. rybinii* Juz. & Bukasov as *I*, the inhibitor), located on chromosome 10, encodes the R2R3 MYB transcription factor (TF), which is highly similar to the product of the previously detected petunias (*Petunia hybrid*) gene PhAN2 [12]. In plants, the R2R3 MYB gene family is the largest group of TF genes which play an important role in the biosynthesis of anthocyanins [13-15].

Data on the genes controlling anthocyanin pigmentation in *S. tuberosum* tubers and flowers facilitate combination of crossing pairs to select hybrids with colored flowers, stems, leaves, and tubers [16-18].

Pigmented *S. tuberosum* cultivars are rich in anthocyanins, in particular, their acylated derivatives [19]. Potato genotypes differ greatly in the content of bioactive substances, including anthocyanins. Pigmented varieties have 1.5-2.5 times higher phenolic activity, 2-3 times higher antioxidant capacity and accumulate higher levels of anthocyanins than non-pigmented genotypes. Anthocyanins were not found in unpigmented potatoes [20].

In the USA, dietary potato breeding has been carried out since the early 2000s. As a result, red and purple potato varieties with a high level of carotenoids and anthocyanins have been produced [4]. In Russia, breeding potato varieties for dietary nutrition is also being in focus. The varieties that have the highest pigmentation index and a 5-fold content of antioxidants compared to white potatoes have been identified.

The program of potato breeding for high anthocyanin content has been performed in the Far East since 2018. Varieties of various origins from Russia, the Netherlands, Germany, Kazakhstan, Ukraine, and Belarus have been assessed to reveal donors of commercial and dietary traits (high yield, red and purple tuber skin, red-violet color of the corolla), which were further crossed [22]. To proceed, it is necessary to study the dietary antioxidant properties of the varieties, the composition and content of anthocyanins in tubers and the relationship between these traits and anthocyanin coloration of other organs, the skin and corolla.

Here, we applied high-performance liquid chromatography (HPLC) and second-order mass spectrometry to identify and quantify anthocyanins in 22 potato varieties of various origins that had not previously been characterized by this trait. Four varieties of Russian origin rich in anthocyanins have been revealed and

recommended for dietary use.

This work aimed to profile and quantify anthocyanins in tubers of dietary potato varieties promising for growing and breeding in the Russian Far East.

Materials and methods. The study included varieties of various origins from the VIR World Collection (the Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg), a collection of the Lorkh All-Russian Research Institute of Potato Farming (Moscow Province), and those bred at the Chaika Federal Research Center of Agricultural Biotechnology of the Far East (FSC ABFE). A total of 22 varieties selected for early maturity (440-650 g tubers per plant on days 60 and 70 after planting) and the tuber color, the Bashkirsky, Vasilek, Dachny, Kazachok, Krepysh, Kuznechanka, Matushka, Mayak, Meteor, Ognivo, Olsky, Pamyati Kulakova, Sarma, Sirenevyi tuman, Fioletovii, Yantar (Russia), Sante (the Netherlands), Vitesse (Germany), Tamyr (Kazakhstan), Povin, Shchedrik (Ukraine), and Manifest (Belarus) were tested at a collection nursery (FSC ABFE, 2016-2018).

The composition and accumulation of anthocyanins were analyzed following a method described by Lewis et al. [20]. Anthocyanins was measured in the tuber flesh together with the skin. The potatoes were collected and stored until the analysis (no more than 2 weeks) in a cool place without direct sunlight. The tubers were rinsed in cold water, weighed, crushed, and placed in a 40% ethanol + 1% formic acid (5 g of the biomass + 25 ml of the resulting solution) mixture, frozen, thawed, disintegrated ultrasonically to destroy the walls and membranes of cells and organelles. Anthocyanins were extracted for 90 min at 40 °C in closed vessels to prevent the access of atmospheric oxygen. The extract was centrifuged (CM-6M, Elmi, Latvia) at 3500 g for 30 min, the supernatant was filtered through syringe filters (pore size 0.45 μm). The extract was stored in a freezer at –20 °C.

Anthocyanins in the extracts were separated and quantified using HPLC analysis (a 5 μl aliquot of each extract, a liquid chromatograph LC-20AD equipped with a high-pressure gradient pump and a CTO-20A column thermostat, Shimadzu, Japan; a Shodex C18-4E reverse phase column 250×4.6 mm I.D., 5 μm sorbent particles, Shodex, Japan; column temperature 50 °C; the rate of mobile phase A:B 0.58 ml/min). Eluent A was acetonitrile (AppliChem GmbH, Germany), eluent B was 1% formic acid solution (Sigma-Aldrich, USA). During gradient elution, B concentration decreased from 100% to 92% (0.00-5.00 min), from 92% to 80% (5.00-45.00 min), and from 80% to 10% (45.00-45.01 min). Peaks were detected at 300-600 nm UV/VIS (a spectrophotometric detector SPD-20A, Shimadzu, Japan). The analysis was performed in 66 biological and 3 analytical replicates. For cultivars Phioletovii and Vasilek, the absorption coefficient of malvidin-3-glucoside (3.02×10^4 at 300-600 nm, a molecular weight of 493.3 g/mol) was used to recalculate the content of anthocyanins, for other varieties, the molar extinction coefficient of pelargonidin-3-glucoside was applied (2.73×10^4 at 300-600 nm, molecular weight of 433.3 g/mol).

Anthocyanins purified by HPLC were identified by direct-injection second-order mass spectrometry method using an amaZon SL trap (Bruker, Germany) equipped with an electrospray ionization source. Detection was carried out in the modes of positive and negative ions, with a mass scanning range from 150 to 2200 u, maximum scanning speed 32000 Da/s, the nebulizing capillary voltage 4500 V, nebulizer pressure 29 psi, dry gas flow 10 l/s, and capillary temperature 180 °C. Ions were fragmented using a 1.5 eV electron beam. The analysis was arranged in 66 biological and 3 analytical replicates.

MS Excel 2007 and Statistica 8 (StatSoft, Inc., USA) were used for data processing, the mean (M) and $t_{0,05}\times$SEM were calculated.

Results. The tested varieties were low-starchy (8.0-12.0%) and had pink or

purple tuber skin. The low-starchy varieties are considered dietary [23].

Anthocyanin coloration of different parts of plants is an important trait that can be effectively used in breeding for a higher level of phenolic pigments [5]. The studied varieties differed in morphological traits (Table 1).

1. Morphological traits of potato (*Solanum tuberosum* L.) varieties involved in the study (a collection nursery, Chaika Federal Research Center of Agricultural Biotechnology of the Far East, 2016-2017)

Varieties	Color		
	tuber flesh	corolla	tuber skin
Sante, Kazachok, Vitesse, Sarma, Meteor	Yellow	White	Yellow
Krepysh	White	Pale red-violet with white brush-stroke markings	
Dachnyi, Shchedrik	White	White	
Yantar	Bright yellow	White	
Tamyr	Light yellow	White	
Olskii	Cream	White	Yellow pink-spotted
Pamyati Kulalova	White	White	
Kuznechanka, Bashkirsky	White	Pale red-violet	Pink
Sirenevyi tuman	Light yellow	Pale red-violet with white brush-stroke markings	
Povin	Yellow	Pale red-violet	
Matushka	Cream	White	
Ognivo	Cream	Red-violet with white brush-stroke markings	
Mayak	Cream	Pale red-violet	Dark pink
Manifest	Cream	Red-violet with white brush-stroke markings	
Vasilek	Cream	Blue-violet with white brush-stroke markings	Violet
Fioletovii	Violet	Blue-violet with white brush-stroke markings	Blue-violet

Five varieties (Kazachok, Meteor, Sarma, Sante, and Vitesse) have white-colored corolla, but yellow skin and flesh. In other varieties, the pigmentation (yellow, shades of red-violet, violet, pink, etc.) of plant parts varied. The morphological features in our studies (the color of the generative organs and the skin of the tubers during the flowering period) correspond to the descriptions [24].

The HPLC-MS/MS detected five different anthocyanins in tubers of the examined potato varieties (Table 2) identified as delphinidin, petunidin, malvidin, cyanidin, and pelargonidin. The biochemical nature of anthocyanins in *S. tuberosum* is the same as in cultured diploid species [25] which anthocyanins include pelargonidin, peonidin, petunidin, and malvidin.

2. HPLC-MS/MS tuber anthocyanin profiles of potato (*Solanum tuberosum* L.) varieties involved in the study (a collection nursery, Chaika Federal Research Center of Agricultural Biotechnology of the Far East, 2018)

Anthocyanin	Molecular ion $[M+H]^+$	HPLC retention time, min	Variety
Delphinidin-3-glucoside	465.3; 303.2	25	Mayak, Povin, Kuznechanka, Manifest, Fioletovii
Delphinidin-3-rhamnosyl-5-glucoside	627.3; 465.3; 303.2	17.5	Mayak, Povin, Kuznechanka, Vasilek, Manifest
Malvidin-3-glucoside	493.3; 331.3	44	Fioletovii
Pelargonidin-3-glucoside	433.3; 271.1	37.5	Mayak, Povin, Kuznechanka, Fioletovii, Vasilek
Petunidin-3-glucoside	479.3; 317.2	35	Vasilek, Manifest, Matushka, Povin, Mayak, Sirenevyi tuman, Pamyati Kulalova, Olskii
Cyanidin-3-glucoside	449.2; 287.2	27.5	Fioletovii
Cyanidin-3-rhamnosyl-5-glucoside	611.3; 499.3; 287.2	19	Matushka, Vasilek, Fioletovii, Povin

The anthocyanins dolphinin and cyanidin were found in both monoglycosylated and diglycosylated forms. The most common anthocyanins were petunidin-3-glucoside and pelargonidin-3-glucoside. They were identified in almost all the samples (Table 3).

Research publications recognize petunidin and pelargonidin as the main anthocyanins in potatoes. Petunidin causes purple color, and pelargonidin causes red-orange color [20, 26]. Pelargonidin-3-glucoside prevailed in the varieties with pink and dark pink tubers, and petunidin-3-glucoside prevailed in violet and blue-violet varieties. In the yellow-skinned varieties anthocyanin compounds were either not detected or detected in an insignificant amount.

3. Concentration of anthocyanins (mg/kg) **in tubers of potato (*Solanum tuberosum* L.) varieties involved in the study** (n = 3, $M \pm t_{0,05} \times$SEM, a collection nursery, Chaika Federal Research Center of Agricultural Biotechnology of the Far East, 2018)

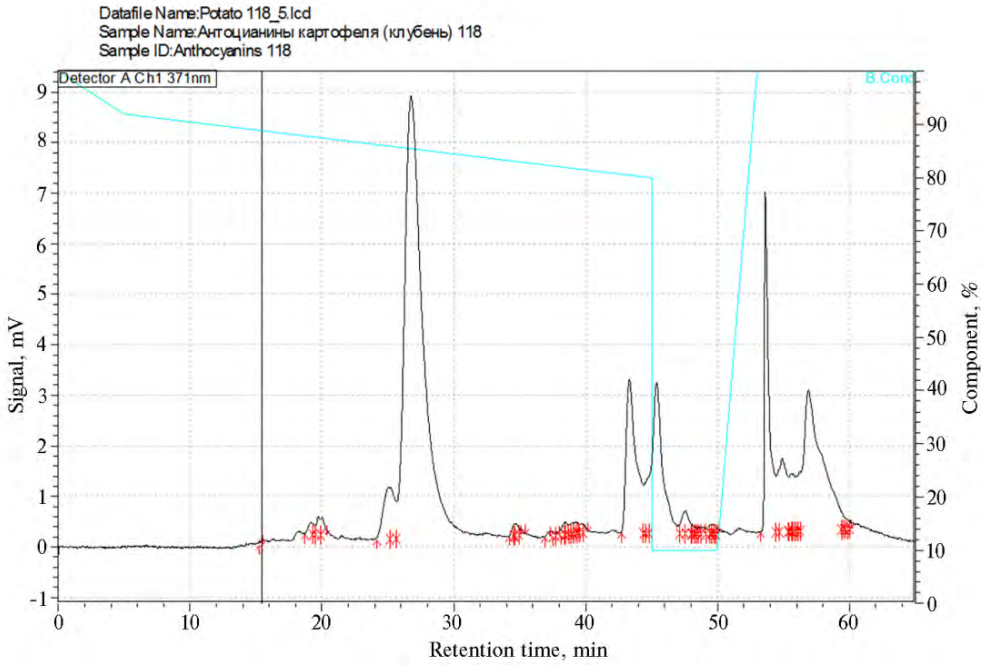
Variety	1	2	3	4	5	6	7
Yellow-skinned tubers							
Sante	< 0.5	< 0,5	1,0±0,1	< 0,5	< 0,5	< 0,5	< 0,5
Kazachok	< 0.5	< 0,5	< 0,5	< 0,5	2,1±0,1	< 0,5	< 0,5
Vitessa	< 0.5	< 0,5	< 0,5	0,9±0,1	< 0,5	< 0,5	< 0,5
Sarma	< 0.5	< 0,5	< 0,5	2,2±0,1	< 0,5	< 0,5	< 0,5
Meteor	< 0.5	< 0,5	1,1±0,1	1,8±0,1	< 0,5	< 0,5	< 0,5
Dachnyi	< 0.5	< 0,5	1,0±0,1	< 0,5	< 0,5	< 0,5	< 0,5
Shchedrik	< 0.5	< 0,5	< 0,5	< 0,5	< 0,5	< 0,5	< 0,5
Yantar	< 0.5	< 0,5	< 0,5	< 0,5	1,8±0,1	< 0,5	< 0,5
Tamyr	< 0.5	< 0,5	< 0,5	< 0,5	2,0±0,1	< 0,5	< 0,5
Krepysh	< 0.5	< 0,5	< 0,5	1,2±0,1	< 0,5	< 0,5	< 0,5
Yellow pink-spotted tuber skin							
Olskii	< 0.5	< 0.5	< 0.5	5.3±0.1	< 0.5	< 0.5	< 0.5
Pamyati Kulalova	< 0.5	< 0.5	< 0.5	3.2±0.1	< 0.5	< 0.5	< 0.5
Pink-skinned tubers							
Kuznechanka	4.1±0.1	9.7±0.1	< 0.5	78.4±0.3	< 0.5	< 0.5	< 0.5
Bashkirsky	0.9±0.1	3.1±0.1	< 0.5	24.8±0.2	2.3±0.1	< 0.5	< 0.5
Sirenevyi tuman	2.7±0.1	< 0.5	12.1±0.1	5.0±0.1	< 0.5	< 0.5	< 0.5
Povin	3.0±0.1	7.3±0.1	<0.5	24.8±0.2	5.0±0.1	< 0.5	10.2±0.1
Matushka	< 0.5	1.1±0.1	<0.5	22.2±0.2	0.7±0.1	< 0.5	< 0.5
Ognivo	1.3±0.1	< 0.5	<0.5	1.9±0.1	< 0.5	< 0.5	< 0.5
Dark pink-skinned tubers							
Mayak	2.1±0.1	2.1±0.1	<0.5	94.6±0.3	9.9±0.1	< 0.5	< 0.5
Manifest	1.0±0.1	1.2±0.1	<0.5	20.1±0.2	1.1±0.1	< 0.5	< 0.5
Violet-skinned tubers							
Vasilek	< 0.5	5.0±0.1	<0.5	38.2±0.2	149.8±0.4	< 0.5	2.0±0.1
Blue-violet-skinned tubers							
Fioletovyi	30.4±0.2	< 0.5	50.1±0.2	< 0.5	5.1±0.1	310.0±0.4	8.4±0.1

note. 1 — delphinidin-3-glucoside, 2 — delphinidin-3-rhamnosyl-5-glucoside, 3 — malvidin-3-glucoside, 4 — pelargonidin-3-glucoside, 5 — petunidin-3-glucoside, 6 — cyanidin-3-glucoside, 7 — cyanidin-3-rhamnosyl-5-glucoside.

The highest anthocyanin levels were characteristic of the varieties Fioletovyi, Vasilek, Mayak, and Kuznechanka. The variety Fioletovyi with blue-violet potato skin and flesh was distinguished by a significant content of four anthocyanins, the cyanidin-3-glucoside (310 mg/kg), malvidin-3-glucoside (50 mg/kg), delphinidin-3-glucoside (30 mg/kg), and cyanidin-3-rhamnosyl-5-glucoside (8 mg/kg). The varieties Mayak and Kuznechanka have pink and dark pink tubers with the highest pelargonidin concentration, 95 and 78 mg/kg, respectively. The variety Vasilek with purple-skinned tubers outstands due to the highest petunidin-3-glucoside content (150 mg/kg). These varieties can be used as a functional food product with high antioxidant properties.

Cyanidin-3-glucoside was found to be the main in the tuber anthocyanin profile of the Fioletovyi variety (Fig.). The most intense signal at 371 nm (retention time 27.5 min) corresponds to the main anthocyanin of this variety, the cyanidine-3-glucoside; in addition, there are significant amounts of malvidin-3-glucoside (peak at 44 min) and dolphinin-3-glucoside (small peak at 25 min). The elution profiles revealed a relationship between the content of specific anthocyanins and the color of tubers, as well as their varietal specificity. Available

publications confirm that the composition of anthocyanins, as a rule, is specific for a particular plant species and is quite stable. However, it depends on the varietal characteristics and growing conditions, which determine the activity of the enzymes involved in synthesis of certain components of the anthocyanin complex [19, 27].



Elution profile of the anthocyanins from **tubers of potato (*Solanum tuberosum* L.) variety Fioloetovyi** (n = 3, a collection nursery, Chaika Federal Research Center of Agricultural Biotechnology of the Far East, 2018). HPLC analysis, a Shodex C18-4E reverse phase column 250×4.6 mm I.D., 5 μm sorbent particles, Shodex, Japan. Eluent A is acetonitrile (AppliChem GmbH, Germany), eluent B is 1% formic acid (Sigma-Aldrich, USA), 0.00-5.00 min gradient (see *Materials and methods*). Peaks at 27.5 min, 44 min, and 25 min are cyanidin-3-glucoside, malvidin-3-glucoside, and delphinidin-3-glucoside, respectively.

Varieties with tuber skin of different shades of purple and pink were distinguished by the tuber anthocyanin concentration compared to varieties with yellow tubers. Therefore, the color of the tuber skin (pink, dark pink, blue-violet, purple) can serve as a visual trait in breeding dietary varieties rich in anthocyanins.

So, a second-order mass spectrometry identified anthocyanins delphinidin, petunidin, malvidin, cyanidin, and pelargonidin in the studied potato varieties. The most common petunidin-3-glucoside was found in almost all the samples. The highest content of anthocyanins was characteristic of the varieties Fioletovyi, Vasilek, Mayak, and Kuznechanka. The Fioletovyi variety with blue-violet tuber skin and flesh outstands for a significant content of four anthocyanins, the delphinidin-3-glucoside (30 mg/kg), malvidin-3-glucoside (50 mg/kg), cyanidin-3-glucoside (310 mg/kg), and cyanidin-3-rhamnosyl-5-glucoside (8 mg/kg). Cvs. Mayak and Kuznechanka have pink and dark pink tubers and an increased content of pelargonidin in flesh, 95 and 78 mg/kg, respectively. Variety Vasilek with purple-skinned tubers has the highest petunidin-3-glucoside content, 150 mg/kg. These findings show that the color of the tuber skin can be a trait for selection and breeding varieties with a high level of anthocyanins. Pink- and dark pink-skinned tubers contain pelargonidine-3-glucoside. Petunidin-3-glucoside and cyanidin-3-glucoside give violet and blue-violet color to the skin. The selected

samples of dietary potatoes are now involved in breeding. To this end, we plan to further study the heritability of the desired anthocyanin pigmentation in the produced potato varieties and determine genes responsible for the anthocyanin composition and content of tubers.

REFERENCES

1. Zhevora S.V., Anisimov B.V., Simakov E.A., Oves E.V., Zebrin S.N. *Kartofel' i ovoshchi*, 2019, 7: 2-7 (in Russ.).
2. Ivanisenko T.V., Saik O.V., Demenkov P.S., Khlestkin V.K., Khlestkina E.K., Kolchanov N.A., Ivanisenko V.A. *Vavilovskii zhurnal genetiki i selektsii*, 2018, 22(1): 8-17 (doi: 10.18699/VJ18.325) (in Russ.).
3. Zhang Y., Cheng S., De Jong D., Griffiths H., Halitschke R., De Jong W. The potato *R* locus codes for dihydroflavonol 4-reductase. *Theor. Appl. Genet.*, 2009, 119(5): 931-937 (doi: 10.1007/s00122-009-1100-8).
4. Wegener C.B., Jansen G. Soft-rot resistance of coloured potato cultivars (*Solanum tuberosum* L.): the role of anthocyanins. *Potato Res.*, 2007, 50(1): 31-44 (doi: 10.1007/s11540-007-9027-4).
5. Simakov E.A., Anisimov B.V., Mityushkin A.V., Zhevora S.V., Starovoitov V.I., Oves E.V., Zhuravlev A.A., Meleshin A.A., Zebrin S.N., Zeiruk V.N., Uskov A.I., Zharova V.A., Mityushkin Al.R.V., Shabanov A.E., Mal'tsev S.V., Salyukov S.S., Ovechkin S.V., Semenov V.A., Gaizatulin A.S. *Sorta kartofelya selektsionnogo tsentra VNIIKKH. Potrebitel'skie i stolovye kachestva, kulinarnyi tip* [Potato varieties of the VNIIKH breeding center. Consumer and dining qualities, culinary type]. Moscow, 2016 (in Russ.).
6. Mulinacci N., Ieri F., Giaccherini C., Innocenti M., Andrenelli L., Canova G., Saracchi M., Casiraghi M.C. Effect of cooking on the anthocyanins, phenolic acids, glycoalkaloids, and resistant starch content in two pigmented cultivars of *Solanum tuberosum* L. *J. Agric. Food Chem.*, 2008, 56(24): 11830-11837 (doi: 10.1021/jf801521e).
7. Cassidy A., O'Reilly È.J., Kay C., Sampson L., Franz M., Forman J.P., Curhan G., Rimm E.B. Habitual intake of flavonoid subclasses and incident hypertension in adults. *The American Journal of Clinical Nutrition*, 2011, 93(2): 338-347 (doi: 10.3945/ajcn.110.006783).
8. Hui C., Bi Y., Xiaopin Y., Lon Y., Chunye C., Mantian M., Wenhua L. Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells in vitro and in vivo. *Nutrition and Cancer*, 2010, 62(8): 1128-1136 (doi: 10.1080/01635581.2010.494821).
9. Sancho R.A.S., Pastore G.M. Evaluation of the effects of anthocyanins in type 2 diabetes. *Food Research International*, 2012, 46(1): 378-386 (doi: 10.1016/j.foodres.2011.11.021).
10. Strygina K.V., Khlestkina E.K. Anthocyanins synthesis in potato (*Solanum tuberosum* L.): genetic markers for smart breeding (review). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(1): 37-49 (doi: 10.15389/agrobiology.2017.1.25eng).
11. Zhang Y., Jung C.S., De Jong W.S. Genetic analysis of pigmented tuber flesh in potato. *Theor. Appl. Genet.*, 2009, 119(1): 143-150 (doi: 10.1007/s00122-009-1024-3).
12. Stushnoff C., Ducreux L.J., Hancock R.D., Hedley P.E., Holm D.G., McDougall G.J., McNicol J.W., Morris W. L., Sugurtas J.A., Verrall S.R., Zuber T., Taylor M.A. Flavonoid profiling and transcriptome analysis reveals new gene-metabolite correlations in tubers of *Solanum tuberosum* L. *Journal of Experimental Botany*, 2010, 61(4): 1225-1238 (doi: 10.1093/jxb/erp394).
13. Feller A., Machemer K., Braun E.L., Grotewold E. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.*, 2011, 66(1): 94-116 (doi: 10.1111/j.1365-313X.2010.04459.x).
14. Allan A.C., Hellens R.P., Laing W.A. MYB transcription factors that colour our fruit. *Trends in Plant Science*, 2008, 13(3): 99-102 (doi: 10.1016/j.tplants.2007.11.012).
15. Dubos C., Stracke R., Groteworld E., Weisshar B., Martin C., Lepiniec L. MYB transcription factors in *Arabidopsis. Trends in Plant Science*, 2010, 15(10): 573-581 (doi: 10.1016/j.tplants.2010.06.005).
16. Andre C.M., Ourfir M., Guignard C., Hoffman L., Hausman J.F., Evers D., Larondelle Y. Antioxidant profiling of native Andean potato tubers (*Solanum tuberosum* L.) reveals cultivars with high levels of beta-carotene, alpha-tocopherol, chlorogenic acid, and petanin. *J. Agric. Food Chem.*, 2007, 55(26): 10839-10849 (doi: 10.1021/jf0726583).
17. Deineka L.A. *Nauchnye vedomosti BelGU. Seriya: Meditsina i farmatsiya*, 2006, 3(4): 92-100 (in Russ.).
18. Andersen Ø.M., Jordheim M. The anthocyanins. In: *Flavonoids: chemistry, biochemistry and applications.* Ø.M. Andersen, K.R. Markham (eds.). London, CRC Press. 2006: 471-552.
19. Eichhorn S., Winterhalter P. Anthocyanins from pigmented potato (*Solanum tuberosum* L.) varieties. *Food Research International*, 2005, 38(8-9): 943-948 (doi: 10.1016/j.foodres.2005.03.011).
20. Lewis C.E., Walker J.R.L., Lancaster J.E., Sutton K.H. Determination of anthocyanins, flavonoids and phenolic acids in coloured potatoes. I: Coloured cultivars of *Solanum tuberosum* L. *J. Sci. Food Agric.*, 1998, 77(1): 45-57 (doi: 10.1002/(SICI)1097-0010(199805)77:1<45::AID-

JSFA1>3.0.CO;2-S).
21. Meleshina O.V., Meleshin A.A. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Sovremennoe sostoyanie i perspektivy razvitiya selektsii i semenovodstva kartofelya»* [Proc. Int. Conf. «Current state and prospects for the development of potato breeding and seed production»]. Moscow, 2018: 129-139 (in Russ.).
22. Kim I.V., Klykov A.G. *Vestnik DVO RAN*, 2018, 3: 12-15 (in Russ).
23. Mityushkin A.A., Zhuravlev A.A., Zharova V.A., Mityushkin A.V., Salyukov S.S., Ovechkin S.V., Gaizatulin A.S., Simakov E.A. *Kartofelevodstvo (sb. nauch. tr.)* [Potato growing]. Minsk, 2018, Iss. 26: 57-64 (in Russ.).
24. Anisimov B.V., Elanskii S.N., Zeiruk V.N., Kuznetsova M.A., Simakov E.A., Sklyarova N.P., Filipov S.N., YAshina I.M. *Sorta kartofelya, vozdelyvaemye v Rossii* [Potato varieties cultivated in Russia]. Moscow, 2013 (in Russ.).
25. Dodds K.S., Long D.H. The inheritance of color in diploid potatoes. 1. Types of anthocyanins and their genetic loci. *Journ. of Genetics*, 1955, 53: 136-149 (doi: 10.1007/BF02981517).
26. Wang H., Race E.J., Shrikhande A.J. Characterization of anthocyanins in grape juices by ion trap liquid chromatography—mass spectrometry. *J. Agric. Food Chem.*, 2003, 51(7): 1839-1844 (doi: 10.1021/jf0260747).
27. Kalita D., Jayanty S.S. Comparison of polyphenol content and antioxidant capacity of colored potato tubers, pomegranate and blueberries. *Journal of Food Processing and Technology*, 2014, 5: 358 (doi: 10.4172/2157-7110.1000358).

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NOVEL APPROACH FOR DESIGNING ROTAVIRUS VACCINE CANDIDATE BASED ON TWO PLANT VIRUSES

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Abstract

Rotavirus A (genus *Rotavirus*, family *Reoviridae*) is still the main cause of viral gastroenteritis in children under 5 years. Existing attenuated vaccines have serious disadvantages, including the risk of potential reversion to pathogenic form, and side effects, the most dangerous of which is intussusception. Moreover, they occurred to be less effective in developing countries, where the most rotavirus-associated deaths are recorded. The development of an effective recombinant rotavirus A vaccine is an actual assignment; herewith the selection of effective and safe adjuvant is the key point for that. Plant viruses are very promising for innovative vaccine designing; they possess high immunostimulating properties, safe for humans and mammals and can serve as a carrier for pathogens' epitopes. Here we suggest an approach for rotavirus A vaccine development that involves two plant viruses: Alternanthera mosaic virus (AltMV) and Tobacco mosaic virus (TMV) as simultaneously epitope carriers and adjuvants. Spherical particles (SPs) generating by the heating of tobacco mosaic virus were used as an adjuvant and platform for presentation of obtained in our previous study chimeric recombinant protein ER6, which is an AltMV coat protein (CP) fused with the epitope RV14 (RLSFQLMRPPNMTP) of rotavirus A antigen VP6. Epitope RV14 are able to induce protective immune response and is conservative for the majority of rotavirus A strains therefore its usage gives hope to the successful overcoming of one of the main difficulties in rotavirus A vaccine development: wide serological diversity. In present work, effective adsorption of ER6 on the SPs surface leading to the SPs-ER6 complexes formation without loss of ER6 antigenic specificity was demonstrated. Two immune antisera with specificity to RV14 epitope within ER6 were obtained. The first serum was obtained via anti-ER6 sera depletion with AltMV CP and recombinant AltMV CP (AltMV rCP), which was expressed in *Escherichia coli* but did not contain RV14 sequence. The second serum was obtained by a direct immunization with synthetic peptide RLSFQLMRPPNMTP. These sera were utilized for studying RV14 within SPs-ER6 complexes. By means of immunofluorescent microscopy, SPs-ER6 complexes were demonstrated to interact with both depleted serum and anti-RV14 serum. Therefore, rotavirus epitope was confirmed to keep its ability to interact with antibodies within obtained complexes. Considering unique adjuvant properties of spherical particles and characteristics of selected epitope obtained SPs-ER6 complexes can be thought as a promising component for recombinant rotavirus A vaccine. Moreover, it can be hoped that the suggested in present work approach, involving the usage of TMV SPs as a platform and adjuvant for chimeric AltMV CP, containing pathogen's epitope, will be useful not only for rotavirus A vaccine development but for designing of vaccines against other pathogens of humans or farm animals.

Keywords: plant viruses, Tobacco mosaic virus, spherical particles, platform-carrier, platform-adjuvant, Alternanthera mosaic virus, chimeric recombinant antigen, recombinant vaccines, rotavirus infection

Rotavirus infection is the main cause of viral gastroenteritis in infants and young children [1]. In 2016, on average, more than 40% of children under the age of five experienced rotavirus diarrhea, while the number of deaths was 128,500 (95% uncertainty interval 104500-155600) [2]. Currently licensed live attenuated vaccines, the monovalent Rotarix® (GlaxoSmithKline Biologicals S.A., Belgium) and pentavalent RotaTeq (Merck & Co., Inc., USA) have proven to be quite effective in high-income countries. However, these oral vaccines can rarely cause serious side effects, the most dangerous of which is intussusception [3-5]. In addition, the majority of deaths associated with rotavirus are recorded in developing countries where the effectiveness of these vaccines is lower than in high-income countries [3, 6-9]. Thus, the designing of a non-replicating new-generation rotavirus vaccine is highly desirable [10, 11].

Recombinant rotavirus antigens (genus *Rotavirus*, family *Reoviridae*) associated with protein carriers or virus-like particles (VLP) are promising candidates for safe and effective rotavirus vaccine [3]. The rotavirus virion is a three-layered particle with icosahedral symmetry [12]. The main rotavirus antigens are the following structural proteins: VP6 forming the intermediate capsid [11, 13, 14], VP7 composing the outer capsid [13, 15], VP4, which forms spikes [15, 16], and VP5* and VP8* derived from the VP4 by proteolysis [17]. VP6 is conserved for rotavirus strains of group A [11, 18] causing human diseases. Despite the fact that VP6 is not located on the surface of the virion it plays a key role in the formation of a protective immune response. The VP6 is able to induce almost 100% protection against rotavirus in mice after the first immunization [11]. CD4+ T cells have been shown to be the main immune cells providing protective immunity [19, 20]. In addition, some studies indicate that VP6-specific IgA, after entering the cell via transcytosis, are able to inhibit transcription. They are supposed to bind rotavirus bilayer particles intracellularly that leads to steric blocking of messenger RNA-releasing channels [21]. The 14-amino acids length epitope of the protein VP6, RV14 (RLSFQLMRPPNMTP, positions 289-302), can induce an immune response comparable to that induced by the whole VP6 molecule, also providing almost 100% protection in mice [20, 22]. Therefore, the epitope RV14 is of particular interest as an antigenic determinant to design rotavirus vaccines.

Plant viruses and their VLPs are a promising tool to design novel vaccine formulations [23-29]. Safety for humans [23] and high adjuvant properties [29-34] provide the attractiveness of virions and VLPs of plant viruses for applying in vaccines as carriers of pathogen recombinant antigens. For the virions and VLPs of the *Alternanthera mosaic virus* (AltMV) (genus *Potexvirus*, family *Alphaflexiviridae*) adjuvant properties have been previously demonstrated, and the AltMV coat protein (CP) has the ability to form RNA-free filamentous VLPs, which are stable under physiological conditions [35]. In our previous studies, we used AltMV CP as a carrier for the RV14 epitope and obtained a 25-kDa chimeric recombinant protein ER6 (Epitope of Rotavirus protein VP6) [36]. However, it is known that individual proteins even with greater molecular weight often demonstrate low immunogenicity in themselves, and the use of an adjuvant is necessary to enhance immune response. In particular, for ~ 88-kDa chimeric protein, which is a rotavirus protein VP6 fused with a maltose-binding protein, the crucial importance of the presence an adjuvant to induce protective immunity was described [18, 19, 37].

We have previously shown that heating the *Tobacco mosaic virus* (TMV) (genus *Tobamovirus*, family *Virgaviridae*) virions leads to the remodeling of this

rod-shaped virus with a helical structure into spherical particles (SPs) that do not contain RNA. The safety and high immunostimulating potency have been demonstrated for SPs [34, 38, 39].

In this paper, we have proposed for the first time an approach to design a vaccine candidate against rotavirus based on two plant viruses with different structures. We used SPs as a platform for adsorption and an adjuvant for the chimeric antigen of rotavirus – ER6. It has been demonstrated that, despite the small size (14 amino acid residues), the antigenic determinant of rotavirus remains available for antibodies in the SPs-ER6 complexes. This gives reason to believe that the presented epitope will induce the production of antibodies, which are necessary to protect against rotavirus infection. The described approach, which includes the use of the plant virus coat protein to create a chimeric antigen containing the pathogen epitope, and the simultaneous TMV SPs application as a platform-adjuvant, can also be expected to allow integrating short epitopes into the compositions of recombinant vaccine candidates against various infectious agents of humans and farm animals.

Our goal was to construct antigenically active complexes based on the chimeric antigen of the rotavirus (ER6) and structurally modified viral particles derived from TMV after thermal remodeling.

Materials and methods. Purified TMV from infected tobacco plants (*Nicotiana tabaccum* L.) and SPs were prepared according to the description [38]. AltMV from infected tobacco plants (*Nicotiana benthamiana* L.) was purified according to Donchenko et al. [35]. For AltMV coat protein isolation, salt deproteinization with 2 M LiCl was applied.

The recombinant rotavirus antigen ER6 was expressed in the *Escherichia coli* system as per the protocols [23]. To produce a recombinant AltMV coat protein (AltMV rCP), the DNA fragments encoding ER6 protein but not containing the RV14 epitope sequences was amplified from the pQE-60-ER6 plasmid carrying an insert for ER6 protein. For amplification, we used a pair of primers 5′-ATACGGATCCAGTACTCCATTTCCTCAAGTCACCCA-3′ and 3′-GTATAGCTTCTCCGGTGGTGGGAGGTATTG-5′ with BamHI and HindIII restriction endonuclease sites at the 5′- and 3′-ends respectively and Encyclo PCR kit (Evrogen, Russia); PCR program: 2 min at 96 °C; 30 s at 95 °C, 30 s at 64 °C, 45 s at 72 °C (26 cycles); 1 min at 72 °C. The BamHI and HindIII (Thermo Scientific, USA) sites were used for cloning the recombinant DNA fragment into pQE-30 vector (Qiagen N.V., Germany). All genetic engineering manipulations were performed using conventional techniques [40]. Further procedures for obtaining AltMV rCP were the same as for the ER6 protein [36].

For electron microscopy, the preparations were loaded on copper mesh grids for electron microscopy covered with carbon-coated collodion support films and kept for 15-20 s. The excess of the preparation was removed by filter paper; in the case of SPs-ER6, the meshes were negatively contrasted with a 2% uranyl acetate solution for 15-20 s. The preparations were analyzed using a JEM-1011 electron microscope (JEOL, Ltd, Japan) equipped with an Orius™ SC1000 W digital camera (Gatan, Inc., USA). The average size of the SPs was measured using the ImageJ software (National Institutes of Health, USA); the mean M and ±SD was calculated for 100 particles.

For immune sera, white outbred mice aged 6-8 weeks were immunized intraperitoneally three times with a 2-week interval. For the first immunization, 10 μg of ER6 protein or 25 μg of synthetic peptide RLSFQLMRPPNMTP (Almabion, Russia) in 0.2 ml sterile PBS (7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl) was mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich Chemie GmbH, Germany) to a homogeneous

suspension. In the second and third immunization, instead of Freund's complete adjuvant, Freund's incomplete adjuvant (Sigma-Aldrich Chemie GmbH, Germany) was used. For obtaining control non-immune serum, the mouse was immunized three times with 0.2 ml PBS. Blood samples were collected 2 weeks after the third immunization. Blood serum was prepared by centrifugation at 10,000 g for 5 min. The antibody titer was determined by indirect enzyme-linked immunosorbent assay (ELISA). Medium sorption plates (Greiner Bio-One GmbH, Austria) were coated with the corresponding antigen at a concentration of 10 μg/ml. Free binding sites were blocked with 1% milk powder (PanReac AppliChem ITW Reagents, Spain) in PBS. All sera were tested in 3-fold dilutions. The secondary antibodies were antibodies against mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., USA) at a dilution of 1:20,000; 3,3′,5,5′-tetramethylbenzidine was a substrate, the reaction was stopped with sulfuric acid added to a final concentration of 1 M. The absorbance in the wells was measured at 450 nm (a Multiskan FC microplate photometer, Thermo Scientific, USA). The titer was expressed as the serum dilution at which the absorbance value corresponds to the mean value of the block +3SD.

For depletion of antiserum, 200 μg AltMV CP was added to 100 μl of anti-ER6 serum, incubated for 30 min at 37 °C, and the precipitate was removed by centrifugation at 10000 g. To 50 μl of the supernatant, 270 μg of AltMV rCP was added, incubated at 4 °C overnight, and centrifuged at 10000 g in the cold, the supernatant was used as depleted serum.

For immunoblotting, proteins were separated by electrophoresis in an 8-20% gradient polyacrylamide gel with sodium dodecyl sulfate (SDS-PAAG) and transferred from the gel to an Amersham™ Hybond® P polyvinylidene fluoride (PVDF) membrane (GE Healthcare — Life Sciences, USA) using a Pierce™ Power Blotter transfer system (Thermo Fisher Scientiific, USA). The membrane was exposed to depleted mouse anti-ER6 serum (1:2000) and secondary antibodies to mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., USA) (1:20,000). After the treatment with Amersham™ ECL™ substrate (GE Healthcare — Life Sciences, USA), a chemiluminescent signal was detected by the ChemiDoc XRS+ gel documentation system (Bio-Rad Laboratories, Inc., USA). Gels were stained with Coomassie® Brilliant Blue G 250 (SERVA Electrophoresis, Germany). PageРишгler Plus Prestained Protein Ladder (Thermo Scientific, USA) was used.

For immunofluorescence microscopy, the SPs-ER6 sample was loaded on formvar-coated coverslips. After 10 min exposure, the excess of the sample was removed, the coverslips were incubated for 1 h with blocking solution (PBS with 1% bovine serum albumin — BSA and 0.05% Tween 20), then for 1 h with 1:50 dilution of the corresponding serum in the blocking solution. The coverslips were washed thrice with a washing solution (PBS with 0.25% BSA and 0.05% Tween 20) and incubated for 45 min with secondary antibodies to mouse IgG conjugated to Alexa 546 fluorophore (Invitrogen, USA; 1:100 dilution in the blocking solution). After secondary antibodies bound, the preparations were washed thrice with the washing solution and once with PBS, then rinsed with water and dried in air. Immediately before the study, the preparations were treated with a photo-protector 1,4-diazabicyclo[2.2.2]octane and examined under an Axiovert 200M fluorescence microscope (Carl Zeiss, Germany) equipped with an ORCAII-ERG2 integrated camera (Hamamatsu Photonics, Japan).

Results. SPs-ER6 complexes obtaining. At the first stage, the previously designed rotavirus recombinant antigen ER6 [36] was adsorbed on the surface of spherical particles.

ER6 is a chimeric AltMV CP fused at the C-terminus with the RV14 epitope (RLSFQLMRPPNMTP) of the VP6 protein of rotavirus A (Fig. 1, A). Figure 1 schematically shows the structure of the ER6 (see Fig. 1, A) and the location of its structural and functional components within the rotavirus (see Fig. 1, B) and AltMV (see Fig. 1, C) virions. The antigenic determinant (RV14) is a fragment of the rotavirus intermediate capsid protein VP6 (see Fig. 1, B), and AltMV CP is the epitope carrier [36].

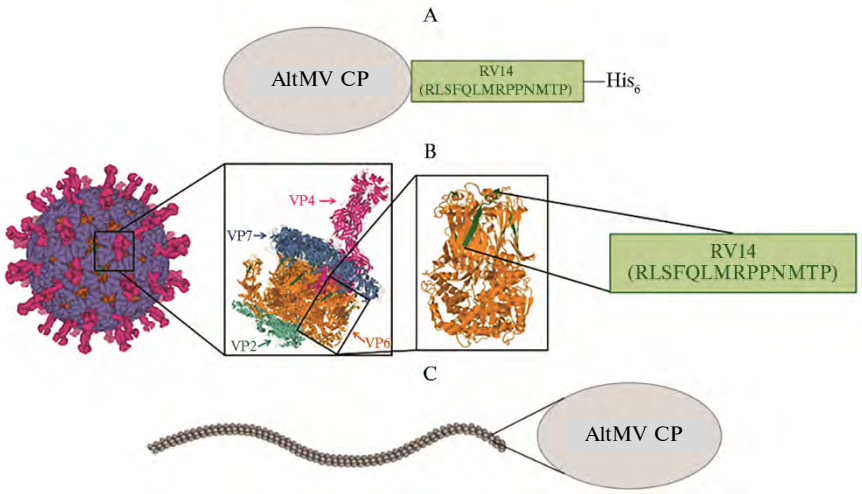

Fig. 1. The scheme of the recombinant antigen ER6 [36] containing RV14 epitope (RLSFQLMRPPNMTP) of the rotavirus A intermediate capsid protein VP6.

A. ER6: AltMV CP — *Alternanthera mosaic virus* coat protein, epitope RV14, His_6 — His-tag.

B. RV14 (RLSFQLMRPPNMTP) epitope position in rotavirus: virion (PDB: 4V7Q) [41-43]; rotavirus capsid proteins structure (PDB: 4V7Q) [41, 43, 44]; VP6 trimer (PDB: 1QHD) [43-45]; RV14 scheme (RV14 within VP6 is marked in green).

C. An AltMV virion (ViralZone) [46] and the AltMV CP schematic image.

Image is not to scale.

SPs prepared from TMV to form complexes with the protein ER6, have a regular spherical shape, are electron-dense and detectable without contrasting (Fig. 2). The average size of the obtained particles measured by electron microscopy was 986±109 nm.

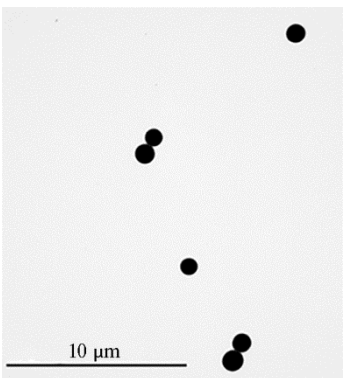

Fig. 2. Spherical particles obtained by thermal treatment of TMV virions. Transmission electron microscopy (JEM-1011, JEOL, Ltd, Japan; digital camera Orius™ SC1000 W, Gatan, Inc., USA).

To obtain SPs-ER6 complexes, the SPs were incubated with the ER6 in MilliQ water overnight at room temperature. Based on previous studies [39], the 10:1 mass ratio of SPs and ER6 was selected.

SPs-ER6 complexes were detected by immunofluorescence microscopy with serum to ER6 (the titer of antibodies to ER6 determined by ELISA was 1:11,284,633). This serum was obtained by immunization of white mice with ER6 in presence of the Freund's adjuvant.

Immunofluorescence analysis clearly revealed the SPs-ER6 complexes (Fig. 3). The image in the fluorescence mode (see Fig. 3, A) fully corresponds to the position of the SPs observed in the phase contrast mode (see Fig. 3, B). Comparison of these images allows us to conclude that all SPs in the field of view are ER6-coated. Despite the large number of SPs are detected in the field of view in

the phase contrast mode in the control sample, which were not treated with serum to ER6 (see Fig. 3, C), nothing detects in fluorescent mode (see Fig. 3, D) that confirms the specificity of the interaction. This indicates that the antigenic specificity of the ER6 protein does not change during adsorption on SPs.

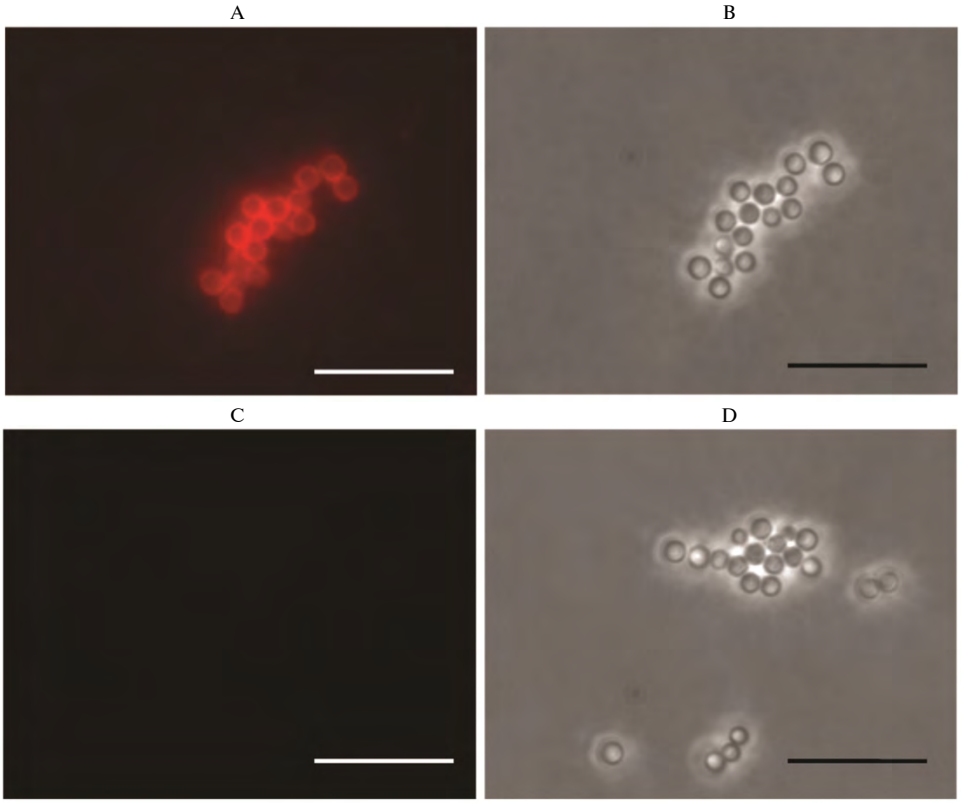

Fig. 3. Recombinant antigen ER6, which is the Alternanthera mosaic virus coat protein fused with RV14 epitope of the rotavirus A protein VP6, can be effectively adsorbed on spherical particles (SPs), derived from the Tobacco mosaic virus coat protein, forming SPs-ER6 complexes. The complexes were treated with immune serum to ER6 and secondary anti-species antibodies conjugated to Alexa 546 fluorophore (A, B) and only secondary antibodies (control) (C, D). Scale bar 10 microns. Immunofluorescence microscopy, the fluorescence (A, C) and phase contrast (B, D) modes (Axiovert 200M microscope, Carl Zeiss, Germany, ORCAII-ERG2 integrated camera, Hamamatsu Photonics, Japan).

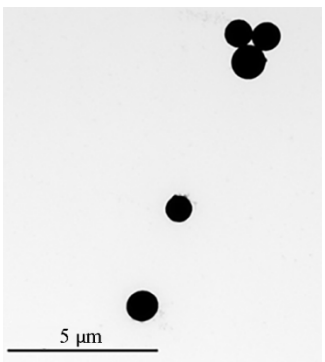

Fig. 4. SPs-ER6 complexes of spherical particles (SPs), derived from the Tobacco mosaic virus coat protein, and the recombinant antigen ER6, which is the Alternanthera mosaic virus coat protein fused with RV14 epitope of the rotavirus A protein VP6 . Transmission electron microscopy, contrasting with 2% uranyl acetate (JEM-1011, JEOL, Ltd, Japan; digital camera Orius™ SC1000 W, Gatan, Inc., USA).

Electron microscopy showed that the formation of complexes with the recombinant protein ER6 does not lead to a change in the morphology of SPs (Fig. 4). The shape and size of the SPs-ER6 complexes turned out to be identical to those of ER6-free SPs (see Fig. 2).

Rotavirus A epitope keeps its antigenic specificity within the SPs-ER6 complexes. We used two antisera specific for the RV14 region of ER6 to assess the availability of the RV14 epitope for antibodies in the SPs-ER6 complexes.

One of the sera is twice depleted serum to ER6. The first stage of depletion was performed with native AltMV CP, and the second one with recombinant AltMV CP expressed in *E. coli* and purified by the same method as for ER6. After removing the precipitate, we assessed the specificity of the interaction of the depleted serum with the antigens ER6, AltMV CP, and AltMV rCP by indirect ELISA. The titer of depleted serum to AltMV CP and to AltMV rCP was low, 1:79424 and 1:85046, respectively, while the titer to ER6 remained relatively high, the 1:1,021,968.

In addition the depleted serum ability to interact with ER6, AltMV CP and AltMV rCP was examined by immunoblot analysis. A clear interaction with the ER6 protein (Fig. 5, lane 2) was demonstrated, while the reaction with AltMV CP (see Fig. 5, lane 1 on the right) and AltMV rCP (see Fig. 5, line 3 on the right) was extremely weak. We did not observe interactions with control samples: TMV, PVX and molecular weight markers (Fig. 5, lanes 4 and M on the right, respectively).

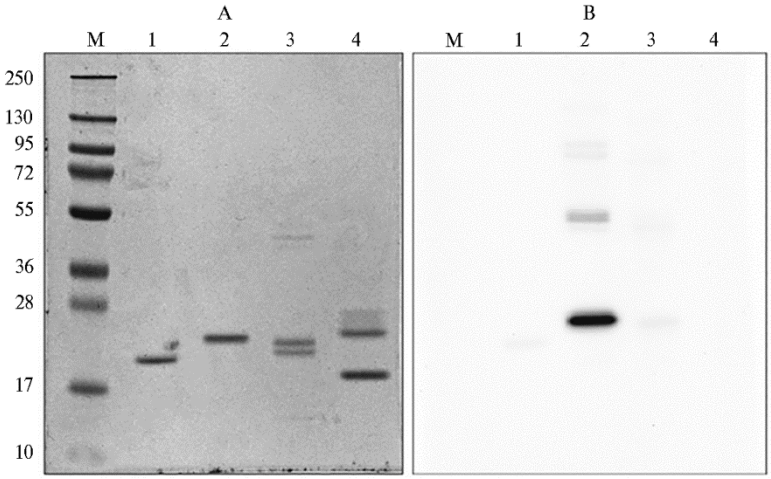

Fig. 5. Immunoblot analysis of interaction specificity between depleted antiserum and recombinant antigen ER6, which is the Alternanthera mosaic virus coat protein fused with epitope RV14 of the rotavirus A protein VP6 (left — electrophoretic separation, right — immunoblotting): 1 — Alternanthera mosaic virus coat protein (AltMV CP), 2 — ER6, 3 —AltMV rCP (the sequence of epitope RV14 is absent in the recombinant protein), 4 — TMV and PVX (controls), M — molecular weight markers (PageRuler Plus Prestained Protein Ladder, Thermo Scientific, USA); molecular weights are indicated in kDa). Serum to ER6 was depleted with AltMVP CP and AltMV rCP; SDS-PAAG electrophoresis (8-20%), staining with Coomassie® Brilliant Blue G 250.

Therefore, the depleted serum remains specific to ER6, while it almost completely lacks specificity to AltMV CP and to AltMV rCP.

The second serum we used to assess antigenic specificity of the SPs-ER6 complexes was the serum against the individual peptide RV14. Short peptides are known to be extremely low immunogenic. Nevertheless, in this work, we attempted to obtain antiserum against synthetic peptide RV14 and for this purpose four mice were immunized. Despite quite a large amount of the peptide (25 μg per mouse) added together with Freund's complete adjuvant, three animals did not develop an immune response. Only one collected serum contained antibodies to the RV14 peptide. The titer determined by indirect ELISA with RV14 as antigen was 1:226143, which is considerably higher compared to 1:3088 in control (non-immune serum).

In order to examine anti-RV14 serum specificity to ER6 antigen, we also used non-immune serum and nonspecific antigen AltMV rCP as controls. The titer of anti-RV14 serum to specific antigen ER6 was 1:183971 while the titer to

nonspecific antigen AltMV rCP was 1:7101. Non-immune serum titer to antigen ER6 was 1:8682, therefore, the efficacy of the interaction of anti-RV14 serum with ER6 was more than 20 times higher compared to controls.

Considering the immune serum against RV14 peptide was proven to react with ER6 specifically, it was also applied in analysis of the SPs-ER6 complexes.

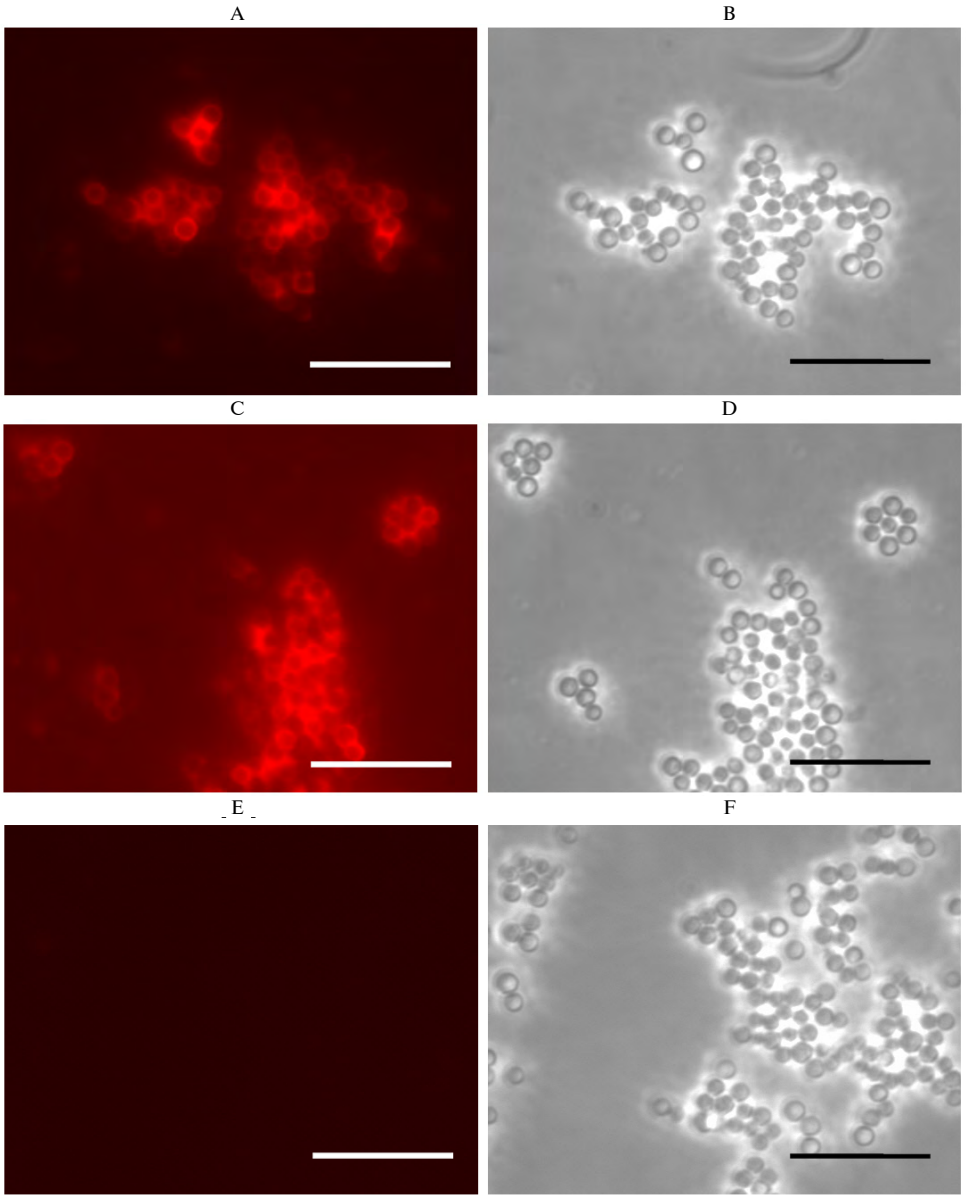

Fig. 6. RV14 epitope of rotavirus A protein VP6 is available for interaction with antibodies within the SPs-ER6 complexes. SPs are spherical particles derived from TMV coat protein. Recombinant antigen ER6 is the Alternanthera mosaic virus coat protein fused with RV14 epitope of the rotavirus A protein VP6. SPs-ER6 complexed were treated with a depleted anti-ER6 serum (A, B), with anti-RV14 serum (C, D), and with secondary antibodies only (E, F) (control). Secondary antibodies are conjugated to Alexa 546 fluorophore. Scale bar 10 μm. Immunofluorescence microscopy in fluorescence (A, C, E) and phase contrast (B, D, E) modes (an Axiovert 200M microscope, Carl Zeiss, Germany, ORCAII-ERG2 integrated camera, Hamamatsu Photonics, Japan).

By means of immunofluorescence microscopy the SPs-ER6 complexes were demonstrated to be recognized by both depleted serum against ER6 (Fig. 6, A) and anti-RV14 serum (see Fig. 6, B). The positions of the detected SPs

corresponded in fluorescence and phase contrast modes (see Fig. 6, B, D). The complete absence of fluorescence in the not-treated with primary antibodies control sample (see Fig. 6, E, F) excludes unspecific binding of secondary antibodies.

These results indicate that the rotavirus epitope RV14 is available for interaction with antibodies and retains its antigenic specificity within the SPs-ER6 complexes.

Various researchers have demonstrated that the designing of plant viruses chimeric proteins fused with pathogens epitopes can lead to the formation of VLPs, which are able to induce immune response to the corresponding epitopes [31, 32, 47, 48]. In particular, based on Papaya mosaic virus (genus *Potexvirus*, family *Alphaflexiviridae*), which is close relative to the AltMV, chimeric VLPs carrying influenza virus [31, 48] or hepatitis C virus [47] epitopes on their surface were obtained. The authors believe that multivalent structure of VLP provides the immunogenicity in this case [30, 31, 47]. In our previous studies, we for the first time used AltMV for designing chimeric recombinant antigen carrying epitope of rotavirus protein VP6. The protein ER6, which is AltMV CP fused to the epitope of rotavirus protein VP6, was obtained. ER6 was recognized by commercial polyclonal antisera to rotavirus A, thus it was proved to be a promising antigen for the development of recombinant rotavirus A vaccines. However, ER6 was unable to form VLPs in the absence of viral RNA [36]. Recombinant proteins themselves possess low immunogenicity and their use requires adding an adjuvant [49, 50]. In this work, we propose SPs derived of TMV virions by thermal remodeling as a platform-adjuvant for a ER6-based vaccine. SPs are stable under physiological conditions, safe for humans and mammals, biodegradable and can adsorb various proteins on their surface that lead to the SPs-antigen complexes formation. Moreover, SPs were previously demonstrated to be able to increase in several times the titer of antibodies to the antigen administrated within complexes with SPs, therefore, SPs have high immunostimulating properties [38, 39]. In this work, rotavirus A recombinant antigen ER6 was efficiently adsorbed on the spherical particles with the SPs-ER6 complexes formation. These complexes were detected by indirect immunofluorescence microscopy with anti-ER6 serum. All SPs were showed to be coated with the ER6. The interaction of primary antibodies with ER6 located on the SPs surface indicates that adsorption does not lead to a loss of the chimeric protein antigenic specificity.

To assess the prospects for using the SPs-ER6 complexes as a component of a rotavirus vaccine, it was also important to make sure that the rotavirus epitope RV14 within such complexes is available for antibodies. To address the problem, two immune sera specific to RV14 were prepared: the depleted anti-ER6 serum and the anti-RV14 serum obtained by immunization with the synthetic peptide. As a result of depletion with AltMV CP and AltMV rCP, the serum retained mainly those antibodies for the production of which the RV14 was necessary. The titer of depleted anti-ER6 serum to the antigen ER6 was more than 10 times higher than to the antigens AltMV rCP and AltMV CP. This serum almost completely lost the ability to recognize both native and recombinant CP of AltMV in immunoblotting but at the same time effectively bounded ER6. The relatively large amounts of antibodies specific to the RV14 region of the protein ER6 allows suggesting that AltMV CP, acting as a carrier of RV14, might also act as adjuvant, enhancing the immune response to the rotavirus epitope. Another serum to RV14 was obtained by direct immunization with the synthetic peptide RV14. Both sera were applied to examine the antigenic specificity of the rotavirus epitope in SPs-ER6 complexes by indirect immunofluorescence microscopy. The SPs-ER6 complexes were proved to react with both depleted anti-ER6 serum and anti-RV14 serum. This indicates that the ER6 keeps the rotavirus antigenic specificity within

the SPs-ER6 complexes, which confirms the correctness of AltMV CP selection as a carrier for the rotavirus epitope. Taking into account the previously shown adjuvant properties of SPs, it can be assumed that the immune response to the antigenic determinant of rotavirus will be greatly enhanced due to formation of complexes with SPs.

Therefore, based on the coat protein of one plant virus, the Alternanthera mosaic virus (AltMV), we have constructed a recombinant antigen ER6 carrying the epitope RV14 of the rotavirus A protein VP6. As well as based on the coat protein of another plant virus, the Tobacco mosaic virus (TMV), we obtained spherical particles (SPs) that form complexes with ER6. Chimeric protein was demonstrated to retain its antigenic specificity during adsorption on SPs. In turn, SPs was previously shown to have high adjuvant activity. This suggests that the obtained SPs-ER6 complexes containing an epitope of the rotavirus protein VP6 and modified coat proteins of simultaneously two plant viruses (AltMV and TMV) can serve as a component of a new safe recombinant vaccine against rotavirus infection. The proposed approach can be further applied to design recombinant vaccines against pathogens of humans and farm animals.

REFERENCES

1. Sicard M., Bryant K., Muller M.L., Quach C. Rotavirus vaccination in the neonatal intensive care units: where are we? A rapid review of recent evidence. *Curr. Opin. Pediatr.*, 2020, 32(1): 167-191 (doi: 10.1097/MOP.0000000000000869).
2. Troeger C., Khalil I.A., Rao P.C., Cao S., Blacker B.F., Ahmed T., Armah G., Bines J.E., Brewer T.G., Colombara D. V., Kang G., Kirkpatrick B.D., Kirkwood C.D., Mwenda J.M., Parashar U.D., Petri W.A., Riddle M.S., Steele A.D., Thompson R.L., Walson J.L., Sanders J.W., Mokdad A.H., Murray C.J.L., Hay S.I., Reiner R.C. Rotavirus vaccination and the global burden of rotavirus diarrhea among children younger than 5 years. *JAMA Pediatrics*, 2018, 172(10): 958-965 (doi: 10.1001/jamapediatrics.2018.1960).
3. Kirkwood C.D., Ma L.F., Carey M.E., Steele A.D. The rotavirus vaccine development pipeline. *Vaccine*, 2019, 37(50): 7328-7335 (doi: 10.1016/j.vaccine.2017.03.076).
4. Weintraub E.S., Baggs J., Duffy J., Vellozzi C., Belongia E.A., Irving S., Klein N.P., Glanz J.M., Jacobsen S.J., Naleway A., Jackson L.A., DeStefano F. Risk of intussusception after monovalent rotavirus vaccination. *N. Engl. J. Med.*, 2014, 370(6): 513-519 (doi: 10.1056/NEJMoa1311738).
5. Carlin J.B., Macartney K.K., Lee K.J., Quinn H.E., Buttery J., Lopert R., Bines J., McIntyre P.B. Intussusception risk and disease prevention associated with rotavirus vaccines in Australia's national immunization program. *Clinical Infections Disease*, 2013, 57(10): 1427-1434 (doi: 10.1093/cid/cit520).
6. Burnett E., Parashar U., Tate J. Rotavirus vaccines: effectiveness, safety, and future directions. *Pediatr. Drugs*, 2018, 20(3): 223-233 (doi: 10.1007/s40272-018-0283-3).
7. Desselberger U., Wolleswinkel-van den Bosch J., Mrukowicz J., Rodrigo C., Giaquinto C., Vesikari T. Rotavirus types in Europe and their significance for vaccination. *Pediatr. Infect. Dis.*, 2006, 25(1): S30-S41 (doi: 10.1097/01.inf.0000197707.70835.f3).
8. Sadiq A., Bostan N., Yinda K., Naseem S., Sattar S. Rotavirus: genetics, pathogenesis and vaccine advances. *Rev. Med. Virol.*, 2018, 28(6): e2003 (doi: 10.1002/rmv.2003).
9. Velasquez D., Parashar U., Jiang B. Decreased performance of live attenuated, oral rotavirus vaccines in low-income settings: causes and contributing factors. *Expert Review of Vaccines*, 2018, 17(2): 145-161 (doi: 10.1080/14760584.2018.1418665).
10. Agarwal S., Hickey J.M., McAdams D., White J.A., Sitrin R., Khandke L., Cryz S., Joshi S.B., Volkin D.B. Effect of aluminum adjuvant and preservatives on structural integrity and physicochemical stability profiles of three recombinant subunit rotavirus vaccine antigens. *Journal of Pharmaceutical Sciences*, 2020, 109(1): 476-487 (doi: 10.1016/j.xphs.2019.10.004).
11. Afchangi A., Jalilvand S., Mohajel N., Marashi S., Shoja Z. Rotavirus VP6 as a potential vaccine candidate. *Rev. Med. Virol.*, 2019, 29(2): e2027 (doi: 10.1002/rmv.2027).
12. Estes M.K., Greenberg H.B. Rotaviruses. In*: Fields virology*. D.M. Knipe, P. Howley (eds.). Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, 2013: 1347-1395.
13. Estes M., Cohen J. Rotavirus gene structure and function. *Microbiological Reviews*, 1989, 53(4): 410-449.
14. Svensson L., Sheshberadaran H., Vene S., Norrby E., Grandien M., Wadell G. Serum antibody responses to individual viral polypeptides in human rotavirus infections. *Journal of General Virology,* 1987, 68(3): 643-651 (doi: 10.1099/0022-1317-68-3-643).

15. Desselberger U., Huppertz H.I. Immune responses to rotavirus infection and vaccination and associated correlates of protection. *Journal of Infectious Diseases*, 2011, 203(2): 188-195 (doi: 10.1093/infdis/jiq031).
16. Kovacs-Nolan J., Dongwan Y., Yoshinori M. Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus. *Biochemical Journal*, 2003, 376(1): 269-275 (doi: 10.1042/BJ20021969).
17. Crawford S., Mukherjee S., Estes M., Lawton J., Shaw A., Ramig R., Prasad B. Trypsin cleavage stabilizes the rotavirus VP4 spike. *Journal of Virology*, 2001, 75(13): 6052-6061 (doi: 10.1128/JVI.75.13.6052-6061.2001).
18. Parbhoo N., Dewar J.B., Gildenhuys S. Sequence analysis and structural implications of rotavirus capsid proteins. *Acta Virologica*, 2016, 60(3): 260-270 (doi: 10.4149/av_2016_03_260).
19. Ward R.L., McNeal M.M. VP6: a candidate rotavirus vaccine. *The Journal of Infectious Diseases,* 2010, 202(Suppl. 1): S101-S107 (doi: 10.1086/653556).
20. Choi A.H.C., Basu M., McNeal M.M., Flint J., VanCott J.L., Clements J.D., Ward R.L. Functional mapping of protective domains and epitopes in the rotavirus VP6 protein. *Journal of Virology*, 2000, 74(24): 11574-11580 (doi: 10.1128/jvi.74.24.11574-11580.2000).
21. Aiyegbo M.S., Sapparapu G., Spiller B.W., Eli I.M., Williams D.R., Kim R., Lee D.E., Liu T., Li S., Woods V.L., Nannemann D.P., Meiler J., Stewart P.L., Crowe J.E. Human rotavirus VP6-specific antibodies mediate intracellular neutralization by binding to a quaternary structure in the transcriptional pore. *PLoS ONE*, 2013, 8(5): e61101 (doi: 10.1371/journal.pone.0061101).
22. Choi A., McNeal M., Basu M., Bean J., VanCott J., Clements J., Ward R. Functional mapping of protective epitopes within the rotavirus VP6 protein in mice belonging to different haplotypes. *Vaccine*, 2003, 21(7-8): 761-767 (doi: 10.1016/s0264-410x(02)00595-9).
23. Balke I., Zeltins A. Use of plant viruses and virus-like particles for the creation of novel vaccines. *Advanced Drug Delivery Reviews*, 2019, 145: 119-129 (doi: 10.1016/j.addr.2018.08.007).
24. Zhao Y., Hammond R. Development of a candidate vaccine for Newcastle disease virus by epitope display in the Cucumber mosaic virus capsid protein. *Biotechnology Letters,* 2005, 27(6): 375-382 (doi: 10.1007/s10529-005-1773-2).
25. Phelps J.P., Dao P., Jin H., Rasochova L. Expression and self-assembly of cowpea chlorotic mottle virus-like particles in *Pseudomonas fluorescens. Journal of Biotechnology,* 2007, 128(2): 290-296 (doi: 10.1016/j.jbiotec.2006.10.005).
26. Mathieu C., Rioux G., Dumas M.C., Leclerc D. Induction of innate immunity in lungs with virus-like nanoparticles leads to protection against influenza and *Streptococcus pneumoniae* challenge. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 2013, 9(7): 839-848 (doi: 10.1016/j.nano.2013.02.009).
27. Lebel M.-E., Daudelin J.-F., Chartrand K., Tarrab E., Kalinke U., Savard P., Labrecque N., Leclerc D., Lamarre A. Nanoparticle adjuvant sensing by TLR7 enhances CD8+ T cell-mediated protection from *Listeria monocytogenes* infection. *The Journal of Immunology,* 2014, 192(3): 1071-1078 (doi: 10.4049/jimmunol.1302030).
28. Acosta-Ramírez E., Pérez-Flores R., Majeau N., Pastelin-Palacios R., Gil-Cruz C., Ramírez-Saldaca M., Manjarrez-Orduco N., Cervantes-Barragán L., Santos-Argumedo L., Flores-Romo L., Becker I., Isibasi A., Leclerc D., Lypez-Macías C. Translating innate response into long-lasting antibody response by the intrinsic antigen-adjuvant properties of papaya mosaic virus. *Immunology*, 2008, 124(2): 186-197 (doi: 10.1111/j.1365-2567.2007.02753.x).
29. Brennan F., Jones T., Hamilton W. Cowpea mosaic virus as a vaccine carrier of heterologous antigens. *Molecular Biotechnology*, 2001, 17(1): 15-26 (doi: 10.1385/MB:17:1:15).
30. McCormick A.A., Palmer K.E. Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expert Review of Vaccines*, 2008, 7(1): 33-41 (doi: 10.1586/14760584.7.1.33).
31. Denis J., Acosta-Ramirez E., Zhao Y., Hamelin M.E., Koukavica I., Baz M., Abed Y., Savard C., Pare C., Lopez Macias C., Boivin G., Leclerc D. Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. *Vaccine*, 2008, 26(27-28): 3395-3403 (doi: 10.1016/j.vaccine.2008.04.052).
32. Lico C., Mancini C., Italiani P., Betti C., Boraschi D., Benvenuto E., Baschieri S. Plant-produced potato virus X chimeric particles displaying an influenza virus-derived peptide activate specific CD8+ T cells in mice. *Vaccine*, 2009, 27(37): 5069-5076 (doi: 10.1016/j.vaccine.2009.06.045).
33. Kalnciema I., Skrastina D., Ose V., Pumpens P., Zeltins A. Potato virus Y-like particles as a new carrier for the presentation of foreign protein stretches. *Molecular Biotechnology*, 2012, 52(2): 129-139 (doi: 10.1007/s12033-011-9480-9).
34. Evtushenko E., Ryabchevskaya E., Nikitin N., Atabekov J., Karpova O. Plant virus particles with various shapes as potential adjuvants. *Scientific Reports*, 2020, 10(1): 1-10 (doi: 10.1038/s41598-020-67023-4).
35. Donchenko E., Trifonova E., Nikitin N., Atabekov J., Karpova O. Alternanthera mosaic potexvirus: several features, properties, and application. *Advances in Virology*, 2018, 2018: Article ID 197370 (doi: 10.1155/2018/1973705).
36. Ryabchevskaya E.M., Evtushenko E.K., Arkhipenko M.V., Donchenko E.K., Nikitin N.A., Atabekov J.G., Karpova O.V. Recombinant rotavirus antigen based on Althernanthera mosaic virus coat

protein. *Molecular Biology* (*Mosk.*), 2020, 54(2): 278-284 (doi: 10.1134/S0026893320020132).
37. Choi A.H.C., Basu M., Mcneal M.M., Clements J.D., Ward R.L. Antibody-independent protection against rotavirus infection of mice stimulated by intranasal immunization with Chimeric VP4 or VP6 protein. *Journal of Virology*, 1999, 73(9): 7574-7581 (doi: 10.1128/JVI.73.9.7574-7581.1999).
38. Trifonova E.A., Nikitin N.A., Kirpichnikov M.P., Karpova O.V., Atabekov J.G. Obtaining and characterization of spherical particles — new biogenic platforms. *Moscow University Biological Sciences Bulletin*, 2015, 70(4): 194-197 (doi: 10.3103/S0096392515040094).)
39. Kondakova O., Trifonova E., Arkhipenko M., Nikitin N., Karpova O., Atabekov J. Development of avian influenza vaccine on the basis of structurally modified plant virus. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(4): 731-738 (doi: 10.15389/agrobiology.2017.4.731eng).
40. Sambrook J., Fritsch E.F., Maniatis T. *Molecular cloning: a laboratory manual.* 2nd edition. Cold Spring Harbor, Cold Spring Harbor Lab. Press, New York, 1989.
41. Settembre E.C., Chen J.Z., Dormitzer P.R., Grigorieff N., Harrison S.C. Atomic model of an infectious rotavirus particle. *The EMBO Journal*, 2011, 30(2): 408-416 (doi: 10.1038/emboj.2010.322).
42. Sehnal D., Rose A.S., Koča J., Burley S.K., Velankar S. Mol*: Towards a common library and tools for web molecular graphics. *Workshop on Molecular Graphics and Visual Analysis of Molecular Data.* J. Byška, M. Krone, B. Sommer (eds.). The Eurographics Association, 2018: 29-33 (doi: 10.2312/molva.20181103).
43. Berman H.M., Westbrook J., Feng Z., Gilliland G., Bhat T.N., Weissig H., Shindyalov I.N., Bourne Ph.E. The Protein Data Bank. *Nucleic Acids Research*, 2000, 28(1): 235-242 (doi: 10.1093/nar/28.1.235).
44. *Jmol: an open-source Java viewer for chemical structures in 3D.* Available: http://www.jmol.org. No date.
45. Mathieu M., Petitpas I., Navaza J., Lepault J., Kohli E., Pothier P., Prasad B.V.V., Cohen J., Rey F.A. Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion. *The EMBO Journal*, 2001, 20(7): 1485-1497 (doi: 10.1093/emboj/20.7.1485).
46. Hulo C., de Castro E., Masson P., Bougueleret L., Bairoch A., Xenarious I., Le Mercier P. ViralZone: a knowledge resource to understand virus diversity. *Nucleic Acids Research*, 2011, 39(Database issue): D576-D582 (doi: 10.1093/nar/gkq901).
47. Denis J., Majeau N., Acosta-Ramirez E., Savard C., Bedard M., Simard S., Lecours K., Bolduc M., Pare C., Willems B., Shoukry N., Tessier P., Lacasse P., Lamarre A., Lapointe R., Macias C., Leclerc D. Immunogenicity of papaya mosaic virus-like particles fused to a hepatitis C virus epitope: evidence for the critical function of multimerization. *Virology*, 2007, 363: 59-68 (doi: 10.1016/j.virol.2007.01.011).
48. Rioux G., Babin C., Majeau N., Leclerc D. Engineering of papaya mosaic virus (PapMV) nanoparticles through fusion of the HA11 peptide to several putative surface-exposed sites. *PLoS ONE*, 2012, 7(2): e31925 (doi: 10.1371/journal.pone.0031925*)*.
49. O'Hagan D.T., De Gregorio E. The path to a successful vaccine adjuvant — "The long and winding road". *Drug Discovery Today*, 2009, 14(11-12): 541-551 (doi: 10.1016/j.drudis.2009.02.009).
50. Del Giudice G., Rappuoli R., Didierlaurent A.M. Correlates of adjuvanticity: a review on adjuvants in licensed vaccines. *Seminars in Immunology*, 2018, 39: 14-21 (doi: 10.1016/j.smim.2018.05.001).

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SOILS OF CHERNEVAYA TAIGA OF WESTERN SIBERIA — MORPHOLOGY, AGROCHEMICAL FEATURES, MICROBIOTA

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Abstract

The soils of Chernevaya taiga are unique in terms of high fertility that was formed not as a result of agricultural practices, but due to the combination of a huge volume of biotic and abiotic resources. This area was able to preserve its "pre-agricultural" level of fertility overtime by avoiding the negative consequences of long-standing agricultural usage. Comprehensive analysis of all related properties within the framework of a metagenomic study and identification of microbial drivers of fertility can become the basis for innovative technologies aimed to increase the productivity of soils and crops. In this work, for the first time were obtained data on the taxonomic structure and features of the microbiota of soils in the Chernevaya taiga and identified taxa, the number of which significantly increases with the transition from the mature zonal soil to the soil of Chernevaya taiga. Analysis of soil samples collected during expeditionary surveys in 2019 showed that the soils in the Western Siberia (Novosibirsk, Tomsk, Kemerovo, and Altai regions) portion of the Chernevaya taiga are texture-differentiated dark gray soils (clay loam and silt clay varieties confined to the deluvial cover of the Holocene and Late Pleistocene) that were formed as a result of a unique combination of geogenic and bioclimatic conditions. These soils are not affected by the permafrost in winter timers and are supplied with enough moisture to precipitate rapid mineralization of litter material and the fixation of mineral nutrients in the upper humus layer of the soil profile. The accumulation of nutrients is an essential property of the soils of the Chernevaya taiga associated with the phenomenon of gigantism and extremely high levels of plant productivity. The soils of Chernevaya taiga contain the maximum amount of carbon in organic compounds compared with soils of oligotrophic habitats (9.85% versus 2.74%). The levels of actual soil fertility in the soils of the Chernevaya taiga are several times higher than in the soils of adjacent biotopes (the maximum content of the exchange forms of phosphorus and potassium is 702 and 470 mg/kg), which, when compared to oligotrophic forests, are poor in terms of agrochemical fertility (the maximum content of the exchange forms of phosphorus and potassium is 113 and 18 mg/kg), do not have a pronounced humus profile and are either gray-humus (Umbrisol) or Podzol types according to substantive-profile classification of Russian soils. The diversity of microorganisms in the studied soils varies depending on the trophic regime of the ecosystem. The soils of the Chernevaya taiga are characterized by an increased diversity of the microbial community (estimated

by the Shannon index), as well as by presence of phyla *Nitrospirae* and *Thaumarchaeota*, that, however, are not dominant. Phyla *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Firmicutes* appeared to be common for all studied soils.

Keywords: soil ecological functions, Chernevaya taiga, microbial communities, NGS, fertility factors, Western Siberia

The global dominance of humanity in the biosphere is determined primarily by agriculture development, which covers more than half of the Earth's land area. It leads to fundamental changes in the environment and climate [1], food and energy shortages, loss of biodiversity and ecosystem stability, and natural water pollution [2-5]. More than 60% of all land changes in the period from 1982 to 2016 are related to human activity, which indicates the controlling role of humanity in the development of the Earth [6]. The problems caused by the anthropogenic transformation of the soil cover and the ecological soil functions are among the most important in the current century [7]. Modern humanity is obliged to preserve and protect the soil since soil resources are the basis of food and environmental security of nations. Long-term use of soils in agricultural production has many negative consequences for soil properties, including organic matter degradation (including dehumidification), increased greenhouse gas emissions, and diversification of its emission products, changes in the acid-base composition of the soil, soil depletion, etc. [8-10]. The use of agricultural technologies (plowing and loosening, application of mineral and organic fertilizers, etc.) significantly changes the biogeochemical cycles, including those involving soil microorganisms. The general trend of these changes is manifested in a decrease in the soil ability to provide agricultural plants with the necessary amount of mineral nutrition elements and nitrogen. In this regard, the agroecosystem cannot function effectively without the introduction of additional doses of nutrients in the form of fertilizers [10].

The introduction of fertilizers into the agroecosystem is accompanied by the emergence of new problems, namely, the significant removal of elements from the agroecosystem with surface and subsurface water runoff [11-13]. Moreover, the return of agriculture from fertilizer application decreases over time [14], and with an increase in the doses of applied nitrogen, the efficiency of its retention by the agroecosystem decreases [13]. These unavoidable processes can be slowed down if a sufficient pool of microbial biomass is created in the soil, which can accumulate elements of mineral nutrition and nitrogen of organic origin in the periods between harvesting and planting of crops. Moreover, some biogeochemical mechanisms that make it possible to retain some of the nitrogen introduced with fertilizers in ecosystems for decades, even under the conditions of existing agricultural practices, are known [15]. It means that the problem caused by a decrease in the effectiveness of fertilizers during their long-term use will be solved by technologies that help maintain and realize the soil fertility potential.

In our opinion, the necessary pool of microbial biomass can be formed bionically, by using models of natural microbiota that can maintain the highest possible biological productivity of vegetation in humid autonomous landscapes as a basis due to the sufficient closure of the biological cycle of substances in the system. In the conditions of the percolative regime, it is ensured by maintaining the cycle volume of mineral nutrition elements. The composition of such a microbiota should develop evolutionarily with a gradual increase in the volume of the biological cycle due to the constant exogenous supply of fertilizers in the case of their weathering and nitrogen in the process of nitrogen fixation. The factors that disrupt these events, by “breaking” the evolutionary cycles, include global natural and anthropogenic cataclysms, catastrophic fires, and plowing. The use of microbiota could become one of the directions of biological soil reclamation, which is extremely important for the agricultural landscapes of Russia [16].

The soils of Chernevaya taiga are a clear example of the key ecological functions of the edaphotope associated with fertility and forest-growing properties. Its study is important for understanding the phenomenon of high soil fertility. The unique combination of soil formation factors in Chernevaya taiga triggers the drivers of intensive soil formation and the circulation of elements. It should be noted that at present, practically no such ecosystems are left in the moderate climate. As a rule, the forest ecosystems of Europe are either located on the place of former agricultural land or were located on their periphery and experienced a powerful influence of the pyrogenic factor [17]. Primary undisturbed ecosystems, which are extremely productive and maximally biogeochemically intensive bioinert formations, should be sought within the barrier-rain landscapes of Siberia.

The main areal of Chernevaya taiga is located in the altitude range of approximately from 200 to 700-800 m on the western, windward macroslopes of the mountains and foothills of the south of Western Siberia. Chernevaya taiga belongs to the type of barrier-rain landscapes and is characterized by a complex of features [26-31]: fir and aspen are dominants in the stand; the grass layer formed by Siberian tallgrass species is well-developed; large shrubs are present in the underwood; the synusia of ground leafy mosses is poorly represented, the epiphytic bryoflora is quite rich; the flora is represented by a complex of relatively thermophilic nemoral species; the spring synusia of ephemeroids is strongly developed; in winter, a deep snow cover is formed (from 80 cm to 2 m or more), due to which the soils do not freeze, which is not typical for the continental climate of the taiga of Western Siberia; there is no stratified ground litter, the decomposition time of the litter is less than 2 years; the number of earthworms is one of the highest in Russia, in general, mesofauna activity is high, including the winter period. Due to the above factors and the soil features of Chernevaya taiga, its bioproductivity in comparison with other zonal types of ecosystems of the temperate zone is maximum.

The tallgrass Chernevaya taiga in the foothills and mountains of southern Siberia is one of the largest preserved massifs of tallgrass forests in Russia. Tallgrass forests are also typical for the Far East. In general, within the taiga zone, tallgrass forests have been preserved in the form of isolated areas, for example, in the Cis-Ural region and on the western slopes of the Urals, on the plains of Western Siberia, in places that have been least exposed to fires and the effects of traditional nature management [21, 22]. The least disturbed areas of tallgrass forests meet the definition of climax ecosystems according to both phytocenotic and soil criteria. The annual litter of tallgrass rapidly decomposes, which over time leads to the accumulation of mineral elements in the humus horizon [18] and supports an active biological cycle of substances, heterogeneity of the intra-cenotic environment, and high biodiversity. The water and climatic conditions and the specific hydrophysical characteristics of the respective soils probably contribute to the stabilization of plant food elements in the soil profile.

The choice of tallgrass forests as a bionic model is associated with the fact that they were the least exposed to exogenous disturbances in the past [19, 20]. In biogeographic studies, it is shown that exogenous disturbances lead to the structure simplification (spatial, species) of communities [23]. There is a point of view that the modern zonal ecosystems of the southern, middle, and northern taiga are seral series of vegetation restoration after exogenous disturbances — fires, logging, plowing, in other words, during demutation shifts [20]. Almost all of these forests are litter-bearing, with a predominance of green mosses, small-grass and low-bush species. Among them, only boreal-nemoral tallgrass forests are considered by some authors as relatively fully corresponding to the final stage of autogenic succession [24].

The given point of view on the nature of boreal forests is debatable, but it well emphasizes the isolation of tallgrass forests. Despite the name, Chernevaya taiga actually differs significantly from the actual taiga, boreal ecosystems in terms of species and coenotic composition, nutrient status, and biogeochemistry. Chernevaya taiga is considered by most researchers as a sub-nemoral, or hemiboreal, ecosystem, that is, it belongs to another class of ecosystems than the boreal forest (the taiga itself) [25].

The uniqueness of the soils of Chernevaya taiga is in exceptionally high fertility, realized at the expense of internal biotic and abiotic resources, and the preservation of microbiota that is not affected by agricultural practices. The soils of Chernevaya taiga show some features, in particular, high forest growth activity with a general low accumulation of humus (effective fertility) [26], and an unusually high rate of decomposition of plant residues [27]. The analysis of such a complex of related properties in the framework of metagenomic research is a serious fundamental task. The result of its solution and the identification of microbial drivers of fertility can be innovative technologies to increase the productivity of soils and crops.

To study the soil microbiota, high throughput sequencing is used [28, 29], which makes it possible to identify components of microbial communities, including uncultivated ones, with previously inaccessible accuracy. It has become clear that the soil microbiota (especially the rhizospheric microbiota) plays an important role in plant nutrition and protection from biotic and abiotic stresses, which is why special attention is currently being paid to the analysis of rhizospheric communities of microorganisms [30].

It is obvious that the phenomenon of high soil fertility in Chernevaya taiga cannot be limited only by agrochemical and agrophysical parameters and must be associated with the characteristics of the soil microbiota. It can also be expected that studies of this potential source of new economically significant strains (for example, cellulolytic microorganisms, producers of antibiotics, and a variety of biologically active molecules) typical for the studied ecosystem will go beyond the limits of soil and agricultural microbiology. However, no data on the soil microbiota of Chernevaya taiga have been available so far.

This paper is the first one dedicated to characterization of the taxonomic structure of prokaryotic microbiota of the Chernevaya taiga soil. It is shown that the differences between the mature zonal soil and the soil of Chernevaya taiga are most likely determined at levels below the phylum. Taxa, the number of which significantly increases in the soil of Chernevaya taiga, were identified. These are predominantly unclassified prokaryotes. Among the identified microorganisms, the order *Chthoniobacterales* is of particular interest, the first representative of which was isolated only recently.

One of the major goals of this study is to determine the main morphological, agrochemical features, and taxonomic composition of the soil microbiota of Chernevaya taiga in Western Siberia in comparison with soils associated with oligotrophic ecosystems of pine forests on sandy soil-forming substrates.

Materials and methods. In the second half of July 2019, expeditionary surveys of soils have been conducted in the Tomsk, Kemerovo, and Novosibirsk Regions, as well as in the Altai. The objects of the study were four soil profiles (different variants of Chernevaya taiga in the Salair and Kuznetsk-Alataus areals), as well as two soils from relatively oligotrophic habitats associated with ancient Aeolian sand massifs covered with pine forests. The southern variants of the studied soils were represented by the following objects: N1 (Dark Gray Soil of Chernevaya taiga, Altai Territory; 54.14070°N, 84.9495°E), N2 (Dark Humus Luvisol, grass pine forest, erosion valley, Novosibirsk Region; 54.37083°N, 82.4393°E), N3 (Gray Humus Soil on aeolian sandy loams under oligotrophic coniferous forest

ecosystem, Novosibirsk Region; 54.40810°N, 82.18420°E); northern variants — T1 (dark gray soil in a tallgrass fir-aspen forest with a shrinking fir stand, Tomsk Region; 56.30693°N, 85.47063°E), T2 (Sod-Podzolic Soil, tallgrass broad grass birch-fir post-logging Chernevoy forest, Kemerovo Region; 55.88619°N, 86.00433°E), T3 (Sod-Eluvial Sandy Loam Soil under the oligotrophic ecosystem of mixed pine forest with an admixture of larch, Tomsk Region, coordinates: 56.48106°N, 84.79860°E).

The soil texture was analyzed according to Kaczynski with pyrophosphate peptization of microaggregates (the sedimentation method). The content of organic carbon and nitrogen was determined using an elemental analyzer (EURO EA-3028-HT, EuroVector S.p.A., Italy; resource center Chemical Analysis and Materials Research Centre of the St. Petersburg State University Research Park). The pH of the water extract was measured at a ratio of 1:2.5, when the pH of the water suspension was below 7.0. The pH of the salt suspension was measured at the same ratio. Mobile compounds of phosphorus and potassium were determined by the Kirsanov method modified by CINAO (GOST R 54650-2011. National standard of the Russian Federation. Soils), exchangeable ammonium — by the CINAO method (GOST 26489-85. State standard of the USSR. Soils), nitrates — by the ionometric method (GOST 26951-86. State standard of the USSR. Soil).

Basal respiration as an indicator of the biological metabolic activity of soils was measured according to the description [37] in closed chambers, taking into account the amount of CO_2 emitted over 7 days by the titration method.

The soil temperature regime was recorded with an automatic device for monitoring climatic parameters SAM-SM (Institute of Monitoring of Climatic and Ecological Systems SB RAS, Russia).

DNA for metagenomic analysis was isolated from soil samples using the NucleoSpin Soil kit (Macherey-Nagel GmbH & Co. KG, Germany) following the manufacturer's instructions.

Preparation of libraries for high throughput sequencing included amplification of the target fragment of the variable V4 region of the 16S rRNA gene using universal primers (515F — 5′-GTGCCAGCMGCCGCGGTAA-3′/806R — 5′-GGACTACVSGGGTATCTAAT-3′) [31] together with linkers and unique barcodes. Polymerase chain reaction (PCR) was performed on a T100 Thermal Cycler device (Bio-Rad Laboratories, USA) in 15 rl of a reaction mixture containing 0.5 units of Q5® High-Fidelity DNA Polymerase (New England BioLabs, USA), 1X Q5 Reaction Buffer, 5 pmol of each of the primers, 3.5 mM dNTP (Evrogen, Russia) and 1-10 ng of the DNA matrix. The PCR program included a denaturation stage at 94 °C − 1 min, product amplification for 35 cycles (94 °C — 30 s, 50 °C — 30 s, 72 °C — 30 s), and final elongation at 72 °C — 3 min. Further sample preparation and sequencing were performed following the Illumina protocol (16S Metagenomic Sequencing Library Preparation) on the Illumina MiSeq instrument (Illumina, Inc., USA) using the MiSeq Reagent Kit v3 (600 cycles) with two-way reading (2×300 n) (Illumina, Inc., USA).

Preprocessing of the received data included removal of service sequences using the Cutadapt program [32], as well as denoising, combining paired reads, and deleting chimeras using the Dada2 package [33] implemented in the R software environment. The taxonomic classification of the obtained amplicon sequence variants was also performed using release SILVA 132 database (https://www.arb-silva.de/documentation/release-132/) containing data for the SSU rRNA genes [34]. Further processing, including the construction of a phylogenetic tree using the SEPP algorithm [35], the calculation of α- and β-diversity, was performed within the QIIME2 package [36], and the plugins implemented in it. The diversity indices reflecting the actual predicted species richness (Chao1),

the degree of evenness according to Shannon and dom-inance according to Simpson were taken into account to assess the α-diversity,

The packages phyloseq [37] and DESeq2 [38] were used to identify differentially abundant taxa. taxa. Based on the results of the performed analysis, a sub-sample of phylotypes was formed, the change in the abundance of which is significant at $\alpha \leq 0.1$ (taking into account the Benjamini-Hochberg correction).

The total organic carbon and nitrogen content, soil basal respiration, and agrochemical parameters were measured in 3-fold analytical repetition and presented as mean (*M*) and standard errors of the mean (±SEM). The soil texture was evaluated in one repetition. To assess the correlation ratio between the parameters of soil availability of nutrients, Spearman's correlation coefficient *r* was calculated. The significance of the differences between the indicators for the soils of Chernevaya taiga and oligotrophic habitats was evaluated by one-way analysis of variance (one-way ANOVA). The taxonomic structure of soil microbiota samples was analyzed in 4 independent repetitions.

Results. Morphology and analytical characteristics. The morphological characteristics and texture of the soils of the selected sites (Fig. 1) are presented in Table 1. The studied sections had covered the main components of the biodiversity of Chernevaya taiga, represented on the surface clays by such contrasting variants as dark gray and sod-podzolic soils [39].

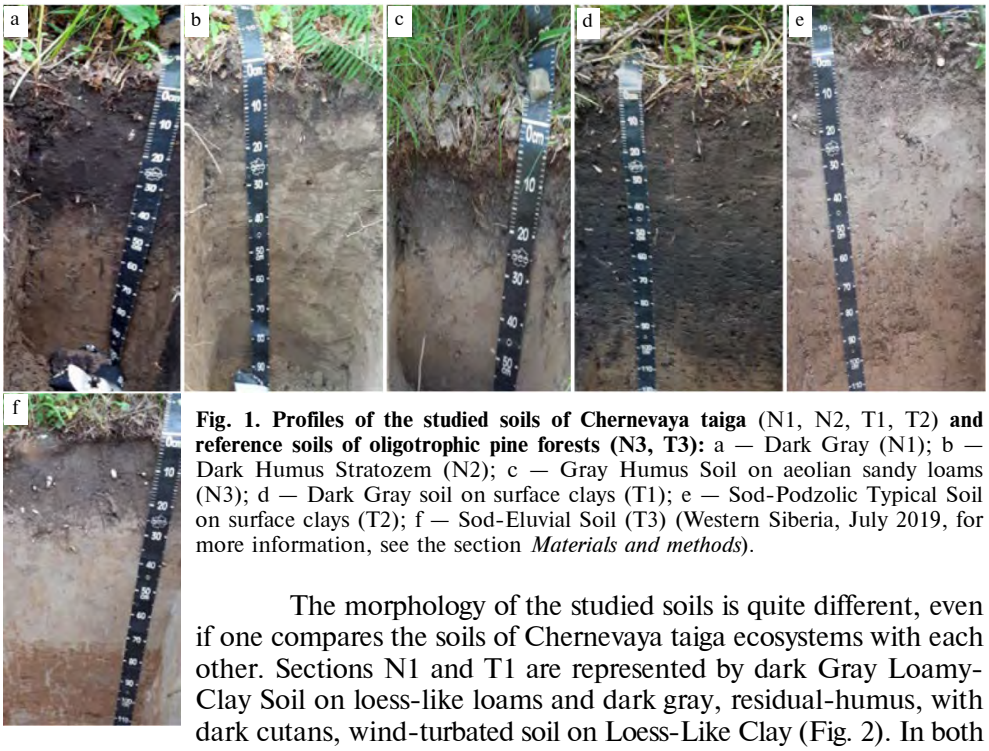


Fig. 1. Profiles of the studied soils of Chernevaya taiga (N1, N2, T1, T2) **and reference soils of oligotrophic pine forests (N3, T3):** a — Dark Gray (N1); b — Dark Humus Stratozem (N2); c — Gray Humus Soil on aeolian sandy loams (N3); d — Dark Gray soil on surface clays (T1); e — Sod-Podzolic Typical Soil on surface clays (T2); f — Sod-Eluvial Soil (T3) (Western Siberia, July 2019, for more information, see the section *Materials and methods*).

The morphology of the studied soils is quite different, even if one compares the soils of Chernevaya taiga ecosystems with each other. Sections N1 and T1 are represented by dark Gray Loamy-Clay Soil on loess-like loams and dark gray, residual-humus, with dark cutans, wind-turbated soil on Loess-Like Clay (Fig. 2). In both soils, there is a well-developed deep dark humus horizon, which has a more complex structure in T1, which is associated with signs of residual humus formation, namely, a decrease in lightness on the Munsell scale in Fig. 1 (see Table 1). This horizon in both soils is characterized by a well-developed three-dimensional lumpy structure, which is gradually replaced by prismatic and nutty-prismatic separations (Fig. 2, A, B). The dark humus horizon in the first case passes into a subeluvial stratum with signs of the EL eluvial horizon in the form of separate small morphons. In the second case, the dark humus horizon is replaced by the flow-humus horizon Ahh, which is characterized by an increased moisture content, the presence

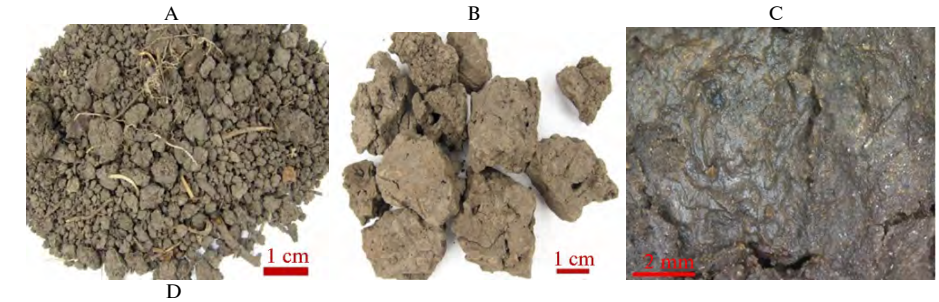


Fig. 2. Mesomorphology of the dark gray soil of Chernevaya taiga: A — forest version of the AU horizon, upper part; B — nutty structure of the lower part of the AU horizon (forest version); C — cutans in the Ahh subhorizon of the lower part of the deep AU (forest version); D — cutans in the BT horizon (Western Siberia, 2019).

of thin dark clay-humus cutans on the faces of structural separations, emphasized by a darker color of the interface surfaces in comparison with the intra-surface mass.

1. Morphological characteristics, the color of soil horizons, the skeletal and fine soil content in the studied soils of Chernovaya taiga (Western Siberia, 2019)

Depth, cm	Horizon	Munsell color code	Skeletal content, %	Fine soil content, %
N1, Dark Gray Soil (Altai Region)				
0-10	O	10 YR 3/1	No data	No data
10-20	AU	10 YR 4/1	11	89
20-30	AU	10 YR 4/1	12	88
30-40	BEL	10 YR 6/2	18	82
40-50	BEL	10 YR 6/2	21	79
60-80	BI	5 YR 6/3	19	81
80-100	BC	5 YR 6/3	21	79
N2, Dark Humus Stratozem (Novosibirsk Region)				
0-2	O	10 YR 3/1	No data	No data
2-10	AU	10 YR 4/1	22	78
10-20	RU	5 YR 2.5/1	11	89
30-40	RU	5 YR 2.5/1	0	100
60-70	RU	5 YR 2.5/1	0	100
80-90	C	7.5 YR 8/1	0	100
N3, Gray-Humus Soil on aeolian sandy loams (Novosibirsk Region)				
0-3	O	10 YR 4/1	No data	No data
3-15	AY	7.5 YR 8/1	6	94
20-30	AC	7.5 YR 8/1	4	96
40-50	C	7.5 YR 8/1	2	98
T1, Dark Gray Soil (Tomsk Region)				
0-1	O	10 YR 4/1	No data	No data
1-15	AU	10 YR 4/1	19	81
15-30	AU	10 YR 4/1	18	82
35-55	Ahh	10 YR 3/3	19	81
70-110	BTtur	5 YR 4/6	21	79
T2, Sod-Podzolic Soil (Kemerovo Region)				
0-3	AY	7.5 YR 6/1	93	7
3-17	EL	5 YR 7/1	25	75
20-30	BEL	5 YR 7/1	18	82
30-40	BT	5 YR 4/6	21	79
40-50	BT	5 YR 4/5	22	78
60-70	BT	5 YR 4/5	23	77
80-90	BCi	5 YR 6/3	5	95
105-120	BCi	5 YR 6/3	10	90
T3, Sod-Eluvial Soil (Tomsk Region)				
0-3	O	10 YR 4/1	No data	No data
10-20	AY	7.5 YR 8/1	5	95
40-50	EL	5 YR 7/1	2	98
50-60	BT	5 YR 4/6	1	99
60-80	BT	5 YR 4/6	1	99

Note. N1, N2, T1, T2 — soils of Chernevaya taiga, N3, T3 — reference soils of oligotrophic pine forests.

Dark Gray Soils are not widely distributed in Chernevaya taiga. They have not been previously described for the low-mountain part, although the authors' studies have shown that there is a high probability of meeting them in the lower parts of the slopes, in hollows, that is, in places with additional moisture. Apparently, the fact that such soils are not mentioned in the works of other authors on Chernevaya taiga is due to the insufficient geographical knowledge of this region. The two examined profiles, N1 and T1, differ in the features of the lower part of the deep dark-humus horizon. In the N1 profile, eluvial morphons appear, which is associated with active subsurface runoff in low-mountain terrain, when in this part there is no accumulation, but the concentration of flows and the removal of a fine fraction, which is enriched with humus. A relative accumulation of skeletal, a dusty fraction that has light tones happens in this place. Such morphons indicate the evolution of this soil towards the dark humus butterburs, which are widely distributed in the foothill sub-taiga of Western Siberia (Prialtaiskaya soil province), where they occupy a reduced position in micro-, less often in mesorelief. In the T1 profile, the Ahh subhorizon is isolated in the lower part of the deep AU horizon. It is the second humus horizon bearing signs of illuvial transformation, which is diagnosed by dark humus-clay cutans on the interface surfaces (see Fig. 2, C). Clay cutans settle due to the slow flow of the topwaters (which is confirmed by a series of field observations in spring and late summer) in the conditions of hollows that drain the gentle slopes relative to the flat terrain of the northern part of the studied area of Chernevaya taiga. Below in the textural horizon, the composition of the cutans is preserved, but their thickness and abundance increase (see Fig. 2, D).

The soils of points N2 and T2 are represented by the next stage of nutrient status, that is, not Dark Gray Soils, but Stratozem and Sod-Podzolic ones, both of which are also typical for Chernevaya taiga. The Dark-Humus Medium-Loamy Stratozem on stratified loams (slide-rocks) is located in the erosional valley of the Karakan River under a grassy pine forest with the presence of aconite. Sod-Podzolic Medium Loam on loess-like loam is located in the post-logging aspen-birch-fir tallgrass forest of Chernevaya taiga.

The soils of points N3 and T3 (reference soils of oligotrophic pine forests) are represented by relatively oligotrophic variants since in their origin they are associated with light soil-forming rocks and pine forests: N3 – Gray-Humus Sandy Loam on aeolian sandy loam in the grass-pine brake-sedge forest, T3 — Sod-Eluvial Sandy Loam under the oligotrophic ecosystem of mixed-grass pine forest with an admixture of larch. At the same time, within the T3 point, there are plant species typical for the Chernevoy forest: *Cacalia hastata* L., *Aconitum septentrionale* (Koelle) Korsh., *Milium effusum* L. However, their abundance and viability are significantly lower than in the studied Chernevye forests. The soil profiles of Chernevaya taiga are usually more differentiated by the content of silt and physical clay compared to the soils of the oligotrophic variants (Fig. 3), which indicates the intensive development of eluvial processes.

Thus, it can be seen that the type of soil and its profile organization largely correspond to the type of forest. Thus, Chernevaya taiga is not formed on soils confined to sandy rocks with light soil texture. Forests of Chernevaya taiga are confined to either dark gray or Sod-Podzolic Soils with a pronounced binomial structure of the soil profile, loam-clay soil texture.

Chemical analysis showed a gradual decrease in the profile distribution of carbon and nitrogen (Table 2), which is in principle typical for texture-differentiated and gray-humus soils. The highest content of organic carbon and nitrogen is typical for the upper organomineral horizons of the soils of Chernevaya taiga, and it is precisely for dark gray soils, which indicates their maximum trophicity. The

soils of oligotrophic habitats have, on average, the lowest humus content. Approximately the same patterns apply to the total nitrogen content. The ratio of carbon to nitrogen [40] indicates an average or high nitrogen content of the soil. The studied soils are close to neutral or slightly acidic, which is typical for texture-differentiated soils. At the same time, the soils of oligotrophic habitats are more acidic, which is associated with less buffering of sandy loam fine soil compared to loam [41]. The intensity of basal respiration was generally higher in oligotrophic soils (points N3 and T3), and the profiles — in the bedding horizons.

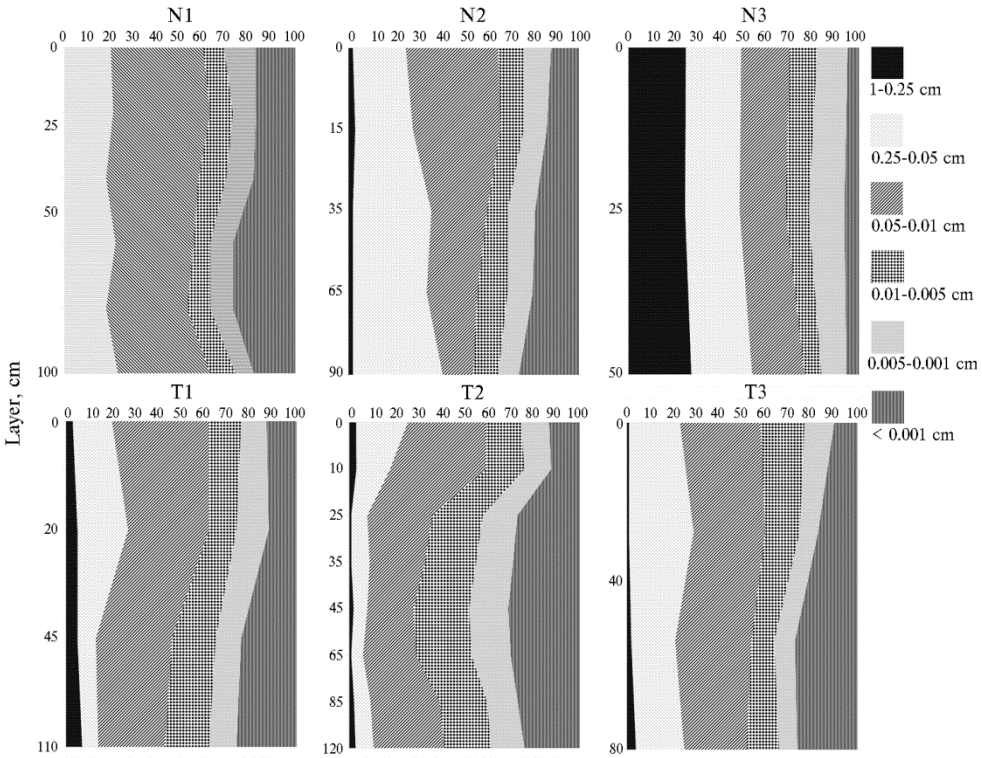


Fig. 3. Profile diagrams of the soil texture of the studied soils: N1, N2, T1, T2 — soils of Chernevaya taiga, N3, T3 — reference soils of oligotrophic pine forests (Western Siberia, 2019). For a description of the soil samples, see the section *Materials and methods* and Table 1.

2. Carbon and nitrogen content, acidity, and basal respiration of the studied soils (n = 3, M±SEM, Western Siberia, 2019)

Code, depth, cm	C, %	N, %	C/N	pH of the extract		Basal respiration, mg of CO_2/(100 g of soil · day)
				water	saline	
N1 0-10	4.76±0.23	0.37±0.05	12.81	6.56	5.82	0.022±0.003
N1 10-20	3.22±0.17	0.27±0.04	11.57	6.13	5.20	0.019±0.002
N1 20-30	2.40±0.17	0.18±0.03	13.00	6.02	4.69	0.013±0.002
N1 30-40	2.25±0.10	0.17±0.03	12.88	5.85	4.64	0.021±0.002
N1 40-50	1.41±0.07	0.11±0.02	12.71	5.44	4.89	0.021±0.002
N1 60-80	0.45±0.05	0.06±0.02	8.14	6.30	4.53	0.020±0.004
N1 80-100	0.24±0.04	0.05±0.01	5.42	6.39	4.57	0.028±0.003
N2 0-2	2.14±0.21	0.16±0.01	13.51	6.95	5.96	0.062±0.001
N2 2-10	2.02±0.19	0.15±0.01	13.59	7.35	No data	0.047±0.009
N2 10-20	0.23±0.04	0.03±0.01	6.45	7.22	No data	0.037±0.008
N2 30-40	0.16±0.04	0.03±0.01	5.68	7.09	No data	0.040±0.002
N2 60-70	0.15±0.02	0.03±0.01	5.18	6.81	5.70	0.041±0.004
N2 80-90	0.46±0.09	0.05±0.01	8.44	6.65	5.65	0.041±0.004
N3 0-3	2.70±0.21	0.17±0.03	15.46	6.70	6.20	0.051±0.004
N3 3-15	0.21±0.04	0.03±0.01	7.49	5.93	5.52	0.029±0.007
N3 20-30	0.05±0.02	0.02±0.01	2.85	6.06	4.91	0.045±0.008
N3 40-50	0.03±0.05	0.01±0.01	6.00	6.39	5.38	0.052±0.007

Continued Table 2

T1 0-1	9.85±0.28	0.62±0.04	15.66	6.59	6.32	0.206±0.006
T1 1-15	3.46±0.25	0.28±0.05	12.31	5.96	5.14	0.048±0.007
T1 15-30	2.37±0.20	0.31±0.02	7.67	5.93	4.71	0.041±0.004
T1 35-55	1.84±0.12	0.14±0.02	12.73	5.28	4.69	0.043±0.001
T1 70-110	0.76±0.09	0.08±0.02	9.00	5.97	4.86	0.048±0.001
T2 0-3	3.97±0.42	0.37±0.02	10.75	6.72	6.16	0.071±0.007
T2 3-17	1.38±0.12	0.14±0.03	9.44	5.33	4.82	0.053±0.002
T2 20-30	0.56±0.07	0.06±0.02	8.08	5.70	4.38	0.028±0.003
T2 30-40	0.38±0.07	0.04±0.01	7.89	5.17	4.26	0.053±0.008
T2 40-50	0.47±0.09	0.07±0.01	6.98	6.03	4.25	0.059±0.008
T2 60-70	0.29±0.04	0.05±0.01	6.30	5.61	4.17	0.055±0.007
T2 80-90	0.20±0.04	0.04±0.01	4.98	5.86	4.30	0.052±0.008
T3 0-3	2.41±0.31	0.20±0.03	12.24	6.96	6.17	0.078±0.001
T3 10-20	0.36±0.08	0.04±0.02	8.62	5.16	4.40	0.053±0.003
T3 40-50	0.07±0.02	0.02±0.01	3.57	5.18	4.54	0.042±0.003
T3 50-60	0.09±0.02	0.03±0.01	3.26	5.69	4.66	0.057±0.004
T3 70-110	0.07±0.01	0.03±0.01	2.25	5.73	4.69	0.063±0.007
One-way ANOVA:						
	$p < 0.04$	$p < 0.03$				$p < 0.04$

N o t e. N1, N2, T1, T2 — soils of Chernevaya taiga, N3, T3 — reference soils of oligotrophic pine forests.

3. Agrochemical parameters of the studied soils (n = 3, M±SEM, Western Siberia, 2019)

Code, depth, cm	P, mg/kg	K, mg/kg	N-NH_4, mg/kg	N-NO_3, mg/kg
N1 10-20	343±21	319±25	11.14±2.15	15.57±1.53
N1 20-30	357±24	266±17	4.33±0.23	10.07±0.58
N1 30-40	702±34	217±14	2.76±0.22	8.59±0.41
N1 40-50	460±22	71±6	0.73±0.06	5.37±0.32
N1 60-80	605±23	133±8	0.41±0.07	5.37±0.42
N1 80-100	682±45	120±8	0.41±0.04	4.97±0.41
N2 0-2	255±20	198±11	10.23±0.97	14.53±1.01
N2 2-10	210±13	174±12	5.42±0.23	11.24±0.85
N2 10-20	178±11	125±8	0.41±0.05	9.54±0.41
N2 30-40	223±11	114±8	0.32±0.04	4.51±0.31
N2 60-70	200±15	95±8	0.25±0.03	7.54±0.52
N2 80-90	210±13	152±11	0.95±0.08	8.56±0.45
N3 3-15	113±8	195±16	0.57±0.05	7.11±0.40
N3 20-30	131±9	71±5	0.49±0.04	4.56±0.25
N3 40-50	243±12	58±4	0.73±0.05	4.03±0.14
T1 1-15	234±15	470±45	7.32±0.54	8.59±0.61
T1 15-30	166±10	355±32	2.20±0.04	7.52±0.45
T1 35-55	231±16	262±22	1.06±0.08	8.19±0.35
T1 70-110	373±16	186±14	0.49±0.05	6.71±0.50
T2 3-17	79±7	200±29	12.69±0.89	18.93±0.33
T2 20-30	76±7	106±15	1.79±0.09	9.66±0.12
T2 30-40	107±8	62±6	0.65±0.05	6.98±0.15
T2 40-50	104±7	98±8	BLD	7.25±0.40
T2 60-70	82±5	142±12	0.24±0.03	12.89±0.60
T2 80-90	87±5	151±14	BLD	9.40±0.10
T2 105-120	184±6	160±12	BLD	8.46±0.20
T3 10-20	44±5	106±11	0.09±0.01	12.21±0.98
T3 40-50	97±9	18±2	0.16±0.02	8.05±0.14
T3 50-60	319±22	22±2	0.08±0.02	6.31±0.50
T3 70-110	234±24	71±3	0.16±0.03	8.32±0.32
One-way ANOVA	$p < 0.05$	$p < 0.04$	$p < 0.04$	$p < 0.03$

N o t e. N1, N2, T1, T2 — soils of Chernevaya taiga, N3, T3 — reference soils of oligotrophic pine forests. BLD — below limit of detection.

The distribution of available forms of phosphorus in the soil profiles was inhomogeneous. Thus, there is a first maximum in the humus horizon, as well as a second maximum in the illuvial formation (Table 3). This distribution is typical for potassium, although sometimes the second maximum is just below the illuvial formation. As for the ammonium and nitrate forms of nitrogen, they are concentrated mainly in the upper horizons. At the same time, the predominance of nitrate forms of nitrogen in soils is typical.

The values of the Spearman's correlation coefficient calculated for the soils of Chernevaya taiga and oligotrophic habitats (Table 4) revealed in the studied

soils a negative correlation between the content of phosphorus and potassium, as well as phosphorus and ammonium and nitrate forms of nitrogen. This correlation was more pronounced in the soils of Chernevaya taiga, which indicates a higher degree of nutrient status in comparison with the soils of oligotrophic habitats. The correlation coefficients for potassium and other compared elements had positive values, and their values were higher for the soils of Chernevaya taiga. The accumulation of total nitrogen correlates well with the accumulation of potassium and nitrate nitrogen. For total nitrogen and ammonium nitrogen, no close relationships were found.

4. Spearman's correlation coefficients (*r*, p = 0.05) for agrochemical indicators of the studied soils (Western Siberia, 2019)

Indicator / Indicator	P	K	N-NH4	N-NO3	C	N
Soils of Chernevaya taiga (N1, N2, T1, T2)						
P	1	–0,75	–0,37	-0,27	–0,48	–0,55
K	–0.75	1	0.78	0.69	0.17	0.20
N-NH4	–0.37	0.78	1	0.93	–0.06	–0.003
N-NO3	–0.27	0.69	0.93	1	0.08	0.11
C	–0.48	0.17	–0.06	0.08	1	0.98
N	–0.55	0.20	–0.03	0.11	0.98	1
reference soils of oligotrophic pine forests (N3, T3)						
P	1	–0.36	0.24	–0.67	–0.17	–0.10
K	–0.36	1	0.19	0.45	0.40	0.46
N-NH4	–0.24	–0.19	1	–0.71	–0.03	–0.04
N-NO3	–0.67	0.45	–0.71	1	0.35	0.37
C	–0.17	0.40	–0.03	0.35	1	0.98
N	–0.10	0.46	–0.04	0.37	0.98	1

Thermal monitoring conducted for the Dark Gray Soil (T1) showed that during the winter period (2019-2020), the soil practically did not freeze. Negative temperatures (from –1 to 0 °C) began to penetrate the soil in early November, reaching a depth of 30 cm by the end of December, after which the zero level (temperatures from +0.1 to –0.1 °C) slowly fell to a depth of 40 cm by mid-February. Within these depths, a great constancy of temperatures was observed in winter. Only in the first centimeters from the surface, the temperature could fall below –0.1 °C, but not reaching –2 °C. In the summer, the soil warmed up to +12 °C to a depth of 50 cm, and the maximum temperature detected was +16 °C at the depth of 10 cm. It is probably due, among other reasones, to the high humidity of the soil.

It should be noted that Chernevaya taiga is characterized by unusually high biological productivity of all components of the ecosystem. No other autonomous landscapes in Siberia can compete with Chernevaya taiga either in terms of the volume of "living matter" or in terms of the intensity of its impact on geogenic and microclimatic factors. According to available data, the mass of annual ground litter of plants is about 55-63 c/ha of dry matter per year, and the biomass is expressed in the following figures: phytomass − up to 4000 dt/ha, zoomass (herpetoria and pedobionts) — 4-8 dt/ha, the biomass of soil-dwelling microorganisms — 80-90 dt/ha [42]. In forests of Chernevaya taiga, tallgrass supplies 28-30 dt/ha of the total annual amount of ground litter of 55-63 dt/ha of dry matter. The content of nitrogen and ash elements in the litter of the grassy layer is significantly higher than in the material of the wood litter (the ash content of the grassy layer is 11.3%, and the ash content of the wood layer is 3.4%, the amount of nitrogen is 2.4 and 1.6 %, respectively) [26]. The tree layer, which has a huge phytomass, returns annually to the biological cycle a disproportionately small amount of ash elements and nitrogen — almost 3-4 times less than their annual intake to the soil surface when the grass stands die off. Phytomass reserves in Chernevaya taiga are 1.5-2 times higher than in the lowland southern taiga of

Western Siberia, nitrogen reserves are 2-2.5 times higher, calcium reserves are 1.4-1.8 times higher, and the annual intake of Ca with litter is 4 times higher [41]. It is known about the study, according to which the content of phosphorus in the soils of Chernevaya taiga is 879-1042 mg/kg [43]. Let us note that it corresponds to the upper range typical for other ecosystems of the Earth's biosphere (including rainforests of the tropical and temperate zones). It has been reported that in terms of the number of actinomycetes and spore-forming forms of bacteria, these soils are close to some steppe soils, black soils, while they are characterized by small absolute and relative numbers of fungi [33].

The rate of litter cycle in Chernevaya taiga is 1-1.5 years [44]. The litter consists of several fractions that differ in the decomposition rate (the lowest is in the needles of fir and cedar, birch leaves, the fall of branches, and the bark of shrubs and trees). The tallgrass fall is labile, decomposing in a year. The absence of litter, the retention of mineral substances from leaching in a humid climate, and the powerful development of tallgrass give the biological cycle some “tropical” features. That's why Chernevaya taiga is also called “Siberian tropics”.

Another feature of Chernevaya taiga is that the total content of calcium in the soil-forming rocks (loess-like clays) in a layer of 1 m is 3300 dt/ha [44]. This amount of calcium passes through the biological cycle in less than 6-7 thousand years. Therefore, in the absence of a reliable recycling mechanism, for which tallgrass parcels are responsible, Chernevaya taiga could not exist. This is what can be observed in the southern part of the forest zone of Western Siberia, where recycling mechanisms were disrupted by forest fires.

The most common component of the soil cover of Chernevaya taiga is texture-differentiated soils (Podzolic, Sod-podzolic, and Light Gray). The trend of the Holocene evolution of these soils consisted of the depletion of the root zone by silt and the periodic mixing of the soil mass by treefalls. At present, within the average depth of the root systems of the main forest-forming plants − fir and aspen, a relatively homogeneous horizon EL or AEL has been formed, with a thickness of 45 to 80 cm, under which the BT horizon lies. Performed measurements of the depths of the tree-fall hollows of fir and aspen in Chernevaya taiga showed that their average depth in normal humid conditions was 51 cm, in case of water-logging — 40 cm. At the same time, no significant differences in the depth of the aspen and fir hollows were detected. Such a soil structure corresponds to the definition of the climax profile of forest soil and indicates the absence of significant exogenous disturbances that interrupted the steady flow of generations of tree species.

The soil cover of Chernevaya taiga is a phenomenon of high-altitude differentiation of landscapes on the mountain macroslopes of southern Siberia, open to western moist air currents. In the mountains and foothills of Chernevaya taiga, there is an upper limit of distribution, above which in the area of medium-mountain terrain, mountain Podzolic Soils, Eluvozem — Pseudopodzolic Soils without an illuvial horizon [42], and Brown Soils [45] dominate in soil profiles. A geographically common feature for all soils of Chernevaya taiga is the presence of well-structured strata, light in the soil texture, with high-quality water-physical properties, represented in the studied soils by the AY, AEL, EL, AU, Ahh horizons. Below these horizons, there is always a water-resistant horizon, whether it is the surface loams and clays transformed into the BT horizon, or the debris-weathering crust and the deeper dense R horizon. The presence of such a binomial structure favors the formation of topwaters and longer retention of moisture, which in the conditions of the sub-boreal belt and high differentiation of the relief supports the possibility of the existence of tallgrass communities with sufficient

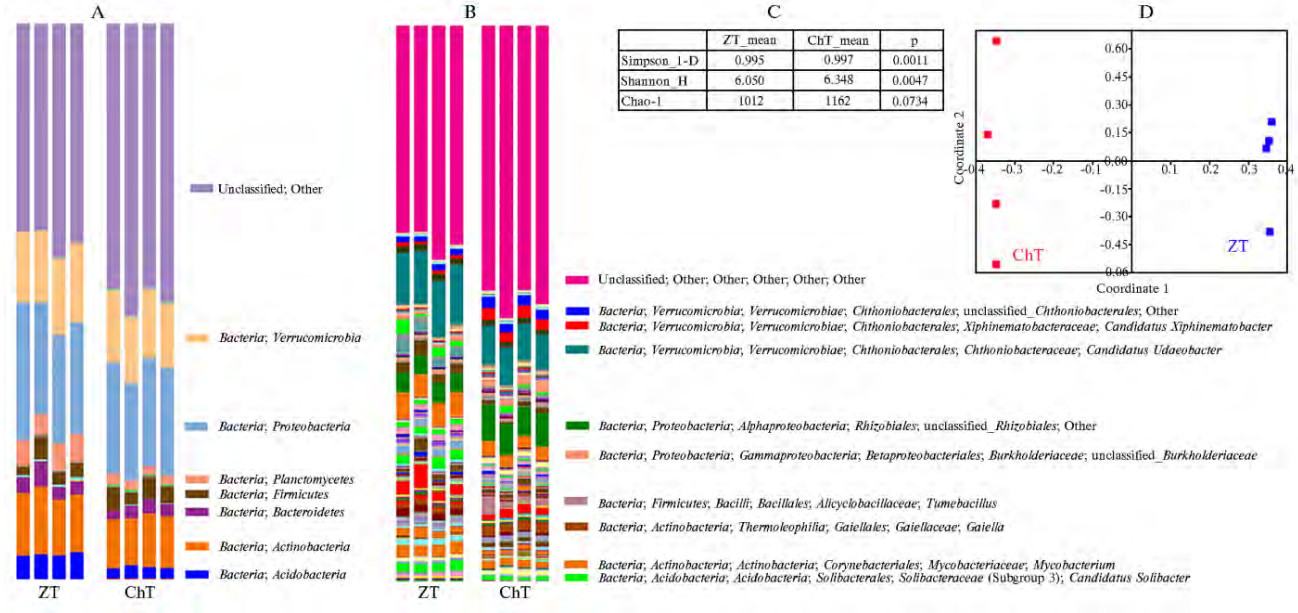
moisture supply in the dry season. Outside of such a binomial structure of soils, the functioning of tallgrass ecosystems is impossible, tallgrass will be replaced by mixed grasses or sedge phytocenoses. In spring and the rainy season, the presence of a well-structured half-meter thick layer contributes to the infiltration of moisture, which is why a favorable air regime is always maintained in the upper root horizon, which contributes to the favorable functioning of root systems. It is also proved by the fact that at the oligotrophic point N3, where there was no water-resistant horizon, there were no tallgrass herbaceous species in the community, and at the point T3, there were these species, since the presence of the horizon D at this point contributes to a favorable water regime, although the lack of nutrient status of the habitat does not allow tallgrass species to maintain their phytocenotic positions.

In the studied soils, the cutan complexes were also very different. Dark Gray Soils that occupy concave slopes have a better-formed cutan complex than Sod-Podzolic Soils.

The two main wood edificators of Chernevaya taiga are Siberian fir *Abies sibirica* Ledeb. and aspen *Populus tremula* L. When moving to the north, climbing into the mountains, in the lower parts of the slopes, aspen may give dominance to birch (*Betula pendula* L.). Chernevaya taiga ecosystems are closely related to Sod-Podzolic Ultra-Deep-Lit Soils, which are changed throughout the areal. As it has been already noted, the most significant are not the soils themselves, but their eluvial horizons in the presence of the underlying water-resistant horizon, as evidenced by the wide distribution of eluvial reservoirs in the mountains.

Soils with a strong eluvial profile dominate not only in the soil profiles of Chernevaya taiga but are also developed on the well-drained plains of Western Siberia [46-49]. The Ultra-Deep Podzolic Soils of the plain, as well as the mountains, are characterized by the spread of fir forests or forests dominated by fir. As it is noted [50], the coefficient of association of fir phytocenoses with Sod-Podzolic Soils is 1.0. During the expeditionary survey, it was confirmed that the fir had a deeply penetrating root system (a large number of anchor roots concentrated in the thickness of 1 m), it reacted to the nature of moisture, and in waterlogged areas, the root distribution becomes surface. With fir, cedar (*Cedrus* Trew, 1757, nom. cons.), which also has a deep root system, dominates as well. During the Holocene period, when the fir formation occupied territories with Deep-Podzolic Soils, there was repeated inversion of the soil layer during treefalls, as evidenced by a complex of signs of treefall disturbances. During successions of wind-turbated soils, the processes that cause eluvial cleavage and lessivage increase [51, 52], which leads to textural differentiation. It can be assumed that where the conditions for deep treefall (with the involvement of a thickness of up to 1 m) were more favorable, the formation of more deeply eluviated soils occurred. The strengthening of eluviation was undoubtedly favored by vigorous intra-soil erosion, which enhances the morphogenetic effect of treefalls.

High humus content is generally typical for Dark Gray Soils [53]. At the same time, the Dark Gray Soils under our study were characterized by a humus profile developed in depth, which indicates their high fertility. The humus content was slightly lower in Sod-Podzolic Soils and even lower in Gray-Humus and Sod-Podzols of oligotrophic pine forests, which corresponds to the literature data [54]. In the studied soils, no eluvial-illuvial differentiation of humus profiles was observed, which is more typical for texture-differentiated soils than for alpha humus soils [55]. According to our data, the acidity of the soils, was quite consistent with their nature and was increased in the case of Gray-Humus and Sod-Podzolic Soils, which is associated with the type of plant litter (pine) and the light soil texture of the soil-forming rocks.



	ZT_mean	ChT_mean	p
Simpson_1-D	0.995	0.997	0.0011
Shannon_H	6.050	6.348	0.0047
Chao-1	1012	1162	0.0734

Fig. 4. Taxonomic structure of the microbiota of the mature zonal taiga soil (ZT) **and Chernevaya taiga soil** (ChT) **at the level of phyla** (A) **and genera** (B)**; diversity indices** (C) **and the results of the β diversity analysis** (principal coordinate analysis, Bray-Curtis distances) (D) (Western Siberia, 2019) are presented.

The dissociation of the illuvial profiles of various nutrition elements is due to the unequal mobility of their forms in the soil environment [55]. The very presence of the second maximum is associated with the role of the eluvial type of soil formation in the formation of biologically active reserves of nutrients in various parts of the soil profile. The predominance of nitrate forms of nitrogen, which are easily accessible to plants (especially in the upper horizons), indicates the important role of nutrient intake from the litter.

Thus, the Dark-Gray Soils of Chernevaya taiga represent a peculiar variant of texture-differentiated soils, which differ from their European counterparts from non-Chernevoy forests by the development of the humus profile and the increased content of fertilizer elements, which contributes to the formation of a special nutrient status with a pronounced phenomenon of gigantism. At the same time, there is a decrease in the degree of morphochromatic differentiation of soil profiles due to the impregnation of humus substances.

Characteristics of the taxonomic structure of soil microbiota. The analysis of the taxonomic structure of the soil prokaryotic microbiota of the mature zonal soil and the soil of Chernevaya taiga, performed in four replications, showed that at the level of prokaryotic phylum, both soils had a similar structure and contained representatives of 15 phyla typical for soil microbial communities. Among them, *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Acidobacteria, Planctomycetes*, and *Firmicutes* dominated, and the proportion of unclassifiable microorganisms was also very high (Fig. 4, A).

A much finer structure can be identified at the genus level (see Fig. 4, B), from which become obvious that the differences between soils are most likely due to differences between taxa of a lower rank than phyla. The revealed dominants of the soil microbiota are typical for the majority of natural soils of the temperate zone [56], which includes the boreal type of soils and forests. The presence of acidobacteria is typical of soils with a slightly acidic reaction of the environment [38], which is one of the characteristics of the studied taiga soils. Representatives of the phylum Firmicutes can participate in the decomposition of organic alkyl fragments of forest litter, which also occurs in the studied soils, where there is a residual accumulation of well-humified organic matter. *Actinobacteri*a can also take part in the degradation of plant polymers, which confirms the high intensity of the biological cycle in the forest ecosystems of Chernevaya taiga and the rapid mineralization of low-molecular components of soil organic matter. Representatives of *Verrucomicrobia* indicate the presence of an intense zoogenic (possibly coprogenic) factor in soil formation, which was also confirmed by the results of our morphological study of soils.

The analysis of the diversity indices (see Fig. 4, C) showed that there was no significant difference in the number of identified taxa between the soils, but according to the Simpson and Shannon indices [57], it can be seen that there are, although small, but significant differences in the parameters due to both evenness and richness of diversity. The total effect can be seen in the analysis of β diversity (see Fig. 4, D), where a clear differentiation between the microbiota of the mature zonal soils and the soils of Chernevaya taiga is visible.

It is clear that possible differences in the taxonomic structure of the microbiotas of the mature zonal soil and the soil of Chernevaya taiga are most interesting to identify prokaryotic taxa, presumably associated with high fertility. As a result of the analysis, taxa were identified, the number of which significantly increases during the transition from the mature zonal soil to the soil of Chernevaya taiga. The total part of such taxa was 5.6% in the mature zonal soil and 32.2% in the soil of Chernevaya taiga. Table 5 shows a list of the identified taxa of the genus rank, grouped at the level of prokaryotic orders, showing a significant increase

in the number during the transition from the mature zonal soil to the soil of Chernevaya taiga.

More than half of the differentially presented taxa belong to unclassified prokaryotes, as, indeed, in the entire original microbiota. However, among the taxa that have been identified, there are several very interesting ones. In Table 5, taxa are sorted by abundance in the soil of the Chernaya taiga. This list is dominated by genera of the orders *Rhizobiales, Chthoniobacterales, Bacillales*, *Myxococcales*, each of which potentially has some connection with soil fertility, although it is difficult to prove it. Nevertheless, the order *Rhizobiales* can undoubtedly be associated with soil, mainly symbiotic, nitrogen fixers. *Bacillales* and *Myxococcales,* as well as the listed *Chitinophagales*, may be related to the decomposition of organic matter.

5. Taxa that increase in abundance during the transition from the mature zonal soil (ZT) **to the soil of Chernevaya taiga** (ChT)**, grouped at the level of prokaryotic orders** (Western Siberia, 2019)

Phylum	Order	Taxon abundance, %		Magnification degree	Number of genera
		ZT average	ChT average		
Unclassified		3.085	18.616	6.0	138
Proteobacteria	*Rhizobiales*	0.648	3.193	4.9	11
Verrucomicrobia	*Chthoniobacterales*	0.581	2.744	4.7	7
Firmicutes	*Bacillales*	0.120	1.456	12.1	7
Proteobacteria	*Myxococcales*	0.289	1.266	4.4	10
Actinobacteria	*Propionibacteriales*	0.199	0.772	3.9	5
Bacteroidetes	*Chitinophagales*	0.044	0.686	15.5	5
Actinobacteria	*Gaiellales*	0.106	0.451	4.3	2
Acidobacteria	*Pyrinomonadales*	0.131	0.382	2.9	1
Actinobacteria	*Micromonosporales*	0.106	0.304	2.9	2
Planctomycetes	*Pirellulales*	0.034	0.255	7.6	2
Actinobacteria	*Frankiales*	0.012	0.200	16.9	1
Verrucomicrobia	unclassified_*Verrucomicrobiae*	0.053	0.197	3.7	2
Actinobacteria	*Microtrichales*	0.014	0.171	12.5	2
Actinobacteria	*Corynebacteriales*	0.012	0.160	13.5	1
Actinobacteria	*Solirubrobacterales*	0.044	0.153	3.5	2
Proteobacteria	*Steroidobacterales*	0.005	0.147	29.3	1
Actinobacteria	unclassified_*Actinobacteria*	0.005	0.146	26.8	1
Verrucomicrobia	*Pedosphaerales*	0.026	0.139	5.4	1
Proteobacteria	*Xanthomonadales*	0.018	0.106	5.8	2
Actinobacteria	*Micrococcales*	0.018	0.094	5.3	1
Gemmatimonadetes	*Gemmatimonadales*	0.024	0.090	3.7	1
Bacteroidetes	*Flavobacteriales*	0.004	0.073	19.7	1
Actinobacteria	unclassified_*Actinobacteria*	0.010	0.066	6.9	1
Planctomycetes	*Isosphaerales*	0.019	0.063	3.3	1
Planctomycetes	*Tepidisphaerales*	0.008	0.063	7.6	1
Actinobacteria	*Streptosporangiales*	0.004	0.059	14.9	1
Actinobacteria	*Streptomycetales*	0.014	0.046	3.3	1

The order *Chthoniobacterales* noted in this list is quite interesting: only recently the first representative of this order was isolated in culture [58], and its genome was sequenced [59]. This taxon is interesting not only because it belongs to the phylum *Verrucomicrobia*, the ecological significance of which (especially in soil communities) has become apparent recently, but also because its "talking" name has some connection with the topic of this study since it comes from the Greek word χθών (earth, soil) and is used in ancient mythological and modern philosophical discourse to refer to "chthonic" creatures and essences that represent the primeval natural power of the earth. Certainly, it is not necessary to take this circumstance too clearly, but it is impossible not to pay attention to it since this taxon is one of the dominant ones, presumably related to the soil fertility of Chernevaya taiga. In any case, the taxonomic and functional composition of a specific component of the soil microbiota of Chernevaya taiga can become a source of new knowledge about the mechanisms of formation and maintenance of soil fertility.

Noteworthy is the actual absence of Archaeal phyla in mature zonal soil,

while the phylum *Thaumarchaeota* (0.1%) is represented in the soil of the Chernevaya taiga.

Thus, our data have clarified the morphological organization, taxonomic position, thermal regime, and texture of the soils of Chernevaya taiga of Western Siberia. It has been found that the soils of the Chernevaya taiga of Western Siberia mainly belong to the division of texture-differentiated soils, of Sod-Podzolic, Gray, and Dark Gray Soil types with clay loam and silt clay soil texture of soil-forming material. These soils are formed in unique combinations of geogenic and bioclimatic conditions, not affected by the permafrost in winter, supplied with moisture to precipitate the rapid mineralization of the litter material and the fixation of mineral nutrients in the upper humus layer of the soil profile. The accumulation of biophilic elements is the most important property of the soils of Chernevaya taiga, which is associated with the phenomenon of gigantism and extremely high plant productivity. Located in adjacent biotopes on light soil-forming material, the soils of oligotrophic forests are poor in terms of agrochemical fertility, do not have a pronounced humus profile, and belong either to the gray humus or to the Al-Fe humus and sod-eluvial variants. At the level of prokaryotic phyla, both soils have a similar structure and contain representatives of 15 phyla typical for soil microbial communities. In general, the taxonomic composition of the microbial phyla corresponds to that in moderately moist soils of the temperate zone. The differences between soils are most likely due to differences between taxa of the rank lower than phyla. The diversity of microorganisms in the studied soils varies depending on the nutrient regime of the ecosystem. The number of phylotypes in soil samples of Chernevaya taiga is increased in comparison with oligotrophic habitats. The soils of Chernevaya taiga are characterized by a greater variety of microbial communities according to the Shannon index, as well as the presence of the phyla *Nitrospirae* and *Thaumarchaeota*, which are not present in the soils of oligotrophic habitats. The Actinobacteria phylum, which is one of the prokaryotic dominants, provides a high intensity of the biological cycle in the forest ecosystems of Chernevaya taiga and rapid mineralization of low-molecular components of soil organic matter. Thus, the soils of Chernevaya taiga have a specific microbiota and the corresponding microbial drivers of soil processes responsible for the productivity of these soils. They are a unique component of the boreal ecosystems of Western Siberia, which allows gaining new knowledge about the mechanisms of increased soil productivity with a unique combination of bioclimatic and geogenic factors.

REFERENCES

1. Vitousek P.M., Mooney H.A., Lubchenco J., Melillo J.M. Human domination of Earth's ecosystems. *Science*, 1997, 277(5325): 494-499 (doi: 10.1126/science.277.5325.494).
2. Clark C. M., Tilman D. Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. *Nature*, 2008, 451(7179): 712 (doi: 10.1038/nature06503).
3. Diaz R.J., Rosenberg R. Spreading dead zones and consequences for marine ecosystems. *Science*, 2008, 321(5891): 926-929 (doi: 10.1126/science.1156401).
4. Marques A., Martins I.S., Kastner T., Plutzar C., Theurl M.C., Eisenmenger N., Huijbregts M.A.J., Wood R., Stadler K., Bruckner M., Canelas J., Hilbers J.P., Tukker A., Erb K., Pereira H.M. Increasing impacts of land use on biodiversity and carbon sequestration driven by population and economic growth. *Nature Ecology and Evolution*, 2019, 3(4): 628-637 (doi: 10.1038/s41559-019-0824-3).
5. Tilman D. Biodiversity and environmental sustainability amid human domination of global ecosystems. *Daedalus*, 2012, 141(3): 108-120 (doi: 10.1162/DAED_a_00166).
6. Song X.-P., Hansen M.C., Stehman S.V., Potapov P.V., Tyukavina A., Vermote E.F., Townshend J.R. Global land change from 1982 to 2016. *Nature*, 2018, 560(7720): 639-643 (doi: 10.1038/s41586-018-0411-9).

7. Foley J.A., DeFries R., Asner G.P., Barford C., Bonan G., Carpenter S.R., Chapin F.S., Coe M.T., Daily G.C., Gibbs H.K., Helkowski J.H., Holloway T., Howard E.A., Kucharik C.J., Monfreda C., Patz J.A., Prentice I.C., Ramankutty N., Snyder P.K. Global consequences of land use. *Science*, 2005, 309(5734): 570-574 (doi: 10.1126/science.1111772).
8. Davidson E.A. The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nature Geoscience*, 2009, 2(9): 659-662 (doi: 10.1038/ngeo608).
9. Guo J.H., Liu X.J., Zhang Y., Shen J.L., Han W.X., Zhang W.F., Christie P., Goulding K.W.T., Vitousek P.M., Zhang F.S. Significant acidification in major chinese croplands. *Science*, 2010, 327(5968): 1008-1010 (doi:10.1126/science.1182570).
10. Gomiero T. Soil degradation, land scarcity and food security: Reviewing a complex challenge. *Sustainability (Switzerland),* 2016, 8(3): article № 281 (doi: 10.3390/su8030281).
11. Kopittke P.M., Menzies N.W., Wang P., McKenna B.A., Lombi E. Soil and the intensification of agriculture for global food security. *Environment International*, 2019, 132: article № 105078 (doi: 10.1016/j.envint.2019.105078).
12. Di H.J., Cameron K.C. Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient Cycling in Agroecosystems*, 2002, 64(3): 237-256 (doi: 10.1023/A:1021471531188).
13. Sebilo M., Mayer B., Nicolardot B., Pinay G., Mariotti A. Long-term fate of nitrate fertilizer in agricultural soils. *Proceedings of the National Academy of Sciences of the United States of America*, 2013, 110(45): 18185-18189. (doi: 10.1073/pnas.1305372110).
14. Wang Y., Ying H., Yin Y., Zheng H., Cui Z. Estimating soil nitrate leaching of nitrogen fertilizer from global meta-analysis. *Science of the Total Environment*, 2019, 657: 96-102 (doi: 10.1016/j.scitotenv.2018.12.029).
15. Tilman D., Cassman K.G., Matson P.A., Naylor R., Polasky S. Agricultural sustainability and intensive production practices. *Nature*, 2002, 418(6898): 671-677 (doi: 10.1038/nature01014).
16. Castellano M.J., David M.B. Long-term fate of nitrate fertilizer in agricultural soils is not necessarily related to nitrate leaching from agricultural soils. *Proceedings of the National Academy of Sciences of the United States of America*, 2014, 111(8): E766 (doi: 10.1073/pnas.1321350111).
17. Kalinicheva E.Yu., Pol'shakova N.V., Kolomeichenko A.S. *Vestnik Orlovskogo gosudarstvennogo agrarnogo universiteta*. 2017, 3(66): 121-128 (in Russ.).
18. Ramankutty N., Foley J.A. Estimating historical changes in global land cover: Croplands from 1700 to 1992. *Global Biogeochemical Cycles*, 1999, 13(4): 997-1027 (doi: 10.1029/1999GB900046).
19. Kryshnyaya S.V. *Vestnik Sakhalinskogo muzeya*, 2011, 17: 338-356 (in Russ.).
20. Bobrovskii M.V. *Lesnye pochvy Evropeiskoi Rossii* [Forest soils of the European Russia]. Moscow, 2010 (in Russ.).
21. Smirnova O.V., Lugovaya D.L., Prokazina T.S. *Uspekhi sovremennoi biologii*, 2013, 2: 164-177 (in Russ.).
22. Smirnova O.V., Shashkov M.P., Korotkov V.N., Shirokov A.I. *Priroda*, 2008, 12: 20-24 (in Russ.).
23. Smirnova O.V., Aleinikov A.A., Smirnov N.S., Lugovaya D.L. *Priroda*, 2014, 2: 54-63 (in Russ.).
24. Tishkov A.A. *Voprosy geografii*, 2012, 134: 15-57 (in Russ.).
25. *Monitoring biologicheskogo raznoobraziya lesov Rossii: metodologiya i metody* /Otvetstvennyi redaktor A.S. Isaev [Monitoring of forest biological diversity in Russia: methodology and methods. A.S. Isaev (ed.)]. Moscow, 2008 (in Russ.).
26. Taranov S.A. V kn.: *Lesnye pochvy gornogo okaimleniya yugo-vostoka Zapadnoi Sibiri (Vostochnyi Altai, Gornaya Shoriya, Salair)* /Otvetstvennyi redaktor R.V. Kovalev [In: Forest soils of mountain bordering in the southeast of Western Siberia (Eastern Altai, Gornaya Shoria, Salair)]. Novosibirsk, 1974: 75-132 (in Russ.).
27. Babenko A.S., Nefed'ev P.S., Nefed'eva Yu.S. *Vestnik Tomskogo gosudarstvennogo universiteta*, 2009, 319:182-185 (in Russ.).
28. Fierer N., Strickland M.S., Liptzin D., Bradford M.A., Cleveland C.C. Global patterns in belowground communities. *Ecology Letters*, 2009, 12(11): 1238-1249 (doi: 10.1111/j.1461-0248.2009.01360.x).
29. Delgado-Baquerizo M., Oliverio A.M., Brewer T.E., Benavent-González A., Eldridge D.J., Bardgett R.D., Maestre F.T., Singh B.K., Fierer N. A global atlas of the dominant bacteria found in soil. *Science*, 2018, 359(6373): 320-325 (doi: 10.1126/science.aap9516).
30. Lundberg D.S., Lebeis S.L., Paredes S.H., Yourstone S., Gehring J., Malfatti S., Tremblay J., Engelbrektson A., Kunin V., del Rio T.G., Edgar R.C., Eickhorst T., Ley R.E., Hugenholtz P., Tringe S.G., Dangl J.L. Defining the core *Arabidopsis thaliana* root microbiome. *Nature*, 2012, 488: 86-90 (doi: 10.1038/nature11237).
31. Bates S.T., Berg-Lyons D., Caporaso J.G., Walters W.A., Knight R., Fierer N. Examining the global distribution of dominant archaeal populations in soil. *The ISME Journal*, 2011, 5: 908-917 (doi: 10.1038/ismej.2010.171).
32. Martin V. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 2011, 17(1): 10-12 (doi: 10.14806/ej.17.1.200).
33. Callahan B.J., McMurdie P.J., Rosen M.J., Han A.W., Johnson A.J.A., Holmes S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 2016, 13: 581-

583 (doi: 10.1038/nmeth.3869).
34. Quast C., Pruesse E., Yilmaz P., Gerken J., Schweer T., Yarza P., Peplies J., Glöckner F.O. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 2013, 41(D1): D590-D596 (doi: 10.1093/nar/gks1219).
35. Janssen S., McDonald D., Gonzalez A., Navas-Molina J.A., Jiang L., Xu Z.Z., Winker K., Kado D.M., Orwoll E., Manary M., Mirarab S., Knight R. Phylogenetic placement of exact amplicon sequences improves associations with clinical information. *mSystems*, 2018, 3: e00021-18 (doi: 10.1128/mSystems.00021-18).
36. Bolyen E., Rideout J.R., Dillon M.R., Bokulich N.A., Abnet C.C., Al-Ghalith G.A., Alexander H., Alm E.J., Arumugam M., Asnicar F., Bai Y., Bisanz J.E., Bittinger K., Brejnrod A., Brislawn C.J., Brown C.T., Callahan B.J., Caraballo-Rodrиguez A.M., Chase J., Cope E.K., Da Silva R., Diener C., Dorrestein P.C., Douglas G.M., Durall D.M., Duvallet C., Edwardson C.F., Ernst M., Estaki M., Fouquier J., Gauglitz J.M., Gibbons S.M., Gibson D.L., Gonzalez A., Gorlick K., Guo J., Hillmann B., Holmes S., Holste H., Huttenhower C., Huttley G.A., Janssen S., Jarmusch A.K., Jiang L., Kaehler B.D., Kang K.B., Keefe C.R., Keim P., Kelley S.T., Knights D., Koeste.r I, Kosciolek T., Kreps J., Langille M.G.I., Lee J., Ley R., Liu Y.X., Loftfield E., Lozupone C., Maher M., Marotz C., Martin B.D., McDonald D., McIver L.J., Melnik A.V., Metcalf J.L., Morgan S.C., Morton J.T., Naimey A.T., Navas-Molina J.A., Nothias L.F., Orchanian S.B., Pearson T., Peoples S.L., Petras D., Preuss M.L., Pruesse E., Rasmussen L.B., Rivers A., Robeson M.S., Rosenthal P., Segata N., Shaffer M., Shiffer A., Sinha R., Song S.J., Spear J.R., Swafford A.D., Thompson L.R., Torres P.J., Trinh P., Tripathi A., Turnbaugh P.J., Ul-Hasan S., van der Hooft J.J.J., Vargas F., Vбzquez-Baeza Y., Vogtmann E., von Hippel M., Walters W., Wan Y., Wang M., Warren J., Weber K.C., Williamson C.H.D., Willis A.D., Xu Z.Z., Zaneveld J.R., Zhang Y., Zhu Q., Knight R., Caporaso J.G. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 2019, 37: 852-857 (doi:10.1038/s41587-019-0209-9).
37. McMurdie P.J., Holmes S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, 2013, 8(4): e61217 (doi: 10.1371/journal.pone.0061217).
38. Love M.I., Huber W., Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology,* 2014, 15(12): 550 (doi: 10.1186/s13059-014-0550-8).
39. Loiko S.V., Geras'ko L.I., Kulizhskii S.P., Amelin I.I., Istigechev G.I. *Pochvovedenie*, 2015, 4: 410-423 (doi: 10.7868/S0032180X15040061) (in Russ.).
40. Orlov D.S., Sadovnikova L.K., Sukhanova N.I. *Khimiya pochv* [Soil chemistry]. Moscow, 2005 (in Russ.).
41. Bazilevich N.I., Titlyanova A.A. *Biologicheskii krugovorot na pyati kontinentakh: azot i zol'nye elementy v prirodnykh nadzemnykh ekosistemakh* [Biological circulation on five continents: nitrogen and ash elements in natural above-ground ecosystems]. Novosibirsk, 2008 (in Russ.).
42. Trofimov S.S. *Ekologiya pochv i pochvennye resursy Kemerovskoi oblasti* [Ecology of soils and soil resources of the Kemerovo region]. Novosibirsk, 1975 (in Russ.).
43. Achat D.L., Bakker M.R., Augusto L., Derrien D., Gallegos N., Lashchinskiy N., Milin S., Nikitich P., Raudina T., Rusalimova O., Zeller B., Barsukov P. Phosphorus status of soils from contrasting forested ecosystems in southwestern Siberia: effects of microbiological and physico-chemical properties. *Biogeosciences*, 2013, 10: 733-752 (doi: 10.5194/bg-10-733-2013).
44. Loiko S.V., Bobrovskii M.V., Amelin I.I. *Materialy dokladov Vserossiiskoi nauchno-prakticheskoi konferentsii «Chelovek i priroda — vzaimodeistvie na osobo okhranyaemykh prirodnykh territoriyakh», posvyashchennoi Godu osobo okhranyaemykh prirodnykh territorii i Godu ekologii (Novokuznetsk, 27-30 sentyabrya 2017 goda)* [Proc. All-Russ. Conf. «Man and nature — interaction in protected natural areas»]. Novokuznetsk, 2017: 81-96 (in Russ.).
45. Smolentsev B.A., Smolentseva E.N. *Vestnik Tomskogo gosudarstvennogo universiteta. Biologiya*, 2020, 50: 6-27 (doi: 10.17223/19988591/50/1) (in Russ.).
46. Korsunov V.M. V sbornike: *O pochvakh Sibiri* [In: About the soils of Siberia]. Novosibirsk, 1978: 122-131 (in Russ.).
47. Korsunova T.M., Korsunov V.M. V sbornike: *Genezis i geografiya lesnykh pochv* [In: Genesis and geography of forest soils]. Moscow, 1980: 85-104 (in Russ.).
48. Korsunov V.M., Vedrova E.F., Ignat'eva L.N. V sbornike: *Pochvy zony KATEKa* [In: Soils of the KATEK zone]. Krasnoyarsk, 1981: 99-113 (in Russ.).
49. Korsunov V.M., Vedrova E.F. V sbornike: *Geografiya i kartografiya lesnykh pochv* [In: Geography and cartography of forest soils]. Novosibirsk, 1982: 66-88 (in Russ.).
50. Gorozhankina S.M., Konstantinov V.D. *Geografiya taigi Zapadnoi Sibiri* [Geography of the taiga of Western Siberia]. Novosibirsk, 1978 (in Russ.).
51. Vasenev I.I., Targul'yan V.O. *Vetroval i taezhnoe pochvoobrazovanie (rezhimy, protsessy, morfogenez pochvennykh suktsessii)* [Windfall and taiga soil formation (modes, processes, morphogenesis of soil successions)]. Moscow, 1995 (in Russ.).
52. Vasenev I.I. *Pochvennye suktsessii* [Soil successions]. Moscow, 2008 (in Russ.).

53. Urusevskaya I.S., Khokhlova O.S., Sokolova T.A. *Pochvovedenie*, 1992, 8: 22-37 (in Russ.).
54. Ponomareva V.V. *Teoriya podzoloobrazovatel'nogo protsessa. Biokhimicheskie aspekty* /Pod redaktsiei M.M. Kononova [The theory of the podzol formation process. Biochemical aspects. M.M. Kononov (ed.)]. Kazan', 1964 (in Russ.).
55. Ponomareva V.V., Plotnikova T.A. *Gumus i pochvoobrazovanie (metody i rezul'taty izucheniya)* [Humus and soil formation (methods and results of the study)]. Leningrad, 1980: 222 (in Russ.).
56. Pershina E.V., Ivanova E.A., Korvigo I.O., Chirak E.L., Sergaliev N.H., Abakumov E.V., Provorov N.A., Andronov E.E. Investigation of the core microbiome in main soil types from the East European plain. *Science of the Total Environment*, 2018, 631-632: 1421-1430 (doi: 10.1016/j.scitotenv.2018.03.136)
57. Rozenberg G.S. *Byulleten' Samarskaya Luka*, 2007, 16(3-21): 581-584.
58. Sangwan P., Chen X., Hugenholtz P., Janssen P.H. *Chthoniobacter flavus* gen. nov., sp. nov., the first pure-culture representative of subdivision two, *Spartobacteria classis* nov., of the phylum *Verrucomicrobia*. *Applied and Environmental Microbiology*, 2004, 70(10): 5875-5881 (doi: 10.1128/AEM.70.10.5875-5881.2004).
59. Kant R., van Passel M.W., Palva A., Lucas S., Lapidus A., Glavina del Rio T., Dalin E., Tice H., Bruce D., Goodwin L., Pitluck S., Larimer F.W., Land M.L., Hauser L., Sangwan P., de Vos W.M., Janssen P.H., Smidt H. Genome sequence of *Chthoniobacter flavus* Ellin428, an aerobic heterotrophic soil bacterium. *Journal of Bacteriology,* 2011, 193(11): 2902-2903 (doi: 10.1128/JB.00295-11).

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FEATURES OF FLUORESCENT PROTEIN APPLICATION TO STUDY THE ROOT SYSTEM DEVELOPMENT OF CUCURBITS (*Cucurbitaceae*)

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Abstract

Modern studies of detailed processes in plant development would not be possible without using a wide range of fluorescent proteins (R. Day and M. Davidson, 2009; D. Chudakov et al., 2010). However, applications of fluorescent proteins are restricted due to problems with their visualization in plant tissues. Plants are difficult objects for microscopic studies. Indeed, even the most advanced methods have significant limitations regarding the depth of light penetration due to the scattering and absorption of light by cell walls. Therefore, to study the distribution of reporter fluorescent proteins in large organs typical for most plants, it is necessary to fix the plant material and prepare thick histological sections with a vibrating-blade microtome. Chemicals traditionally used for fixing, dehydrating, and embedding of plant tissues samples lead to changes in the structure of fluorescent proteins and, as a result, often to the loss of their fluorescence. Therefore, it is important to optimize the protocols for fixing plant tissues, preparing sections, and studying the distribution of fluorescent proteins by laser scanning confocal microscopy. In this work, we propose a novel, integrated, and potentially universal approach to fixation of tissues of transgenic plants and preparation of sections in the course of studying the patterns of cellular response to auxin and expression of transcription factors using laser scanning confocal microscopy. Our aim was to sum up modern approaches to the application of this technique for visualization of tissue and cellular patterns of fluorescent reporter proteins distribution on sections of large non-model plants. The first step for using fluorescent proteins in plants is the generation of genetic constructs that carry the promoter of the gene of interest fused to a reporter gene encoding a fluorescent protein. For this, a transformation protocol has to be available for the selected plant species. We have described the use of Gateway® cloning technology for the construction of vectors for plant transformation that meet modern experimental requirements. To study auxin localization *in vivo* we developed a series of vectors with genes encoding various fluorescent proteins (eGFP, tdTomato, mRuby3) under the control of the auxin-sensitive *DR5* promoter (E. Ilina et al., 2012). We now demonstrate the advantage of nuclear-targeted fluorescent proteins (mNeonGreen-H2B, tdTomato-H2B, mRuby3-H2B), as well as the possibility of their application for additional visualization of cell nuclei in combination with highly specific cell wall staining using SCRI Renaissance2200. An effective method is presented for constructing vectors to study cell-specific expression patterns of developmental regulators using the transcription factor genes *GATA24* (A. Kiryushkin et al., 2019) and *LBD16* in some *Cucurbitaceae* species as examples. We also applied an expression cassette, *pAtUBQ10::DsRED1* (E. Limpens et al., 2004), carrying a gene encoding red fluorescent protein under the control of a constitutive promoter, to demonstrate the advantages of the use of fluorescent proteins in screening for transgenic vs. wild type roots. A new method of fixation and clearing of plant tissues containing reporter fluorescent proteins and preparation of sections is presented, using transgenic roots of *Cucurbitaceae* as an example. The advantage of using melted agarose compared to embedding media for

orienting plant parts during the preparation of samples is revealed. The increased photostability of fluorescent proteins in sections due to the use of clearing reagent ClearSee (D. Kurihara et al., 2015) as a mounting medium is demonstrated. In sum, we apply a complex of several modern methodological approaches of sample preparation and laser scanning confocal microscopy that offers significant advantages for studying the developmental biology of large non-model plants.

Keywords: agrobacterial transformation, fluorescent proteins, confocal microscopy, plant development, transcription factors

In recent decades, significant advances in knowledge concerning the structure and functions of plant parts have allowed us to better understand specific events of plant life. This has occurred through use of model plants *Arabidopsis thaliana*, rice, tobacco, corn, *Medicago truncatula*, and *Lotus japonicus*. In recent years, omix technologies and the next top models in plant sciences [1] have contributed to a significant breakthrough in understanding of molecular mechanisms of plant development and functioning. The range of model plant species and sequenced genomes in the plaBiPD (https://www.plabipd.de/plant_genomes_pa.ep) and Phytozome (http://phytozome-next.jgi.doe.gov) databases has increased, and the transcriptomes and proteomes of these plants are receiving much attention. This has led to renewed interest in in-depth studies of more complex non-model plants, including members of the *Cucurbitaceae* family [2-4].

Nowadays, various reporter fluorescent proteins (FP) are used to study molecular and physiological mechanisms of plant development [5]. The areas of FP application are diverse, including tissue patterns of gene expression; visualization of protein distribution, transport, and interaction; labelling of organelles, cells, and tissues; search for new drugs; breeding and selection. [6-8]. However, reporter fluorescent proteins have significant limitations when considering the tissues of many plants.

As a convenient model organism, *Arabidopsis* was applied in most microscopic studies employing fluorescent proteins. *Arabidopsis* has thin roots of ~ 100 μm in diameter, which allows examination of fluorescent protein location in root tissues without sectioning of plant samples, e.g., by noninvasive *in vivo* confocal microscopy. The leaves of *Arabidopsis* are also thin but optically opaque due to chlorophyll, making only the surface layers available to study the distribution of fluorescent proteins. For *Arabidopsis*, the ClearSee tissue optical clearing technique has been developed. ClearSee reagent allows for clearing while maintaining fluorescence of some reporter proteins to permit the visualization of fluorescent protein distributions in optically non-transparent organs [9]. Combining visualization of fluorescent proteins and cell walls stained with nonspecific Direct Red 23 or Direct Yellow 96 dyes [10], the ClearSee method can be leveraged to explore patterns and localization of gene expression in a specific cell or tissue. ePro-ClearSee, an immunohistochemical method that also does not require sectioning of plant organs, was adapted for epigenetic investigations to detect modifications of histones and DNA [11]. In addition, Schiff staining of organs followed by clearing has been modified to study the distribution of indigo (a product of GUS staining) over the entire thickness of any *Arabidopsis* organ [12]. However, this staining is incompatible with the use of fluorescent proteins.

The vast majority of plants, including important crops, have a more complex anatomical structure compared to *Arabidopsis*, their organs consist of a greater number of cell layers in tissues and, accordingly, are larger in size. The maximum penetration depth of the confocal microscope laser into plant tissue is relatively shallow due to light scattering and absorption by cell walls and reaches a maximum value of 100 μm. Multiphoton laser microscopy is rather difficult to use, although

it allows deeper penetration into plant tissues using a near-infrared (900-1000 nm) femtosecond laser [13, 14]. The latest technology of light-sheet fluorescence microscopy based on wide-field fluorescence microscopy allows penetration to a depth of 500 μm [15-17] but is only applicable to transparent or cleared animal and plant objects. Even for *Arabidopsis*, this technology, successfully combined with image deconvolution and reconstruction using a Fiji Multiview-Reconstruction plug-in (https://imagej.net/Multiview-Reconstruction), has significant limitations in terms of light penetration depth and resolution like traditional microscopy methods [18].

Despite the undoubted recent progress in microscopic technologies, investigations into the distribution of reporter fluorescent proteins or fusion of their genes with the genes of target proteins (fusion proteins as reporters) in the thick organs of plants requires an optimized protocol for fixing and sectioning to produce thick histological sections of plant material. Chemicals traditionally used for fixing, dehydrating, and embedding plant tissue samples alter the structure of fluorescent proteins and, as a result, lead to a loss of fluorescence. Some researchers use cryomicrotomy to obtain thick (25-50 μm) sections of plant tissues where the fluorescence of such proteins is preserved [19]. However, such approaches have not become widespread.

In plants, the brightest and most photostable fluorescent proteins should be used (e.g., mNeonGreen, mRuby3, or mScarlet) [20, 21] which provide sample scanning to a greater depth. Plant cell autofluorescence in different spectral regions should also be considered. Therefore, vectors for plant transformation must correspond to the objectives of the experiment and the object. The existing sets of vectors for marking of plant cell organelles [22] or commercial genetic constructs are not always best suited to a specific scientific problem, and there is a need for a flexible and efficient methodology to design an optimal vector. Cloning technologies Gateway® [23] and Golden Gate [24] are efficient, provide targeted cloning, and allow the assembly of almost any combination of DNA fragments (e.g., a promoter, a coding part, and a terminator) in a vector suitable for the plant transformation.

This paper reports a novel methodology which leverages fluorescent reporter proteins for investigating the molecular genetic mechanisms of development of large organs of non-model plants, using cucurbits as an example. More specifically, an integrated approach to fixation of transgenic plant tissue and sample sectioning for laser scanning confocal microscopy is put forward to study patterns of cellular auxin response and expression of transcription factors. The paper also describes an application of Gateway® cloning technology to produce new genetic constructs (vectors) for plant transformation that overcomes experimental challenges.

Our aim was to sum up modern approaches to the application of an effective universal technique for visualization of tissue and cellular patterns of fluorescent reporter proteins distribution on sections of large organs of non-model plants (on the example of cucurbits).

Methods. Design of DNA constructs using Gateway® recombination cloning technology. *Gateway® cloning guidelines*. To deliver the genes encoding reporter fluorescent proteins into the plant cells, DNA constructs (vectors) are required. In constructing vectors suitable for plant transformation, we used Gateway® cloning technology (Thermo Fisher Scientific, USA) [25]. For the LR clonase plus reaction, three different entry vectors containing either the promoter, the coding part of the target gene, or the transcription terminator were mixed with the destination vector in a tube containing LR Clonase™

II Plus enzyme mix (Thermo Fisher Scientific, USA; hereinafter LR clonase plus) that catalyzes the *in vitro* recombination between att-sites of entry clones and a destination vector (Fig., A).

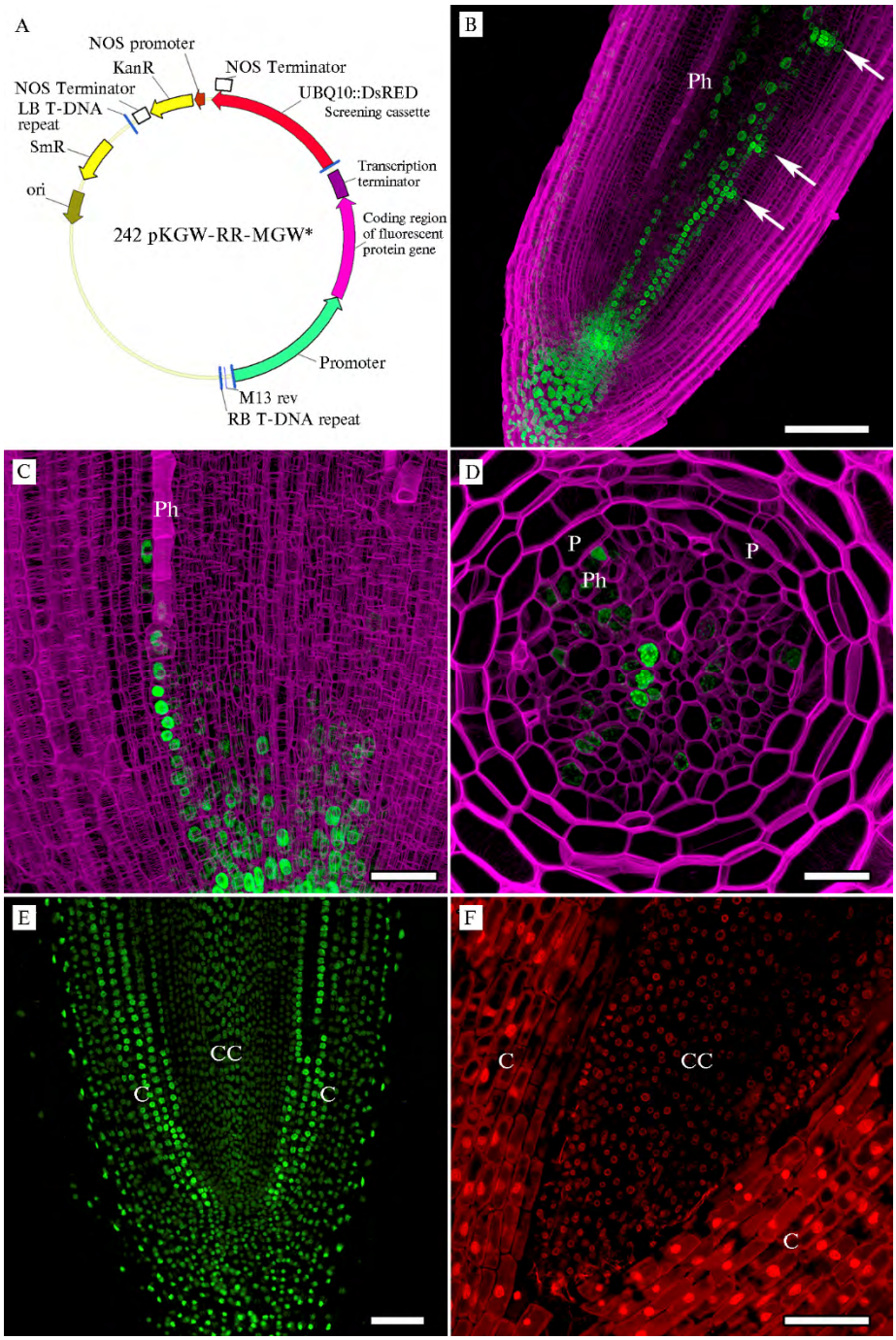
To construct vectors for plant transformation, we also used LR Clonase™ II enzyme mix which transfers one DNA fragment from the entry vector to the destination vector via *att*L-*att*R recombination. This technology is based on the bacteriophage lambda recombination system [26]. The entry and destination vectors contain heteronymous homologous regions of the bacteriophage lambda DNA, the *att*L (left), and *att*R (right) sites capable of site-specific recombination. The recombination is mediated by LR plus clonase which is a mixture of integrase and excisionase enzymes of the bacteriophage lambda and the Integration Host Factor protein of *Escherichia coli* [26]. Divergent *att*L regions flank the edges of DNA fragments cloned into the entry vector, which allows them to be transferred into the destination vector in a specific order. To generate entry vectors, a BP Clonase™ enzyme mix was used, which catalyzes recombination between the attB sites flanking the target DNA fragment and the *att*P sites of the donor vector. Also the target DNA fragment was conventionally cloned into the entry vector by restriction and ligation performed sequentially.

The entry vectors used for the experiments were 369_pENTRattL4attR1_BSAI (Thermo Fisher Scientific, USA) to clone the promoter region, pUC18-entry 8 [27] to clone the reporter DNA fragment, and the pDONR P2-P3 donor vector (Thermo Fisher Scientific, USA) to clone the transcription terminator fragment. pKGW-GG-RR, the destination vector for the LR clonase reaction [28], contained the fused reporter gene encoding for green fluorescent protein eGFP and β-glucuronidase (GUS) *eGFP-GUS* [25] adjacent to *att*-sites [28] and the *pAtUBQ10::DsRED1* cassette [29] to screen transgenic roots by fluorescence of the DsRED1 protein. The destination vectors for the LR plus clonase reaction were 242 pKGW-RR-MGW containing the same cassette *pAtUBQ10::DsRED1* (see Fig., A), and 236 pKGW-RR-MGW without such a cassette for selection of transgenic material.

LR plus, LR, and BP clonase reactions were undertaken according to the manufacturer's recommendations (Thermo Fisher Scientific, USA). The presence and correct assembly of the cassette in the destination vector was verified by PCR and sequencing. The destination vector was transformed into *Rhizobium rhizogenes* R1000 and then used to transform squash and cucumber seedlings according to a previously developed method [3] to generate composite plants with wild-type shoot and transgenic root system.

Construction of a vector plasmid family to study the in vivo localization of the cellular response to auxin. Auxin is one of the most important factors affecting root development in higher plants [30]. To study the role of auxin in lateral root initiation and development in cucurbits, a family of vectors was developed containing the synthetic auxin-sensitive *DR5* promoter [31] and a reporter gene for detecting the cellular response to auxin. The pKGW-RR-MGW-*DR5::eGFP-GUS* vector was produced by an LR clonase-mediated reaction between the entry vector pUC18-entry8-*DR5* carrying the *DR5* promoter and the destination vector pKGW243-GG-RR [3]. The cellular response to auxin was studied on whole transgenic squash roots (*DR5::eGFP-GUS*) stained for β-glucuronidase activity (GUS assay) and on longitudinal and cross sections of the transgenic squash roots. A limitation of GUS staining is caused by diffusion of indigo, a product of the β-glucuronidase reaction, which can lead to erroneous results on tissue localization of the response to the plant hormone. Therefore, fluorescent proteins with nuclear localization are most suitable for a more detailed study of

the role of auxin in the lateral root initiation in squash.



The use of reporter fluorescent proteins to study patterns of cellular response to auxin and expression of transcription factors in *Cucurbitaceae* plants (confocal laser scanning microscopy, LSM 780 microscope, Zeiss, Germany; longitudinal sections of the tips of fixed roots, the section thickness is 65 μm; maximum intensity projections of optical sections are shown).

A. Chart of vector 242 pKGW-RR-MGW including promoter, coding region of fluorescent protein gene, transcription terminator, and *pUBQ10::DsRED* cassette for screening transgenic material.

B. Visualization of auxin response maxima (nuclear localization of mRuby3 fluorescence, green channel) in transgenic roots of cucumber seedlings carrying the *DR5::mRuby3-H2B* insert. Cell walls are counter stained with SCRI Renaissance2200 (purple channel), white arrows indicate lateral root primordia. Ph — protophloem. Scale bar, 100 μm.

C, D. Visualization of the *CpLBD16b* gene expression pattern in the root tip of squash carrying the *CpLBD16b::mNeonGreen-H2B* insert, a longitudinal (C) and cross (D) sections. *CpLBD16b* is detected by mNeonGreen fluorescence (nuclear localization, green channel), cell walls are counter stained with SCRI Renaissance2200 (purple channel). Ph — protophloem, P — pericycle. Scale bar, 50 μm.

E. Visualization of cell nuclei (Venus fluorescence, green channel) in transgenic roots of squash seedlings carrying the *UBQ10::H2B-Venus* insert. C — cortex, CC is central cylinder. Scale bar, 100 μm.

E. Visualization of cell nuclei (tdTomato fluorescence, red channel) in transgenic roots of squash seedlings carrying the *pAct::tdTomato-H2B* insert. C — cortex, CC — central cylinder. Scale bar, 100 μm.

We also developed constructs 242 pKGW-RR-MGW-*DR5::mRuby3-H2B* and 242 pKGW-RR-MGW-*DR5::tdTomato-H2B* which provide nuclear localization of the reporter protein. The cellular response to auxin was analyzed on longitudinal and cross 60 μm sections of tips of transgenic squash and cucumber roots bearing constructs *DR5::NLS-eGFP-GUS*, *DR5::mRuby3-H2B* and *DR5::tdTomato-H2B*. The use of reporter proteins tdTomato and mRuby3 fused to human histone H2B [32] allows for the most accurate data on the spatial patterns of the cellular response to auxin in dividing cells during lateral root initiation in the meristem of the parental root in cucumber (see Fig., B) compared to the *DR5::NLS-eGFP-GUS* reporter construct [3] encoding the NLS signal of the nuclear localization. Importantly, the brightness of the tdTomato and mRuby3 proteins is three times higher than that of the eGFP protein. In addition, mRuby3 exhibits increased photostability [33, 34]. The auxin response maximum associated with the initiation site of the lateral root primordium in cucumber occurs at a distance of 200-300 μm from the initial cell prior to the first anticlinal division of the pericycle and endoderm. In addition, auxin is involved in further development of the lateral root primordium.

Therefore, the auxin-sensitive promoters combined with reporter constructs are appropriate tools for investigating the involvement of the hormone in a particular morphogenetic process. The proposed approach requires the use of the brightest photostable fluorescent proteins with nuclear localization to increase the brightness of fluorescence probes by tens of times compared to proteins with cytoplasmic localization, since when studying the early stages of lateral root initiation or similar processes, it is necessary to detect a weak signal from several cells of the pericycle in the thickness of the root. Fluorescent tags of nuclear localization should also correspond to the objectives of the experiment. Thus, the NLS nuclear localization signal did not allow reliable detection of the presence or absence of a marker in a dividing cell, since the nuclear envelope is disassembled during cell division, and the fluorescent protein diffuses into the cytoplasm. Nevertheless, NLS visualizes nuclei in a shape close to that of native nuclei. The combination of reporter proteins with H2B histone visualizes the figures of mitosis, since histone remains bound to nuclear DNA during mitosis. However, in cucurbit plants, uneven distribution of the fluorescent tags in aldehyde-fixed chromatic nuclei may be a disadvantage of fusion of the reporter with the histone protein (see Fig., D).

Construction of vectors for studying the tissue expression pattern of developmental regulator genes. To study a fine coordination of the developmental processes in a plant organ, for example, the initiation of a lateral root, it is necessary to identify the tissues and cells in which the gene of interest is expressed. Asymmetric cell division often mediates cell differentiation in multicellular organisms and plays an important role in the development of new organs. In most flowering plants, lateral roots are formed from pericycle cells located at the xylem pole. These cells are asymmetrically divided into small central and larger flanking cells with different fates. In *Arabidopsis*, LATERAL ORGAN BOUNDARIES DOMAIN 16 (LBD16) and other members of the LBD family play an

important role in establishing the asymmetry of the lateral root founder cells. *LBD16* is specifically expressed in a pair of pericycle cells at the xylem pole prior to the first anticlinal division during lateral root initiation. The pericycle cells at the xylem pole expressing LBD16 are presumably the lateral root founder cells [35], the specification of which occurs in the basal part of the root meristem and depends on the oscillatory expression of genes, including the *GATA23* gene encoding for transcription factor [36].

Using the NCBI (https://www.ncbi.nlm.nih.gov/), Phytozome (https://phytozome.jgi.doe.gov), Cucurbit Genomics Database (http://cucurbitgenomics.org), and PlantTFDB (http://planttfdb.gao-lab.org) databases, we identified the *CpGATA24* [37] and *CpLBD16b* genes in the *Cucurbita pepo* genome, which are the orthologs of *Arabidopsis* auxin-inducible genes *GATA23* and *LBD16* which are involved in specification of pericycle cells for lateral root initiation.

Two vectors, 242 pKGW-RR-MGW-*pCpLBD16b::mNeonGreen-H2B* and 242 pKGW-RR-MGW-*pCpGATA24::mNeonGreen-H2B*, were developed by an LR plus clonase reaction to localize the expression pattern of *CpLBD16b* and *CpGATA24* orthologous genes. The promoter sequences of the *CpLBD16b* and *CpGATA24* genes were amplified using a squash genomic DNA template and cloned into the 369_pENTRattL4attR1_BSAI vector. mNeonGreen, one of the brightest and most photostable proteins with a short maturation time [38, 39] fused to histone H2B, was used as a reporter for the localization of the promoter activity of these genes. The *mNeonGreen-H2B* nucleotide sequence was amplified on a plasmid template (Allele Biotechnology and Pharmaceuticals, Inc., USA, plasmid # H2B-213) [20] and cloned into the pUC18-entry8 vector. The 373_pENTRattR2attL3-TermAct vector containing the *Actin2* gene terminator sequences from *Arabidopsis* was used as a transcription terminator [24]. The resulting binary vectors were used for plant transformation to localize activity of the *CpGATA24* [37] and *CpLBD16b* promoters in the squash plant tissues by confocal laser scanning microscopy (see Fig. C, D). It was revealed for the first time that the *CpGATA24* gene is involved in lateral root primordium initiation during the first anticlinal divisions in the pericycle. *CpLBD16b* is not expressed in the pericycle cells at the xylem pole of the root but it occurs in the initial cells and in the developing protophloem cell file (see Fig., C, D).

To sum up, the expression of reporter genes encoding fluorescent proteins (especially those with nuclear localization) under the control of species-specific promoters of the genes of interest provides reliable data on the expression pattern of these genes in plant tissues and on their involvement in morphogenesis processes, even in large plant organs.

Constitutive promoters as a visualization tool. Selection of transgenic plant organs is essential during plant transformation. During transformation of squash plants with *R. rhizogenes*, a callus formed at the site of inoculation, consisting of cells carrying the T-DNA insert of the binary vector, of non-transformed cells, and, possibly, of cells in which the insert has undergone silencing. The roots developing from such a callus are either transgenic, or wild-type, or chimeric, i.e., formed by both transformed and non-transformed cells. This causes difficulties in the analysis of transgenic roots. A cassette consisting of a gene encoding a fluorescent protein under the control of a constitutive promoter, located within the T-DNA in a binary vector, provides effective screening of transgenic roots. There should be differences in the emission spectra of the screening and reporter proteins. Traditionally, these are pairs with fluorescence in red and green spectral regions. In vector 242 pKGW-RR-MGW, the *pAtUBQ10::DsRED1* cassette containing gene for the red fluorescent protein DsRED1 under the control of constitutive promoter of the ubiquitin gene *AtUBQ10* from *Arabidopsis* provided

the selection of transgenic roots (see Fig., A). In this case, the reporter genes encoded fluorescent proteins from the non-red part of the spectrum (see Fig. B-D).

In most microscopic applications, it is necessary to visualize cell nuclei. Usually, cell nuclei are stained with dyes for DNA (for example, DAPI or propidium iodide), however, such dyes penetrate unevenly into the sample and stain all nucleic acids, including mitochondrial and chloroplast DNA, and sometimes RNA. An advanced approach to nuclear imaging is the use of constitutive promoters which control a gene encoding a fluorescent protein with nuclear localization. The presence of such a cassette in the T-DNA of the vector allows simultaneous screening of transgenic roots and visualization of nuclei in cells on sections.

We have developed new vectors for nuclei visualization in the root tissues of cucurbits, both containing genes for screening proteins under the constitutive promoters. In 242 pKGW-RR-MGW-*pUBQ10::H2B-Venus*, the yellow fluorescent protein reporter gene *Venus* is fused to the human histone gene *H2B* under control of ubiquitin gene *pUBQ10* promoter from *Arabidopsis*. The Venus reporter protein is fast-maturating, highly photostable, and is brighter compared to eGFP [33, 40]. Vector 236 pKGW-RR-MGW-*pACT2::tdTomato-H2B* contains the reporter gene *tdTomato* fused to the histone gene *H2B* under the constitutive promoter of the actin gene *pACT2* from *Arabidopsis*.

It was found that promoters of the ubiquitin and actin genes provide high expression of genes encoding fluorescent proteins but exhibit different activities depending on localization in root tissues (see Fig. E, F). Both promoters were more active in cortex and rhizodermis cells than in the central cylinder, which creates difficulties in studying lateral root initiation and other processes occurring in the pericycle and other tissues of the central cylinder.

Thus, constitutive promoters in combination with a gene encoding a fluorescent protein are a valuable tool both for screening transgenic material and for visualizing cell nuclei by confocal laser microscopy. Nevertheless, the constitutive promoter should be chosen carefully. In the screening cassette, many vectors for plant transformation contain the strong constitutive promoter *35S* of the cauliflower mosaic virus CaMV. We have previously shown that, upon transformation of squash plants with the "empty" vector pMDC162-GFP [23], the *35S* promoter located in the T-DNA in *p35S::GFP* cassette drives ectopic expression of the *GUS* gene which lacks a promoter and, therefore, should not be expressed in root tissues [3]. Cases when the *35S* promoter affects the expression of target cassettes, possibly leading to erroneous results, have hitherto been described [41, 42]. Native promoter *pAtUBQ10* in the *pAtUBQ10::DsRED1* cassette of the vector 242 pKGW-RR-MGW provides robust expression of the target cassette [3]. Uneven activity of constitutive gene promoters in different tissues and plant organs is also known to occur. The *35S* promoter is more active in the central cylinder, leading to brighter fluorescence of the cell nuclei, compared to the cortex [43]. The promoters of constitutively expressed ubiquitin and actin genes of *Arabidopsis* are most popular [18, 29, 44, 45]. However, other constitutive gene promoters are also known. For example, the *PtMCP* has been cloned from poplar *Populus tomentosa* [46]. The promoter of the *RIBOSOMAL PROTEIN S5A* (*RPS5A*) gene, which is selectively expressed in proliferating cells [47], can be used as a marker of proliferative activity.

Sample preparation technique for localization of fluorescent proteins on sections of fixed organs. In experiments using reporter proteins, preserving their fluorescence ability for as long as possible represents an important task. Accordingly, we modified the method of fixing plant tissues and preparing sections, using transgenic roots of squash and cucumber plants as examples.

Root cells are known to undergo plasmolysis upon fixation in a 100 mM

phosphate buffer. For this reason, we changed the composition of the fixative [48] proposed by Brian Lin (Tufts University, Boston, MA, USA). A 5-fold decrease in the molarity of the phosphate buffer (up to 20 mM) addresses the problem of plasmolysis. A similar approach was also used for immunolocalization of the cytoskeleton on sections of fixed symbiotic nodules [49, 50]. For most fluorescent proteins (GFP, Venus, tdTomato, mRuby3), the optimum pH is 7.2. However, to prevent rapid decay of mNeonGreen in the roots of transgenic plants, we used a buffer with a higher pH, 8.0. The final fixative composition was as follows: 1 % paraformaldehyde, 5 % dimethyl sulfoxide (DMSO), 75 mM L-lysine, 10 mM sodium m-periodate in 20 mM phosphate buffer, pH 7.2-8.0. The tips of the main root (6-8 mm long) were fixed. To remove air from tissues, containers with plant material and the fixative were left to stand in an exicator under vacuum (–1 atm) for 3-5 min, then the vacuum was removed. The procedure was repeated 3-4 times. The roots were kept in the fixative for 1 h at room temperature. After fixation, the material was washed three times for 15-20 min with 20 mM phosphate buffer containing 75 mM L-lysine.

Fixed roots were placed in rubber molds and mounted in molten 2 % LE agarose (Lonza Group, Switzerland) at 40-50 °C. The agarose blocks were glued onto flat metal holders using Super Moment cyanoacrylate glue (Henkel, Russia). An automatic precision microtome with a vibrating blade HM650V (Thermo Fisher Scientific, Microm International GmbH, Germany) was used to prepare 65-μm-thick sections of roots. The sapphire knife (Delaware Diamond Knives, Inc., USA) provided significantly improved section quality. Distilled water was the optimal cutting medium because different buffers, e.g., phosphate, Tris buffered saline (TBS), and microtubule stabilizing buffer (MTSB) significantly deformed root sections. The cell walls were counter stained for 1 h with fresh 0.1 % solution of an SCRI Renaissance 2200 dye (SR2200, Renaissance Chemicals, Ltd, UK) [51] in dH_2O (pH 8.0) without subsequent washing. If necessary, cell nuclei were alternatively stained for 30-50 min with 0.3 μg/ml DAPI.

Sections were placed into embedded medium under coverslips. It was revealed that the majority of fluorescent proteins (GFP, Venus, tdTomato), after the proposed fixation, retain fluorescence in the CFMR2 liquid final medium (Citifluor, Division of Electron Microscopy Sciences, USA). However, clearing reagent ClearSee [9] in our modification (13.7 g/l L-lysine monochloride, 100 g/l xylitol, 150 g/l sodium deoxycholate, 240 g/l urea in 20 mM phosphate buffer, pH 8.0) turned out to be optimal for embedding sections, including those of transgenic roots carrying mNeonGreen.

Thus, many methodological problems associated with reporter fluorescent proteins must be considered to preserve their fluorescence. Plant material should be embedded into 2-3 % agarose convenient to hold small plant parts during cutting. It has hitherto been shown that neither dehydration in a series of alcohols nor embedding media should be applied for immunolocalization of cytoskeleton in nitrogen-fixing root nodules of legumes [49, 50]. When the transgenic material carrying the reporter fluorescent proteins is embedded in paraffin or Steedman's wax (a medium with a more gentle sample preparation suitable for the immunolocalization of sensitive proteins) [52-54], dehydration in alcohols is required which leads to rapid decay of the fluorescence of such proteins. When mounted in molten agarose, short-term exposure to 40 °C does not lead to denaturation of the fluorescent protein. The agarose concentration also requires optimization. Specifically, the softer the object, the lower the agarose concentration should be. If the agarose is too dense it can cause strong compression and dehydration of the soft object, which appears as a compression of the stretch zone of the root tip. Instead of the traditionally used propidium iodide (PI) or FM4-64 (plasmalemma

staining), we propose a simplified staining protocol with SCRI Renaissance 2200 cell wall specific dye, which allows a clear visualization of the anatomical structure on tissue sections (see Fig., C, D). Our findings also testify to the importance of optimal embedding medium for the sections, as this parameter affects the stability and duration of fluorescence.

Laser scanning confocal microscopy for visualization of fluorescent proteins in fixed tissues. Analysis of sections and imaging were performed using a confocal laser scanning microscope LSM 780 (Zeiss, Germany; objectives 10×/0.45 and 20×/0.8 of the Plan-Apochromat series with a high numerical aperture, and 40×/1.3 with oil immersion). Reporter fluorescent proteins eGFP and mNeonGreen were detected at the laser wavelength of 488 nm, yellow Venus protein at 514 nm, red proteins mRuby3 and tdTomato at 561 nm. Cell walls (SCRI Renaissance 2200) or nuclei (DAPI) were detected by excitation at 405 nm. To visualize unstained anatomical structures, differential interference contrast (DIC) in an additional transmitted light channel was applied. For imaging and image processing, we used the ZEN v. 2.3 software package (Zeiss, Germany).

Currently, fluorescent proteins cannot be visualized in thick sections of plant tissues in any other way than using confocal microscopy. The possibilities offered by a laser scanning microscope include a significant increase in the spatial resolution of the signal, which makes it possible to separate structures that merge when using wide-field microscopy due to out-of-focus light. The spatial structure of a tissue or organ site is difficult to determine using relatively thin sections (7-16 μm), however, only thin sections can be examined by traditional light microscopy. Thick sections (50-70 μm) in combination with confocal microscopy makes it possible to obtain a series of optical sections (z-stack). Further, depending on the purpose of the study, it is possible to represent the z-stack in the form of a two-dimensional image using the maximum intensity projection algorithm or to obtain a 3D reconstruction. This approach has been successfully used to study the development of various plant organs, in particular, the apical meristems of shoots and roots and the development of lateral roots [55, 56].

An essential problem in studying the development of plant organs is the overlap of the spectra of several fluorescent proteins and autofluorescence of cell and tissue elements [57-59]. The main sources of autofluorescence in plant tissues are chlorophyll (red region of the spectrum) and lignin (green and yellow regions). Autofluorescence masks and complicates the registration of the fluorescence of reporter proteins. Autofluorescence can be strong enough to be mistaken for reporters' fluorescence, leading to erroneous data. The use of confocal microscopy allows the separation of autofluorescence and reporter signal. A linear spectral unmixing algorithm can be used to eliminate autofluorescence, as well as to separate the overlapping spectra of several fluorescent reporter proteins [58, 60]. It is convenient to assess the nature of autofluorescence using emission spectra on unstained sections [61]. Usually, the samples contain several dyes/fluorescent proteins, each of which marks the cell structure or individual cells. Upon spectral imaging, fluorophores are detected either individually or as a mixture, depending on their spatial distribution within the object. The linear spectral unmixing algorithm allows the relative contribution of the intensity of each fluorophore to be determined for each pixel [60]. If the spectra of all fluorophores in the sample are known, then their signals within a pixel can be calculated from the spectral curve of each pixel, which is determined and recorded during the lambda scan. After the spectral contribution of each fluorophore has been determined, the lambda stack can be divided into separate channels for each fluorophore. In particular, the autofluorescence spectrum can be represented as a separate channel and eliminated

from the final image of the sample. Thus, using a complex of modern approaches in confocal laser microscopy addresses the problems of large and dense samples by high resolution three-dimensional imaging for cell or tissue distribution of fluorescent proteins.

Altogether, we have successfully developed and applied a new complex of techniques that allows us to study the localization of reporter fluorescent proteins on fixed sections of large organs of non-model plants. It is shown that the genetic constructs containing screening and reporter cassettes should be carefully chosen. It is also important to consider particularities of the plants, primarily their autofluorescence. The optimal solution is to construct vectors in accordance with the tasks of a specific experiment. Therefore, a necessary condition for the application of the proposed approach is the availability of a transformation technique for the species of interest, which allows the T-DNA of the vector to be delivered to the plant genome. The critical factors are the composition and pH of the fixative, which must be adapted with consideration for both the plant species and the applied fluorescent reporter protein. The method of section preparation is also important to preserve the fluorescence of the fluorescent proteins. Embedding plant material into agarose is optimal. For visualization of cell walls and tissue morphology, we have proposed a simplified protocol for staining with SCRI Renaissance 2200. Correct selection of the medium for embedding sections is also important, since its properties affect the photostability of the protein during the study of preparations. Finally, the accuracy of the final result will depend on the resolution of the selected microscopy method. Applications of fluorescent reporter proteins for studying plant development are not limited to localization of the cellular response to phytohormones, tissue localization of promoter activity of the gene encoding a developmental regulator, or to detection of the protein itself. Expression of fluorescent protein genes under the control of promoters of genes of interest is an extremely specific fluorescent label produced directly by the plant. Therefore, not only cell structures, but also physiological processes are marked, which cannot be achieved by simple staining a sample with fluorescent dyes. However, all studies using fluorescent proteins require careful experimental design and sample preparation to ensure sample integrity.

REFERENCES

1. Cesarino I., Ioio R.D., Kirschner G.K., Ogden M.S., Picard K.L., Rast-Somssich M.I., Somssich M. Plant science's next top models. *Annals of Botany*, 2020, 126(1): 1-23 (doi: 10.1093/aob/mcaa063).
2. Che G., Gu R., Zhao J., Liu X., Song X., Zi H., Cheng Z., Shen J., Wang Z., Liu R., Yan L., Weng Y., Zhang X. Gene regulatory network controlling carpel number variation in cucumber. *Development*, 2020, 147(7): dev184788 (doi: 10.1242/dev.184788).
3. Ilina E.L., Logachov A.A., Laplaze L., Demchenko N.P., Pawlowski K., Demchenko K.N. Composite *Cucurbita pepo* plants with transgenic roots as a tool to study root development. *Annals of Botany*, 2012, 110(2): 479-489 (doi: 10.1093/aob/mcs086).
4. Osipowski P., Pawełkowicz M., Wojcieszek M., Skarzyńska A., Przybecki Z., Pląder W. A high-quality cucumber genome assembly enhances computational comparative genomics. *Mol. Genet. Genomics*, 2020, 295(1): 177-193 (doi: 10.1007/s00438-019-01614-3).
5. Day R.N., Davidson M.W. The fluorescent protein palette: tools for cellular imaging. *Chemical Society Reviews*, 2009, 38(10): 2887-2921 (doi: 10.1039/B901966A).
6. Chudakov D.M., Matz M.V., Lukyanov S., Lukyanov K.A. Fluorescent proteins and their applications in imaging living cells and tissues. *Physiological Review*, 2010, 90(3): 1103-1163 (doi: 10.1152/physrev.00038.2009).
7. Mathur J. The illuminated plant cell. *Trends in Plant Science*, 2007, 12(11): 506-513 (doi: 10.1016/j.tplants.2007.08.017).
8. Berg R.H., Beachy N.R. Fluorescent protein applications in plants. *Methods in Cell Biology*, 2008, 85: 153-177 (doi: 10.1016/S0091-679X(08)85008-X).
9. Kurihara D., Mizuta Y., Sato Y., Higashiyama T. ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. *Development*, 2015, 142(23): 4168-4179 (doi: 10.1242/dev.127613).

10. Ursache R., Andersen T.G., Marhavý P., Geldner N. A protocol for combining fluorescent proteins with histological stains for diverse cell wall components. *Plant J.*, 2018, 93(2): 399-412 (doi: 10.1111/tpj.13784).
11. Nagaki K., Yamaji N., Murata M. ePro-ClearSee: a simple immunohistochemical method that does not require sectioning of plant samples. *Scientific Reports*, 2017, 7: 42203 (doi: 10.1038/srep42203).
12. Truernit E., Bauby H., Dubreucq B., Grandjean O., Runions J., Barthélémy J., Palauqui J.-C. High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of phloem development and structure in *Arabidopsis*. *Plant Cell*, 2008, 20(6): 1494 (doi: 10.1105/tpc.107.056069).
13. Mizuta Y., Kurihara D., Higashiyama T. Two-photon imaging with longer wavelength excitation in intact *Arabidopsis* tissues. *Protoplasma*, 2015, 252(5): 1231-1240 (doi: 10.1007/s00709-014-0754-5).
14. Feijy J.A., Moreno N. Imaging plant cells by two-photon excitation. *Protoplasma*, 2004, 223(1): 1-32 (doi: 10.1007/s00709-003-0026-2).
15. Girkin J.M., Carvalho M.T. The light-sheet microscopy revolution. *Journal of Optics*, 2018, 20(5): 053002 (doi: 10.1088/2040-8986/aab58a).
16. Ovečka M., Vaškebová L., Komis G., Luptovčiak I., Smertenko A., Šamaj J. Preparation of plants for developmental and cellular imaging by light-sheet microscopy. *Nature Protocols*, 2015, 10: 1234 (doi: 10.1038/nprot.2015.081).
17. Valuchova S., Mikulkova P., Pecinkova J., Klimova J., Krumnikl M., Bainar P., Heckmann S., Tomancak P., Riha K. Imaging plant germline differentiation within *Arabidopsis* flowers by light sheet microscopy. *eLife*, 2020, 9: e52546 (doi: 10.7554/eLife.52546).
18. Ovečka M., von Wangenheim D., Tomančák P., Šamajová O., Komis G., Šamaj J. Multiscale imaging of plant development by light-sheet fluorescence microscopy. *Nature Plants*, 2018, 4(9): 639-650 (doi: 10.1038/s41477-018-0238-2).
19. Knapp E., Flores R., Scheiblin D., Modla S., Czymmek K., Yusibov V. A cryohistological protocol for preparation of large plant tissue sections for screening intracellular fluorescent protein expression. *BioTechniques*, 2012, 52(1): 31-37 (doi: 10.2144/000113778).
20. Shaner N.C., Lambert G.G., Chammas A., Ni Y., Cranfill P.J., Baird M.A., Sell B.R., Allen J.R., Day R.N., Israelsson M., Davidson M.W., Wang J. A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nat. Methods*, 2013, 10(5): 407-409 (doi: 10.1038/nmeth.2413).
21. Bindels D.S., Haarbosch L., van Weeren L., Postma M., Wiese K.E., Mastop M., Aumonier S., Gotthard G., Royant A., Hink M.A., Gadella Jr T.W.J. mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nat. Methods*, 2016, 14: 53 (doi: 10.1038/nmeth.4074).
22. Ivanov S., Harrison M.J. A set of fluorescent protein-based markers expressed from constitutive and arbuscular mycorrhiza-inducible promoters to label organelles, membranes and cytoskeletal elements in *Medicago truncatula*. *Plant J.*, 2014, 80(6): 1151-1163 (doi: 10.1111/tpj.12706).
23. Curtis M.D., Grossniklaus U. A Gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology*, 2003, 133(2): 462-469 (doi: 10.1104/pp.103.027979).
24. Engler C., Youles M., Gruetzner R., Ehnert T.-M., Werner S., Jones J.D.G., Patron N.J., Marillonnet S. A Golden Gate modular cloning toolbox for plants. *ACS Synthetic Biology*, 2014, 3(11): 839-843 (doi: 10.1021/sb4001504).
25. Karimi M., Inze D., Depicker A. GATEWAY(TM) vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science*, 2002, 7(5): 193-195 (doi: 10.1016/S1360-1385(02)02251-3).
26. Landy A. Dynamic, str2uctural, and regulatory aspects of lambda site-specific recombination. *Annu. Rev. Biochem.*, 1989, 58(1): 913-941 (doi: 10.1146/annurev.bi.58.070189.004405).
27. Hornung E., Krueger C., Pernstich C., Gipmans M., Porzel A., Feussner I. Production of (10*E*,12*Z*)-conjugated linoleic acid in yeast and tobacco seeds. *Biochimica et Biophysica Acta (BBA) — Molecular and Cell Biology of Lipids*, 2005, 1738(1-3): 105-114 (doi: 10.1016/j.bbalip.2005.11.004).
28. Op den Camp R.H.M., De Mita S., Lillo A., Cao Q., Limpens E., Bisseling T., Geurts R. A phylogenetic strategy based on a legume-specific whole genome duplication yields symbiotic cytokinin type-A response regulators. *Plant Physiology*, 2011, 157(4): 2013-2022 (doi: 10.1104/pp.111.187526).
29. Limpens E., Ramos J., Franken C., Raz V., Compaan B., Franssen H., Bisseling T., Geurts R. RNA interference in *Agrobacterium rhizogenes*-transformed roots of *Arabidopsis* and *Medicago truncatula*. *Journal of Experimental Botany*, 2004, 55(399): 983-992 (doi: 10.1093/jxb/erh122).
30. Tsai F.Y., Coruzzi G. Light represses transcription of asparagine synthetase genes in photosynthetic and nonphotosynthetic organs of plants. *Mol. Cell. Biol.*, 1991, 11(10): 4966-4972 (doi: 10.1128/mcb.11.10.4966).
31. Ulmasov T., Murfett J., Hagen G., Guilfoyle T.J. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell*, 1997, 9(11): 1963-1971 (doi: 10.1105/tpc.9.11.1963).
32. Nam H.-S., Benezra R. High levels of Id1 expression define B1 type adult neural stem cells. *Cell*

Stem Cell, 2009, 5(5): 515-526 (doi: 10.1016/j.stem.2009.08.017).
33. Shaner N.C., Steinbach P.A., Tsien R.Y. A guide to choosing fluorescent proteins. *Nat. Methods*, 2005, 2(12): 905-909 (doi: 10.1038/nmeth819).
34. Bajar B.T., Wang E.S., Lam A.J., Kim B.B., Jacobs C.L., Howe E.S., Davidson M.W., Lin M.Z., Chu J. Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting. *Scientific Reports*, 2016, 6(20889): 1-12 (doi: 10.1038/srep20889).
35. Goh T., Joi S., Mimura T., Fukaki H. The establishment of asymmetry in *Arabidopsis* lateral root founder cells is regulated by LBD16/ASL18 and related LBD/ASL proteins. *Development*, 2012, 139(5): 883-893 (doi: 10.1242/dev.071928).
36. De Rybel B., Vassileva V., Parizot B., Demeulenaere M., Grunewald W., Audenaert D., Van Campenhout J., Overvoorde P., Jansen L., Vanneste S., Möller B., Wilson M., Holman T., Van Isterdael G., Brunoud G., Vuylsteke M., Vernoux T., De Veylder L., Inzé D., Weijers D., Bennett M.J., Beeckman T. A novel Aux/IAA28 signaling cascade activates GATA23-dependent specification of lateral root founder cell identity. *Current Biology*, 2010, 20(19): 1697-1706 (doi: 10.1016/j.cub.2010.09.007).
37. Kiryushkin A.S., Ilina E.L., Puchkova V.A., Guseva E.D., Pawlowski K., Demchenko K.N. Lateral root initiation in the parental root meristem of cucurbits: Old players in a new position. *Front. Plant Sci.*, 2019, 10: 365 (doi: 10.3389/fpls.2019.00365).
38. Hostettler L., Grundy L., Käser-Pébernard S., Wicky C., Schafer W.R., Glauser D.A. The bright fluorescent protein mNeonGreen facilitates protein expression analysis *in vivo*. *G3: Genes, Genomes, Genetics*, 2017, 7(2): 607-615 (doi: 10.1534/g3.116.038133).
39. Balleza E., Kim J.M., Cluzel P. Systematic characterization of maturation time of fluorescent proteins in living cells. *Nat. Methods*, 2017, 15: 47 (doi: 10.1038/nmeth.4509).
40. Nagai T., Ibata K., Park E.S., Kubota M., Mikoshiba K., Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.*, 2002, 20(1): 87-90 (doi: 10.1038/nbt0102-87).
41. Yoo S.Y., Bomblies K., Yoo S.K., Yang J.W., Choi M.S., Lee J.S., Weigel D., Ahn J.H. The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. *Planta*, 2005, 221(4): 523-530 (doi: 10.1007/s00425-004-1466-4).
42. Zheng X.L., Deng W., Luo K.M., Duan H., Chen Y.Q., McAvoy R., Song S.Q., Pei Y., Li Y. The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters. *Plant Cell Rep.*, 2007, 26(8): 1195-1203 (doi: 10.1007/s00299-007-0307-x).
43. Samac D.A., Tesfaye M., Dornbusch M., Saruul P., Temple S.J. A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*). *Transgenic Res.*, 2004, 13(4): 349-361 (doi: 10.1023/B:TRAG.0000040022.84253.12).
44. An Y.-Q., McDowell J.M., Huang S., McKinney E.C., Chambliss S., Meagher R.B. Strong, constitutive expression of the Arabidopsis ACT2/ACT8 actin subclass in vegetative tissues. *The Plant Journal*, 1996, 10(1): 107-121 (doi: 10.1046/j.1365-313X.1996.10010107.x).
45. Vermeer J.E.M., von Wangenheim D., Barberon M., Lee Y., Stelzer E.H.K., Maizel A., Geldner N. A spatial accommodation by neighboring cells is required for organ initiation in *Arabidopsis*. *Science*, 2014, 343(6167): 178-183 (doi: 10.1126/science.1245871).
46. Chen Z., Wang J., Ye M.-X., Li H., Ji L.-X., Li Y., Cui D.-Q., Liu J.-M., An X.-M. A novel moderate constitutive promoter derived from poplar (*Populus tomentosa* Carrière). *Int. J. Mol. Sci.*, 2013, 14(3): 6187-6204 (doi: 10.3390/ijms14036187).
47. Benjamins R., Quint A., Weijers D., Hooykaas P., Offringa R. The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. *Development*, 2001, 128(20): 4057-4067.
48. McLean I.W., Nakane P.K. Periodate-lysine-paraformaldehyde fixative a new fixative for immunoelectron microscopy. *Journal of Histochemistry & Cytochemistry*, 1974, 22(12): 1077-1083 (doi: 10.1177/22.12.1077).
49. Kitaeva A.B., Kusakin P.G., Demchenko K.N., Tsyganov V.E. Key methodological features of tubulin cytoskeleton studies in nodules of legume plants. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2018, 53(3): 634-644 (doi: 10.15389/agrobiology.2018.3.634eng).
50. Kitaeva A.B., Demchenko K.N., Tikhonovich I.A., Timmers A.C.J., Tsyganov V.E. Comparative analysis of the tubulin cytoskeleton organization in nodules of *Medicago truncatula* and *Pisum sativum*: Bacterial release and bacteroid positioning correlate with characteristic microtubule rearrangements. *New Phytol.*, 2016, 210(1): 168-183 (doi: 10.1111/nph.13792).
51. Musielak T.J., Schenkel L., Kolb M., Henschen A., Bayer M. A simple and versatile cell wall staining protocol to study plant reproduction. *Plant Reprod.*, 2015, 28(3): 161-169 (doi: 10.1007/s00497-015-0267-1).
52. Zdyb A., Demchenko K., Heumann J., Mrosk C., Grzeganek P., Göbel C., Feussner I., Pawlowski K., Hause B. Jasmonate biosynthesis in legume and actinorhizal nodules. *New Phytol.*, 2011, 189(2): 568-579 (doi: 10.1111/j.1469-8137.2010.03504.x).

53. Stumpe M., Göbel C., Demchenko K., Hoffmann M., Klösgen R.B., Pawlowski K., Feussner I. Identification of an allene oxide synthase (CYP74C) that leads to formation of α-ketols from 9-hydroperoxides of linoleic and linolenic acid in below-ground organs of potato. *The Plant Journal*, 2006, 47(6): 883-896 (doi: 10.1111/j.1365-313X.2006.02843.x).
54. Demchenko K., Zdyb A., Feussner I., Pawlowski K. Analysis of the subcellular localisation of lipoxygenase in legume and actinorhizal nodules. *Plant Biology*, 2012, 14(1): 56-63 (doi: 10.1111/j.1438-8677.2011.00480.x).
55. Ilina E.L., Kiryushkin A.S., Semenova V.A., Demchenko N.P., Pawlowski K., Demchenko K.N. Lateral root initiation and formation within the parental root meristem of *Cucurbita pepo*: is auxin a key player? *Annals of Botany*, 2018, 122(5): 873-888 (doi: 10.1093/aob/mcy052).
56. Prunet N., Jack T.P., Meyerowitz E.M. Live confocal imaging of *Arabidopsis* flower buds. *Developmental Biology*, 2016, 419(1): 114-120 (doi: 10.1016/j.ydbio.2016.03.018).
57. Dickinson M.E., Bearman G., Tille S., Lansford R., Fraser S.E. Multi-spectral imaging and linear unmixing add a whole new dimension to laser scanning fluorescence microscopy. *BioTechniques*, 2001, 31(6): 1272-1278 (doi: 10.2144/01316bt01).
58. Kraus B., Ziegler M., Wolff H. Linear fluorescence unmixing in cell biological research. In: *Modern research and educational topics in microscopy*. A. Méndez-Vilas, J. Díaz (eds.). Formatex, Badajoz, Spain, 2007: 863-872.
59. Mylle E., Codreanu M.-C., Boruc J., Russinova E. Emission spectra profiling of fluorescent proteins in living plant cells. *Plant Meth.*, 2013, 9(1): 10 (doi: 10.1186/1746-4811-9-10).
60. Zimmermann T., Rietdorf J., Pepperkok R. Spectral imaging and its applications in live cell microscopy. *FEBS Letters*, 2003, 546(1): 87-92 (doi: 10.1016/S0014-5793(03)00521-0).
61. Korzhevskii D.E., Kirik O.V., Sukhorukova E.G., Kolos E.A., Karpenko M.N., Sufieva D.A., Nazarenkova A.V. *Molekulyarnaya morfologiya. Metody fluorestsentnoi i konfokal'noi lazernoi mikroskopii* [Molecular morphology. Methods of fluorescence and confocal laser microscopy]. St. Petersburg, 2014 (in Russ.).