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SYMBIOTIC NODULE SENESCENCE IN LEGUMES: MOLECULAR-GENETIC AND CELLULAR ASPECTS

(review)

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Abstract

Senescence is the natural stage in development of symbiotic nodule. As a result of senescence, reutilization of different nutrients from nodule to the other plant organs occurs. Generally senescence in legumes is triggered after flowering finishing, although the first traits of senescence can be observed very early during nodule development. A delay of the triggering of senescence program will allow to prolong the active nitrogen-fixating period and therefore to increase the amount of symbiotrophic nitrogen in plants and, finally, to elevate legume productivity. That is why no wonder that in the recent years the senescence of nitrogen-fixing nodules is actively studied. In this review the main developmental stages of nitrogen-fixing symbiotic nodule of legumes, particularities of symbiotic nodule development of determinate and indeterminate types are considered. In legumes with indeterminate nodules, the symbiosomes are not long-leaving as the infected tissues are permanently renewing due to apical meristem. There are two subsequent stages identified in an indeterminate nodule senescent. First a bacteroid degradation and the death of some infected cells occur, and then both symbiosomes and all infected cells are destroyed. In determinate nodules, the senescence initiated in the central part of a nodule, then extends to the peripheral zone. In this review morphological characters of nodule senescence at ulatrstructural level are analyzed. The role of cysteine and threonine proteases is discussed. Reutilization of nitrogen and other products of protein degradation are probably the most important during senescence. There are the evidences that in the root nodules of legumes the cysteine proteases are involved into nodule functions, adaptation of the host plant cells to physiological stresses, and the nodule senescence control. By a large-scale analysis of nodule transcriptome of *Medicago truncatula* Gaertn. several groups of genes expressed at successive stages of the senescence of indeterminate nodule are revealed. In this review the role of phytohormones, such as ethylene, abscisic acid, jasmonic acid, gibberellins and nitrogen monooxide in senescence of symbiotic nodule is considered. Nevertheless, until recent days our knowledge about hormonal control of nodule senescence is still incomplete. The oxidative stress, accompanying the process of nodule senescence is discussed. On the nodule aging, the concentrations of peroxides, protein carbonyls, modified DNA nucleotides and catalytic Fe increase. Iron activates lipids peroxidation in a peribacteroid space, resulting in degradation of the peribacteroid membrane in senescent nodules. The concentrations of oxidized glutathione and homoglutathione rise significantly during the nodule development, and the reduced forms decrease under senescence, indicating an oxidative stress in the senescing nodules. In this review the role of genes, encoding proteins involved in transport of wide-range of molecules, and genes, whose products are involved in regulatory and signal functions in cell; differences between stress-induced senescence and natural senescence are considered. Using model legumes, *Lotus japonicus* (Regel) K. Larsen and *M. truncatula*, several genes were cloned the mutations of which caused early senescence. It is emphasized that these genes encode different proteins involved into functioning of a symbiotic nodule. Until now, two transcription factors in *M. truncatula* are described, which are involved into nodule senescence. An induced senescence is more rapid, comparing to natural senescence, and manifests the signs of an oxidative stress and programmed cell death.

Keywords: legume-*Rhizobium* symbiosis, nodule development, genetic control, oxidative stress, proteases, ethylene, abcisic acid, jasmonic acid, gibberellins, nitrogen monoxide, rhizobia.

Senescence is the natural stage in development of symbiotic nodules. As a result of senescence, reutilization of different nutrients from nodule to the

other plant organs occurs. Generally senescence in legumes is triggered after the flowering has finished, although the first signs of senescence can be observed very early during nodule development.

Development of a symbiotic nodule. The development starts with a signal interaction between legumes and bacteria known as rizobia. Various aspects of the development of symbiotic nodules were widely discussed in numerous reviews (1-5). In brief, the legumes produce flavonoids, which trigger the synthesis of lipochitooligosaccharides, or Nod-factors, in rhizobia. The Nod-factors are perceived by specific receptors of plants, and activate the signal transduction mechanisms. As a result, the infection of host plant by rhizobia occurs and the formation of symbiotic nodule primordium is induced (1, 5). The infection process starts with the deformation and curling root hairs, which form an infection pocket containing rhizobia (2, 5). Hence they move deeper into the root hairs and root cells by special channel, an infection thread, formed by the plant (3-5). Simultaneously, in the root cortex and pericycle the cell divisions are induced, leading to formation of a nodule primordium. The infection thread reaches primordium, where the special outgrowths appear. They are surrounded by a plasma membrane but have no wall, which normally forms the border of an infection thread (3, 4). Due to a process resembling endocytosis the rhizobia are released from these outgrowths, or the infection droplets, into the plant cell cytoplasm (4), wherein the bacteria are surrounded by a symbiosome (peribacteroid) membrane of plant origin with the inclusion of bacterial proteins (1, 4). Then the differentiation of bacteria into a specialized form, the bacteroid, occurs. The bacteroid with symbiosome membrane is the main nitrogen-fixing unit, a symbiosome (1, 4, 5). In legumes from temperate latitudes (i.e. *Pisum sativum* L., *Medicago sativa* L.), the meristematic activity is maintained over the life of nodules (they earned the name «indeterminate»). Due to permanent meristematic activity in the nodule, there are different zones: I — meristem; II, III and IV — zones of infection, nitrogen fixation and senescence, respectively (Fig. 1, A, C). The indeterminate nodules are typically elongated in shape. In the nodules of the southern legumes (*Glycine max* L., *Phaseolus vulgaris* L.) the meristem remains active for a short period, leading to no zone formation and spherical shape of nodules, earned the name «determinate» (1, 4, 5).

Infected plant cells from the nitrogen-fixing zone remain functionally active for a limited time, and then senescence begins as a programmed cell death.

In the legumes with indeterminate nodules the symbiosomes are short-lived, because the central infected tissues are constantly renewed due to apical meristem. Thus, the first signs of senescence of the sybiosomes in pea and alfalfa were observed 14 days after the inoculation (6), while the intensive senescence started much later, after the end of flowering. In the legume plants that have determinate nodules the senescence also coincides with the end of flowering and early ripening, as it was shown in *G. max* (7) and *Vigna mungo* (L.) Hepper (8). Often the senescence is accelerated if the symbiosis is not effective due to mutations in plants (9, 10) or in bacteria (11). Senescence can be also induced by different abiotic stresses (12).

Morphological signs of senescence. Visually the senescence in nodules manifests in change of color in the nitrogen-fixing zone from pink, associated with the leghemoglobin functioning, to green due to the destruction of the heme group of the protein and the formation of biliverdin (13).

During senescence the cells of both symbiotic partners are destroyed (Fig. 1, B-D, 2, A, B). But in case of indeterminate nodules the zone of nitrogen fixation is constantly renewed due to cell transition from the meristem. Nev-

ertheless, the senescence zone increases with age, and the ultrastructural changes can be found in symbiosomes and cell organelles. Thus, the electron density of cytoplasm decreases, and there are vesicles and membrane fragments due to destruction of the plant cells and symbiosomes (14, 15).

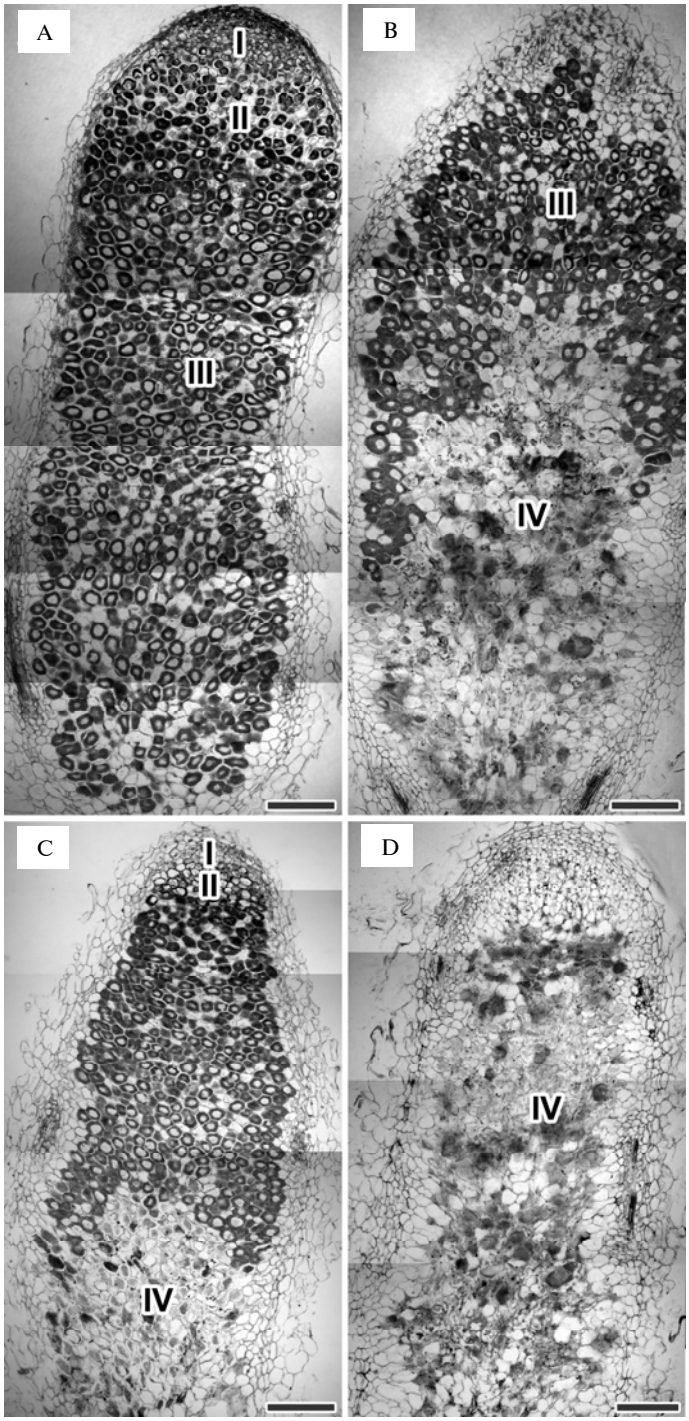

Fig. 1. Symbiotic nitrogen-fixing nodules in *Pisum sativum* L., the parental line SGE (A, B, 4 and 6 weeks, respectively) **and its derivate, the symbiotic mutant SGEFix$^-$-8 (*sym25*) with early senescent nodules** (C, D, 4 and 6 weeks, respectively)**:** I — meristem, II, III, IV — zones of infection, nitrogen fixation, and senescence, respectively (Scale bars: 200 μm).

In determinate nodule the senescence starts in its center and extends to a peripheral zone (16).

In young nodules of *Medicago truncatula* Gaertn., the model plant, it was shown (15), that in the central part of nitrogen-fixing zone there are several senescent infected cells of type I, in which the symbiosome degradation occurs with an increase of vesicular activity and amount of rough endoplasmic reticulum. Plant cells thus remain intact and have no visible signs of aging. In the same zone there are cells of type II with more severe senescence signs, in which integrity of a cell wall is disrupted. In older nodules, additionally to the cells of types I and II there are the cells of type III with destroyed symbiosomes and the signs of plant cell death (e.g. loosening of plasma membrane from the cell wall).

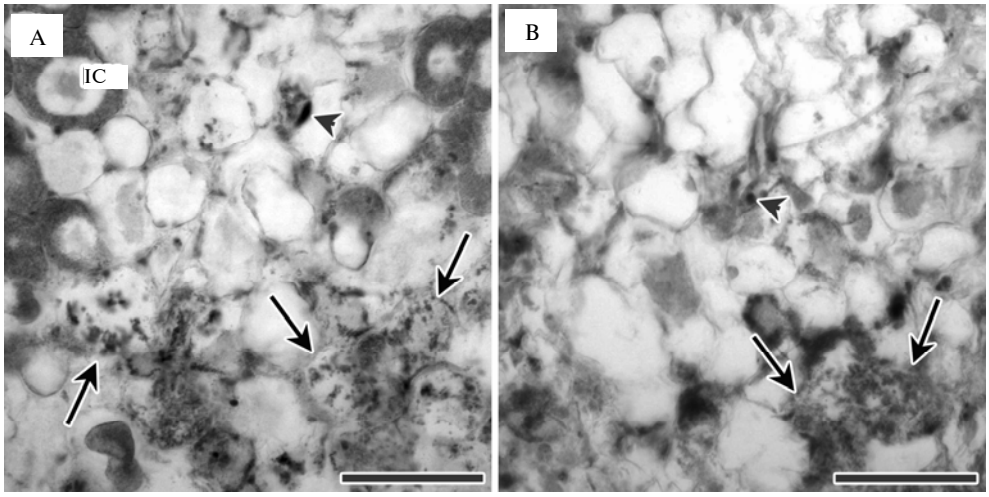

Рис. 2. Zone of senescence in nitrogen-fixing nodules in *Pisum sativum* L. of the parental line SGE (A) **and its derivate, a symbiotic mutant SGEFix$^-$-8 (*sym25*) with early senescent nodules** (B)**: IC** — infected cell; early degradation of bacteroids and host plant cells (type II, small arrows) and their full degradation (type III, big arrows) are shown (Scale bars: 100 μm).

Based on ultrastructural analysis, two stages can be indicated in the senescence of indeterminate nodules, namely an initial degradation of bacteroids and death of several plant cells, and full degradation of both symbiosomes and infected cells (12, 15).

In the nodules of ineffective pea mutants with early aging in the senescent cells there were the lysosome-like compartments with degrading bacteroids (9).

A senescence zone in mature indeterminate nodules of *M. truncatula* is cone-shaped and oriented towards direction of the nodule growth. The infected cells are destroyed before uninfected cells, which are involved into the transport of substances to the vascular tissues. The cone shape probably optimizes a remobilization of catabolites, because the cells with a peripheral localization near the vascular bundles can remain viable for longer time. Thus, a central position of the point where the senescence is initiated resulted from the largest distance from peripheral vascular system and environment that probably is due to the role of concentration gradients of oxygen or molecules delivered from the vascular bundles (12).

In alfalfa nodules after 7 weeks, the zone V located proximally to the senescence zone was described besides of zone IV (14). In zone V the next stage of releasing rhizobia from the remaining infection threads was observed that leads to reinvasion of old plant cells. This zone is an ecological niche where intracellular rhizobia act as exclusively saprophytic partners (14).

P r o t e a s e s. Senescence functionally means a reutilization of substances accumulated in tissues and then transported into another parts of the plant to be used for new organ formation. Protein degradation that allows reutilizing nitrogen is probably the most important during senescence. Therefore, it is not surprising that the majority of genes with positive regulation that are involved in senescence processes are the protease genes (17).

There are several proteases involved into senescence in plants. Thus, it was shown that the cysteine and threonine proteases mainly participate in the senescence of nodules (12, 15, 18).

There are evidences that in the root nodules of legumes the cysteine proteases are involved in the functioning of the nodule, in the adaptation of the host cells to physiological stress and in the control of nodule senescence. Proteases and their inhibitors were identified in the infected cells of the nodules (7, 19). Cysteine proteases with the acidic optimal pH were described in the nodules of *P. vulgaris* (19), and their activity increased to the beginning of senescence. Similar investigations were performed on *G. max* (7), *V. mungo* (8) and *M. sativa* (10).

In *P. sativum* an expression of the genes of cysteine protease 1 (*PsCyp1*) and cysteine protease 15a (*PsCyp15a*) was examined (18), using *Rhizobium leguminosarum* bv. *viciae* 3841, the effective strain, and *R. leguminosarum* bv. *viciae* B661, defective in the lipopolysaccharide synthesis and forming early senescent nodules under inoculation. Expression of both genes, *PsCyp1* and *PsCyp15a*, increased during nodule development (18).

By using wide-range transcriptome analysis of alfalfa *M. truncatula* nodules, few groups of genes specifically activated at successive stages of indeterminate nodule senescence were found. An increased expression was identified for genes from protease gene family during the later stages of senescence under a significant degradation of both symbiotic partners (15).

Among the identified molecular markers of the later stages of senescence, there was the vacuolar processing enzyme (VPE) gene from family C13 of cysteine protease group, responsible for maturation of vacuolar proteins, and genes encoding threonine proteases involved in F-box-specific ubiquitin/26S proteosome pathway (15).

H o r m o n e s. Development of a legume-*Rhizobium* symbiosis is also subject to hormonal regulation by the host plant. Among the phytohormones, ethylene, abscisic and jasmonic acids, gibberellins and nitrogen oxide NO were shown to be involved in the senescence of symbiotic nitrogen-fixing nodules. Nevertheless, it should be noted that to date our knowledge about the hormonal regulation of this process remains quite incomplete.

Ethylene acts as an activator of senescence in the symbiotic nodules of legume plants that is confirmed by upregulation of transcription factors (ERF) and the genes of ethylene biosynthesis, such as S-adenosylmethionine (SAM) synthetase, and 1-aminocyclopropane-1-carboxylate oxidase (15).

Induction of lipoxygenase genes suggests the involvement of jasmonic acid in different stages of aging symbiotic nodule. The oxidation of polyunsaturated fatty acids, with the participation of lipoxygenase enzymes is the first step in the biosynthesis of oxylipins such as jasmonate (15, 20).

During the natural senescence of the nodule, there is a decrease of ascorbate-glutathione antioxidant pool, combined with a reduction in carbon-to-nitrogen ratio in the nodule tissues. These changes can be perceived and transmitted by signaling mechanisms associated with abscisic acid (ABA), mobilizing proteolytic activity in the senescent symbiotic nodule (15, 21). ABA may be involved in the induction of the enzyme, which enhances the acceptor capability,

and thus contribute to the early development of individual nodules (22). However, an increased rate of synthesis of ABA during aging can cause the death of nodule. The content of ABA in the nodules of pea (*P. sativum*) is quite high during the first 2 weeks, but then reaches a plateau, and again increases at the later stages of development (23).

Gibberellic acid can inhibit senescence in nodules as it was illustrated by activation of genes which encode gibberellin-2-oxidase converting active phytohormone to inactive state (15, 24).

Recently, using the model legume plant *M. truncatula*, the regulation of aging in symbiotic nodule by nitrogen monoxide was revealed (25). Bacterial flavogemoglobin has been shown to play an important role in NO control in the nodules. In *hmp* mutant defective in flavogemoglobin biosynthesis, early senescence was observed with an increased amount of NO indicated in nodule tissues, while the hmp^{++} strain with flavogemoglobin overexpression was characterized by slower senescence and decreased amount of NO in nodules. Endogenous NO also caused a premature senescence of nodules (25).

Oxidative stress under senescence. Functioning of Legume-*Rhizobium* symbiosis contributes to the formation of reactive oxygen species (ROS). A slow autooxidation of oxyhemoglobin was shown to occur resulting in formation of superoxide anion O_2^- which disproportionates and produces hydrogen peroxide H_2O_2 (26). Besides, leghemoglobin reacts with H_2O_2, as a result the oxidized forms, particularly proteins with ferric iron and protein radicals, are produced (27). Hydrogen peroxide can also cause the protein destruction with releasing catalytic iron (i.e. iron in a molecular form) which is capable to activate lipid peroxidation and hydroxyl radical formation (28). ROS also are produced in reduction processes under conditions required for nitrogen fixation and due to activity of some proteins, including ferredoxine, uricase and hydrogenase (29).

Nevertheless, in nodules there are high concentrations of antioxidants (e.g. ascorbate, glutathione, superoxyde dismutases, catalases and enzymes of ascorbate-glutathione pathway, peroxiredoxine) (30). Antioxydants provide for keeping ROS in low concentrations. Free radicals were detected in the nodules (31, 32), and their concentrations increased during nodule development. Different rhizobial antioxidative systems play important role in ROS control. Moreover, the extracts from senescent nodules can cause the lipid peroxidation in plant cell membranes and peribacteroid membranes (26). In the cytochemical analysis with $CeCl_3$ the generation of cerium perhydroxide precipitates was observed in the senescent cell walls and around the peribacteroid and bacteroid membranes, confirming H_2O_2 involvement in the senescence of a symbiotic nodule (33, 34).

Study of 2- and 10-weeks nodules of *G. max* showed the oxidative stress during symbiotic nodule senescence in legumes. During nodule aging, concentration of peroxides, protein carbonyls, modified DNA, and catalytic iron increased. The iron from peribacteroid space provides for activation of lipid peroxidation, that can contributed to periacteroid membrane degradation in the senescent nodules. The amounts of glutathione and homoglutathione increased significantly in the course of development of the nodules, while the concentration of their reduced forms decreased during senescence, indicating the development of oxidative stress. For the first 24 hours of a nodule development, a significant DNA and protein destruction was found, thus there is the likelihood of oxidative stress during the formation of symbiosis (35).

Diffusion barrier responsible for an oxygen gradient in active nodules is probably lower under senescence. This may cause an increased oxygen flow to the tissues and an accelerated ROS generation (35).

Genetic control. Using experimental mutagenesis, in different legume plants a number of mutants unable to fix atmospheric nitrogen (Fix^- phenotype) were obtained. The histological and ultrastructural study indicated their Nop^- phenotype (no nodule persistence) as they were unable to maintain a stable structure and function of nodules. As a result, the early senescence is triggered in the nodules (Fig. 1, C, D; 2, B). In pea, the early senescence is characteristic for mutants in the genes *sym13*, *sym25*, *sym26*, *sym27* and *sym42* (36). Early senescence may be obviously considered as a specific reaction of the host plant to ineffective symbiosis.

In the experiments on model legume plants, *Lotus japonicus* (Regel) K. Larsen and *M. truncatula*, some genes have been cloned. Being defective, they led to early senescence. It must be emphasized that these genes encode quite different proteins involved in a symbiotic nodule functioning.

The first of them, *LjSST1*, encodes a nodule-specific tranporter which is located on symbiosome membrane and transfers sulphates from a plant cell cytoplasm to bacteroids (37). In mutants defective in this gene the early senescent nodules and lytic vacuoles are observed (37).

The gene *LjIGN1* encodes an ankyrin-repeat membrane protein containing transmembrane regions, necessary for differentiation and keeping up bacteroids, although it exact role remains unknown (38). In mutant in gene *LjIGN1*, the aggregation of bacteroids was observed in infected cells with disintegrated intracellular compartments, and the senescence starts much earlier than in the other Fix^- *L. japonicus* mutants (38).

The gene *LjSEN1* encodes the integral membrane protein, homologous to nodulin-21 of *G. max*, Fe/Mn transporter CCC1 of *Saccharomyces cerevisiae* and Fe transporter VIT1 of *Arabidopsis thaliana* L. *LjSEN1* expresses only in infected cells of the nodule (39), and the mutants demonstrate early senescence (40).

In *M. truncatula* the gene *MtDNF2* is cloned, for which, due to alternative splicing, the five mRNA transcripts can be transcribed. A predominant transcript encodes PI-PLC-like protein, similar to phosphoinositide phospholipase C. Presumably it can bind phosphatidylinositol or phosphorilated phosphatidylinositol, preventing their decay into inositol phosphate and diacylglycerol, which are the secondary messengers or the precursors of secondary messengers that trigger defense reactions. The mutant plants have nodules with the signs of early senescence (41).

In *M. truncatula* the mutant *esn1* with early senescence is recently reported. For this mutant, the differentiation of bacteroids, *nifH* (a nitrogenase subunit) gene expression, and *LHG* of leghemoglobin are characteristic (42).

It should be noted that to date there are no induced mutants with later senescence. Nevertheless, the transgenic alfalfa *M. truncatula* plants were obtained defective in expression of cystein protease 15a (gene *Cyp15a*), which are characterized by a delayed senescence of the symbiotic nodules (43).

Transcriptome analysis is one more fruitful approach to detect genes involved in nodule senescence. In 2006, the transcriptome was studied in *M. truncatula* plants of different age to find genes associated with the nodule senescence (15). Using cluster analysis, three groups of genes were revealed according to subsequent stages of senescence. To the first cluster the regulatory genes were assigned which are active when the senescence is initiated. These are the genes of APETALA/Ethylene Response Factor (AP2/ERF), also involved in defense against diseases and stresses; the gene homologous to that of abscisic acid-insensitive transcription factor with DNA-binding domains for AP2/ERF and B3; the genes of protein kinases and MAP-kinases encoding products involved in

signal transduction associated with stresses and reaction to external conditions; the DEAD-box genes of RNA-helicase, participating in mRNA export at stresses and plant development (15). The genes from second and third clusters are involved in senescence at stage I, when the bacteroids are destroyed, and at stage II, when plant cell degradation occurs, respectively. In both these clusters there are genes with regulatory and signal functions. Besides, the degradation of proteins, nucleic acids, membrane lipids and carbohydrates is subject to transcriptional regulation. Intensification of catabolic gene expression results in destruction of symbiosomes at early stage of senescence and plant cells at a later stage. Induction of genes encoding proteins that are involved in transfer of different molecules (e.g ATP-binding proteins and specific transporters of phosphates, amino acids, ions of metals) suggests that the degradation of macromolecules and mobilization are closely related, and under senescence of nodules the reutilization of metabolites occurs. Due to these catabolic events associated with transport of metabolites, the nodule is reversed from a carbon recipient to the donor of nutrients (15).

To date in *M. truncatula* two transcription factors are found involved in the senescence of symbiotic nodule. Thus, an increased expression of *MtATB2* gene encoding transcription factor of bZIP-type is described. The transcripts were found in apical zone and in vascular bundles of nodules. *MtATB2* transcription is repressed by saccharose, and MtATB2 protein is involved in the regulation of metabolism of amino acids (44). Another transcription factor, MtNAC969, was involved in repression of the genes activated in roots at saline stress, and also it was shown that RNA interference of transcripts led to manifestation of early senescence signs in symbiotic nodules (45).

The responses associated with nodule senescence are also subject to regulation during translation, since some of the studied genes (e.g., the gene encoding 40S ribosomal protein S8) encode ribosomal proteins, elongation factors and other proteins involved in regulation of translation (15).

Stress-induced and natural senescence. Premature nodule senescence can be induced by stresses. An induced senescence is faster and has the signs of oxidative stress and programmed cell death (15).

In a comparative ultrastructural study of natural and stress-induced nodule senescence in *M. truncatula*, some characteristic features were revealed, in particular, the bacteroids were more dense, peribacteroid space increased, and symbiosomes often fused. Nevertheless, the peribacteroid membrane remains intact even if internal part of bacteroid disappears, thus being evidently different from natural senescence when the symbiosomes are completely destroyed. Vesicular transport is less, too. After vacuole destruction, a complete degradation of cytoplasm occurs, mitochondria disappear, with the remnants of peribacteroid membranes and bacteroids being observed. Saprophytic bacteria from infection threads colonize the old cells at an early stage of degradation (12).

The darkness-induced senescence is faster and accompanied by destruction of the symbiosome contents in absence of remobilization cellular signals, and also by rapid colonization by saprophytic bacteria (12).

Comparing natural and darkness-induced senescence, not only characteristic morphological features but specific gene expression was found. At a transcriptional level, 50 % of genes activated under natural senescence are not involved in stress-induced senescence. They are genes responsible for regulation and transport, degradation of membranes and proteins, and also for stress tolerance (e.g., the genes encoding syntaxin protein and two phosphatidylinositol-4-phosphate 5-kinases, necessary for specific vesicle trafficking) (46). In certain other genes, just temporary positive regulation under darkness-induced nodule senescence was reported. Genes that are not likely to be involved in such in-

duced senescence encode many functions related to the degradation of proteins by means of the proteasomes, and some cysteine proteases, probably due to a simplified degradation at stress-induced senescence if compared to natural senescence. Recently a sharp acidification of peribacteroid space was shown in plants after nodule senescence induced by darkness (47).

Thus, currently the molecular, genetic and cell mechanisms underlying symbiotic nodule senescence are actively studied. Their understanding allows to start the programs for creating varieties of legumes with long nitrogen fixation to improve providing the soils with biological nitrogen and to increase yields with decreased application of mineral fertilizers.

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DIVERSITY OF PLANT VIRUSES IN THE EAST-ASIAN RUSSIA: 50 YEARS OF STUDYING

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Abstract

In ornamental plants, vegetables, fruits, berries, cereals and legumes the viral infections causes a decrease of productivity and yield quality, especially in the southern regions of agriculture in the Russian Far East where an infectious background is one of the highest. Besides, the viral infection leads to more impact from other phytopathogens, particularly under bacterial and fungal infections, and provokes degradation of varieties. Breeding varieties and hybrids resistant to viruses is now considered the most effective approach to antiviral plant protection. Therefore, the characteristic features of viruses are the key factors for plant protection strategy. For more than 50 years, in the Asian Russia more than 50 viruses have been found in agrocenoses of vegetables, cereals, legumes, ornamental plants, berries and potato plants, and also in biocenoses of wild plants and weeds. More than 10 of them have not been identified earlier not only in Far East and Siberia, but also in Russia. Basin on biological traits, physicochemical properties of viral polypeptides and nucleic acids, as well as antigenic characteristics of capsid proteins, a taxonomic status of the Asian Russian viral isolates has been identified, and their areal, pathogenicity and the impact have been studied. In Far East Russia, there have been revealed, described and identified the following viruses: *Brome mosaic virus*, Vicia unijuga mosaic virus, *Alfalfa mosaic virus*, *Cucumber mosaic virus* IA and IB East-Asian isolates, Soybean stunt virus, *Tomato aspermy virus*, *Cauliflower mosaic virus* Far-East Russian isolates, *Dahlia mosaic virus*, *Radish mosaic virus* type isolate, *Red clover mottle virus*, *Arabis mosaic virus*, *Raspberry ringspot virus*, *Tobacco ringspot virus*, *Tomato ringspot virus*, *Potato leafrole virus*, *Barley yellow dwarf virus — PAV*, *Pea enation mosaic virus 1*, *Bean common mosaic virus*, *Bean yellow mosaic virus*, Dahlia mild green mottle virus, *Hippeastrum mosaic virus*, *Onion yellow dwarf virus*, *Potato virus A*, *Potato virus Y*, *Soybean mosaic virus*, *Tobacco etch virus*, *Tradescantia mosaic virus*, Trifolium montanum (clover) mosaic virus, *Turnip mosaic virus*, *Watermelon mosaic virus* (*WMV-W*), Cereal (Oat) Russian pupation (pseudo-roset) virus, *Northern cereal mosaic virus*, *Lily symptomless virus*, *Potato virus M*, *Potato virus S*, Vicia pseudorobus mosaic virus, *Hydrangea ringspot virus*, *Plantago asiatica mosaic virus*, *Potato aucuba mosaic virus*, *Potato virus X*, *White clover mosaic virus*, *Tobacco necrosis virus*, *Rice stripe virus*, *Tobacco rattle virus*, *Tobacco mosaic virus*, *Tomato mosaic virus*, *Cucumber green mottle mosaic virus*, *Barley stripe mosaic virus*, *Rice mottle virus*, *Grapevine plum line virus*, *Pea streak virus*, *Potato yellow dwarf virus*, *Carnation ringspot dianthovirus*, *Carnation mottle virus*. A few of them (marked as ordinary typed) are not still registered and entered into the list of International Committee on Taxonomy of Viruses (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/). Using routine and molecular methods, the isolates were attributed to 18 genera from 10 families of 87 genera and 20 families described presently. Considering distance of Far East from the Central Russia, specific ecological factors, the local climate and unstable weather, a strategy for phytomonitoring of plant viruses has been worked out. In the southern and central zones, the most attention was paid to viruses on rice and soybean plants. The phytomonitoring of potato, vegetable plants and other crops was carried out all over the Asian Russia in both field conditions and greenhouses.

Keywords: plant viruses, methods of identification, taxonomy, East-Asian territory of Russia.

Viruses, the pathogenic agents, cause a significant decrease of plant productivity, especially in the Russian Far East where the levels of infections are rather high. Viral infection leads to worse exterior parameters and decreased quality of ornamental, fruit and berry plants, vegetables, grain and legume crops. Besides, viral infection contributes to invasions by other pathogens, such as bacteria and fungi, and also can provoke a degeneration of valuable varieties. Nowadays, the breeding of tolerant varieties and hybrids are considered the most

Genome, order, family	Genus	Species
ssRNA(+)	*Bromovirus*	*Brome mosaic virus*
Bromoviridae		Vicia unijuga mosaic virus[1]
	Alfamovirus	*Alfalfa mosaic virus*
	Cucumovirus	*Cucumber mosaic virus* IA and IB East-Asian isolates
		Soybean stunt virus[1]
		Tomato aspermy virus
dsDNA-RT	*Caulimovirus*	*Cauliflower mosaic virus* Far-East Russian isolates
Caulimoviridae		*Dahlia mosaic virus*
ssRNA(+)	*Comovirus*	*Radish mosaic virus* type isolate
Pecornovirales		*Red clover mottle virus*
Comoviridae	*Nepovirus*	*Arabis mosaic virus*
		Raspberry ringspot virus
		Tobacco ringspot virus
		Tomato ringspot virus
ssRNA(+)	*Polerovirus*	*Potato leafrole virus*
Luteoviridae	*Luteovirus*	*Barley yellow dwarf virus — PAV*
	Enamovirus	*Pea enation mosaic virus 1*
ssRNA(+)	*Potyvirus*	*Bean common mosaic virus*
Potyviridae		*Bean yellow mosaic virus*
		Dahlia mild green mottle virus[1]
		Hippeastrum mosaic virus
		Onion yellow dwarf virus
		Potato virus A
		Potato virus Y
		Soybean mosaic virus
		Tobacco etch virus
		Tradescantia mosaic virus
		Trifolium montanum (clover) mosaic virus[1]
		Turnip mosaic virus
		Watermelon mosaic virus (*WMW-W*)
ssRNA(–)	*Cytorhabdovirus*	Cereal (Oat) Russian pupation (pseudo-roset) virus[1]
Rhabdoviridae		*Northern cereal mosaic virus*
ssRNA(+)	*Carlavirus*	*Lily symptomless virus*
Tymovirales		*Potato virus M*
Betaflexiviridae		*Potato virus S*
		Vicia pseudorobus mosaic virus[1]
ssRNA(+)	*Potexvirus*	*Hydrangea ringspot virus*
Tymovirales		*Plantago asiatica mosaic virus*
Alphaflexiviridae		*Potato aucuba mosaic virus*
		Potato virus X
		White clover mosaic virus
ssRNA(+)	*Necrovirus*	*Tobacco necrosis virus*
Tombusviridae		
ssRNA(+)	*Tenuivirus*	*Rice stripe virus*
«floating» genus		
ssRNA(+)	*Tobravirus*	*Tobacco rattle virus*
Virgaviridae	*Tobamovirus*	*Tobacco mosaic virus*
		Tomato mosaic virus
		Cucumber green mottle mosaic virus
	Hordievirus	*Barley stripe mosaic virus*
ssRNA(+)		*Rice mottle virus*
Not classified viruses		*Grapevine plum line virus*
		Pea streak virus
		Potato yellow dwarf virus
		Carnation ringspot dianthovirus
		Carnation mottle virus

Comments. 1 — viruses not registered in the List of International Committee on Taxonomy of Viruses — ICTV, http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/).

effective approach to antiviral defense. This is why the characteristic features of the viruses become a key factor to determine a strategy for plant protection from these pathogens. In many European countries the creation of varieties tolerant to viral diseases, and drafting adaptive agroecosystems benefit the economy and allow to improve environment. To grow tolerant varieties and obtain the high yield, there is no need to apply high doses of pesticides.

First investigations of phytopathogenic viruses in the Far East are associated with the study of potato diseases. The vegetative mode of potato propagation resulted in fast accumulation of the viruses during several vegetation seasons, leading to degradation of the crop. Yield losses made 10-50 %, and the tuber quality was deteriorated, in particular, starch and vitamin C concentration decreased. It was important not only to study a severity and pathogenicity of mono- and mixed infections, but also to obtain healthy seed material and cultivate the varieties tolerant to viral infection. To start with, the most severe and spread potato viruses such as *Potato virus X* (PVX), *Potato leafroll virus, Potato virus Y and Potato virus A* (*PVY and PVA*), *Potato aucuba mosaic virus, Potato virus M and Potato virus S* (PVM and PVS) (Table) have been detected. Further, the list lengthened, including *Tobacco rattle tobravirus, Alfalfa mosaic virus, Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV*)*. A significant biodiversity of PVX, PVY, PVM and PVS species was characteristic (1, 2). By the beginning of XXI century, a virus-free potato seed reproduction based on the recovery of varieties and accelerated propagation of the initial seed material in the conditions precluding re-infection, allows to solve most problems of potato cultivation in the region due to application the apical meristem method and culling infected plants in the tests with antiviral serum (3, 4). After 1990, as new pathogens, viroids and mycoplasmas, were revealed, their investigation and the development of protective measures became actual. Particularly, in potato, *Potato spindle tuber viroid* (5) and *Potato leaf edge yellowing virus* (6) were described.

In vegetables cultures, mainly of *Brassicaceae* Burnett., *Solanaceae* Juss., *Cucurbitaceae* Juss., *Liliaceae* Juss., *Apiaceae* Lindl., *Chenopodaceae* Vent. and *Fabaceae* Lindl. families, the identified viruses formed the most numerous group of more than 20 species (7). The most frequent viral infections were registered on plants of *Cucurbitaceae* family, e.g. zucchini, pumpkin, melon, cucumber, squash, watermelon et al., *Solanaceae* family, e.g. tomatoes, eggplants, peppers et al., *Brassicaceae* family, e.g. different cabbages, cauliflower, radish, loba, daikon et al., and *Fabaceae* family, e.g. vegetable beans, peas vegetable, beans faba et al.. Phytomonitoring in 2000-2013 showed the CMV and TMV being the most widespread and harmful in *Cucurbitaceae* and *Solanaceae* plants, respectively. They both, the CMV and TMV, demonstrate a significant biodiversity, since over 20-30 TMV isolates and over 40 CMV isolates were described in drag, ornamental and other plants only in the Far East (8).

For CMV, a tripartite (+)RNA genome is characteristic, being a reason to study a genetic variability of its numerous Far East isolates. In Chabarovskii krai, in the Amur River basin, they infected tomato *Lycopersicon esculentum* L., pepper *Capsicum annuum* L., pumpkin *Cucurbita maxima* spp., melon *Cucurbita melo* spp. and cucumber *Cucumis sativus* spp. plants, in the Primorskii krai the infections were mostly found on tomato, cucumber, pepper, sometimes on watermelon, zucchini, squash, eggplant and pumpkin (9, 10). CMV also was identified on Kamchatka. No CMV infections were found in cabbage, horseradish, lettuce and parsley. Rather often the mixed infections with TMV could be observed resulting in significant yield losses that could sometimes reach 80 to 100 %. The obtained data for the Far East isolates were the same as for a conventional CMV strain (11). The isolates were easy transmitted mechanically and by aphids, but not by mean of seeds. According to the antigenic specificity of capsid proteins we identified the CMV isolates as a Far East serotype, and basing on PCR they were assigned to the IB subgroup of CMV East Asian isolates, or the vegetable isolate of Khabarovs, and IA isolates, or the ornamental isolates of Primorskii krai (12). It should be noted that in recent decades there were constant attempts to offer a rational classification of CMV isolates. First, basing on the biological characteristics they were divided into two groups, I and II, that has

been confirmed by molecular methods. Then, two groups, the Fny (I) and Q (II), varing on nucleotide sequences at 60-70 %, were formed of CMV population. Further, two subgroups, the IB and IA (the East Asian and the most common world isolates, respectively) were identified in I group.

Recently the genetic variability of 7 the most unique East Asian TMV isolates, infecting vegetables is under consideration to clarify their belonging to genus *Tobamovirus* (13). Earlier the *Tobamovirus* genus was not assigned to any known family, and only in 2009, together with other five «floating» genera, it was assigned to *Virgaviridae* family. RNA-sequencing showed the tight similarity of tobamoviruses comparing to other genera of the family. Thus, the International Committee on Taxonomy of Viruses, recommended to revise attributing known and newly isolated tobamoviruses to clarify their taxonomy according to the genome sequences (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/). It was suggested that in case the homology is below 10 %, the isolates are the strains of the same species but not different species. In the course of comparative phylogenetic analysis of gene sequences in isolated obtained in our investigations, and TMV from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/), the virus envelope protein (VEP) gene of 480 bp was identified in the eggplant isolate from Priamur'e (TMV-eggA) and in North Korean isolate (TMV-tNK) found on tomato plants in greenhouses (Institute of Experimental Biology, Academia of Science, DPRK, Pyangyang) and used as a control. With respect to biological and antigenic properties TMV-eggA was assigned to the tomato group, and TMV-tNK was assigned to the tobacco group. These isolates demonstrated high similarity to TMV and were less similar to *Rehmannia mosaic virus* (RMV) and *Tomato mosaic virus* (ToMV). Genome sequences of TMV-tNK and most TMV isolates demonstrated 97-99 % homology. As to amino acid composition of VEP, there was 94-95 % homology to RMV and only 83-85 % homology to ToMV. To standard isolate TMV U1, TMV-eggA is 95 % similar on the VEP amino acid sequence and 89 % similar on the genome sequence. The highest similarity to Mx isolates from Mexico (96 and 96 %, respectively), pet-TW from Taiwan (97 and 96 %, respectively), Ohio V from USA (99 and 98 %, respectively) was indicated allowing them to from a cluster. To them, TMV-eggA also demonstrates high degree of identity, e.g. 99 % homology of VEP gene to Ohio V, indicating the probability of common origin. Wherein, there was 90 % similarity on VEP gene and 96 % similarity on VEP between Ohio V and TMV U1, and the same data were obtained for TMV-eggA. Besides, eggplant Far East Russian isolates, similar to other isolates from its cluster, clearly differed from other TMV isolates due to extreme number of silent mutations. Comparing to TMV U1, only 5 of 50 point mutations in VPE resulted in the amino acid replacement. In further investigations of East Asian TMV isolates, it should be found out whether it is an adaptation to a group of test plants, or this phenomenon resulted from long geographic isolation from the main TMV population. According to our data, the genetic diversity of nucleotide sequences in most TMV isolates is very low (up to 6 %), thus, the 11 % divergence seems to be rather significant. Ohio V is the only isolate with reported sequence, while the others are only deposited in GenBank. The data obtained for genome of eggplant Far East Russian isolates could be helpful to clarify the taxonomy of TMV from this cluster.

On brassica vegetable plants and dahlias the DNA *Cauliflower mosaic virus* (CaMV) and *Dahlia mosaic virus* were found. Molecular study of eight CaMV isolates from daikon, cauliflower, cabbage, radish pants suggests them to be the Russian Far East population different from foreign isolates deposited in GenBank (14). To discuss their relationships in more detail, the genome sequencing should be completed.

According to data obtained, the members of *Potyvirus* genus of *Poty-*

viridae family are mostly distributed on vegetable crops (10). In Primor'e region on pepper plant the Tobacco etch virus was found for the first time in Russia. A Siberian isolate of *Onion yellow dwarf virus* detected in Novosibirskaya and Irkutskaya provinces has been studied. Another potyviruse, *Bean yellow mosaic virus* (BYMV) before recent time infected few plants of *Fabacea* family, such as soybean, red clover, pea, bean. To date, BYMV is frequently detected in biocenosis on different clover species, but unexpectedly for the first time in Russia it was also found on *Cucurbitaceae* vegetable plants. One more virus not found in Russia before, the *Watermelon mosaic virus* (WMV) was also isolated, and two its pathotypes, WMV-P and WMV-A with pawpaw and watermelon as test plants, respectively, are currently described. The Far East WMV isolate according to its properties could be assigned to WMV-A pathotype. It is extracted from squash and watermelon in Primorskii krai, and from squash on Sakhalin. In vegetable crops, *Alfalfa mosaic virus*, *Radish mosaic virus, Turnip mosaic virus*, *Tomato mosaic virus*, PVY, *White clover mosaic virus*, PVX, TMV, *Cucumber green mottle mosaic virus* and PVS have been also described.

The ornamental plants, e.g. petunia, hollyhock, snapdragon, the last being described as a CMV test plant for the firs time, delphinium, ficus, faskheda, dahlias, orchids were mostly infected by CMV, while, according to publications, in southern countries the orchids are rarely infected by CMV (15). On dahlia, apart from CMV and *Dahlia DNA mosaic virus*, we detected Dahlia mild green mottle virus which was not observed until recent time either as a member of *Potyvirus* genus, or as a *Dahlia* ssp. pathogen (16). Of *Potyvirus* on dahlia, only *Beet mosaic virus* has been earlier reported (17). For *Lily symptomless virus* and *Tulipa virus 1* the host plant range was mainly restricted by the species of *Liliaceae* family. The *Tradescantia mosaic virus*, a new potyvirus in Russia and in the Russian Far East, particularly, was also identified. This virus initially infected only *Commelinaceae* plants. A recent comparison of our results and databases (18) evidences that, according to ICTV, this Far East isolate is to be identified as *Tradescantia mosaic virus* from *Potyvirus.*

Daffodils were infected by *Tobacco necrosis virus* and the two TMV original strains. PVX was isolated from petunias, henbane and chrysanthemums, *Hydrangea ringspot virus* was found on hydrangea, *Carnation ringspot dianthovirus* was identified on carnation, commonly mixed with *Carnation mottle virus*, BYMV was isolated from gladiolus, Japanese aucuba, iris and Tigridia pavonia (28). *Tomato aspermy virus* was detected in Primorskii krai and Khabarovskii krai on chrysanthemums in greenhouses (8).

Unfortunately, with accordance to ICTV regulations, only four viruses of those infecting grain crops in the Russian Far East can be registered by the ICTV rules, but in fact the range of these viruses is much wider. They are *Barley stripe mosaic virus* from barley and whet plants in Primorskii krai, *Brome mosaic virus* first found in Amur Province on cultivated and wild cereals (8), and two cytorhabdoviruses, *Northern cereal mosaic virus* (NCMV) not detected previously in Russia, and Cereal (Oat) Russian pupation (pseudo-roset) virus (CRPV). CRPV may be considered the exclusive Russian virus as it is found only in Russia, similar to *Russian winter wheat mosaic virus* characteristic for the European Russia. Both properties of these two rhabdoviruses and biology of their the only vector, *Laodelphax striatteleus* F., were studied (19). Areas of these viruses differ (19), with CRPV frequently found in Siberia, and NCMV detected in the Amur River Basin in Primorskii krai, Khabarovskii krai and Chitinskaya Province. Due to the industrial cultivation of crops, NCMV is the most spread in the South of the Amur region, where the epiphytotics often cause a significant losses of yields. Natural NCMV circulation is related to biological peculiarities of its vector, *L. striatteleus* F., and crop loses depend on effectiveness of *L. stri-*

atteleus as a source of infection and NCMV number.

In rice, *Rice stripe virus* and *Rice mottle virus* have been found (8).

The southern Far East is the main Russian territory for soybean cultivation, thus, in the region the undiverted attention is paid to viral patholodies. According to detected symptoms, the viruses infecting *Fabacea* plants can be divided into the groups with stringent and wide-range specificity. Those from the first group demonstrate a restricted range of host plants, while those from the second one are characterized by high-effective transmittion of viruses in different ways, including infected seeds, and a dependence on natural foci of infection, e.g. perennial weeds and cultivated plants). In the world, more than 30 viruses infecting legumes have been currently described; of them 10 viruses have been studied in the Russian Far East. *White clover mosaic virus* was isolated from pea, red and white clover plants in Primor'e and on Sakhalin. For *Pea enation mosaic virus 1* found on pea plants in Primor'e, a narrow range of host plants, the *Fabacea* species mainly, is characteristic (20). *Bean common mosaic virus* (BCMV) was firstly identified in bean plants. Its host plants are the members of *Fabacea* genera, *Phaseolus*, *Pisum*, *Trifolium*, *Vicia*, *Vigna* mainly. BCMV is a non-persistant virus transmitted mechanically and by different aphids, and the high level of seed transmission is also characteristic. Due to comparative study of biological and antigenic characteristics of three BCMV isolates from the Russian Far Eats, China and North Korea, a lot of their similarities has bee found out (8). BCMV was isolated from pea and bean plants. The bean isolate from Primor'e infected legumes only, and could be easy transmitted mechanically and by aphids, also low seed transmission was detected depending on the plant variety and species. There is a single report about *Red clover mottle virus* found in the Russian Far East on pea plants (21). *Tobacco ringspot virus* isolated from soybean plants can be easy transmitted to *Nicotiana tabacum* L., pea, cowpeas, and through seed infection. Besides, the virus was extremely sensitive to external conditions, and at 28-30 °C no visible symptoms of infection could be detected (20).

For soybean in Primorskii krai and Khavarovskii krai, and later in Amur Rovince, the *Soybean mosaic virus* (SMV) is the most common and harmful. Its host plant range is rather limited, and for the long time the virus was considered to infect *Glycine max* (L.) Merr. only. To date there are described more than 30 plant species that can be infected, and for 10 of them a local reaction to SMV infection or latent infection are characteristic. SMV is easy transmitted via inoculation and through seed infection being commonly occurred. More than 20 aphids can transmit SMV. As to symptoms of infection, there are SMV of strong, low and moderate pathogenicity. Low-pathogenic SMV strain is spread on soybean crops in Amur Rovince. Nevertheless, soybean varieties are mostly infected by the strain of moderate pathogenicity similar to Japanise strain B. The high-pathogenic strain caused yellowing and necrotisation in leaves and apexes in the plants, they often died or were unable to yield production. SMV also infected some bean species.

Soybean stunt virus (SSV) was isolated in Amur Region from soybean plants (20). It can be easy transmitted under juice inoculation, by different aphids and via seeds. Soybean plants are its natural host. In infected plants a ring-like pigmentation of seed coat can be observed. The experimental host plant range is wide including plants of *Amaranthaceae*, *Compositae*, *Chenopodiaceae*, *Fabaceae*, *Solanaceae*, *Polygnaceae* families, etc. The virus is spread in the South-East Asia, particularly in China, Indonesia, Japan, etc. It is not yet included into the list of ICTV, but, considering properties, we assigned SBS to *Cucumovirus* genus of *Bromoviridae* family.

The viruses that affect berry crops are poorly studied. On raspberries and

currants the *Tomato ringspot virus*, *Arabis mosaic virus*, *Raspberry ringspot virus* of *Nepovirus* genus were identified (8). The same investigators also found CMV in berry crops.

Due to monitoring both agro- and biocoenoses in the Far East, not only infected plants, but cultivated vegetables, ornamentals, legumes, potato plants, natural herbaceous plants (e.g. different kinds of clover, plantain, etc.) were shown to be a reservation of viral infection. The pathogens were also detected on weeds and wild plants. They are *White clover mosaic virus*, *Red clover mottle virus*, *Plantago asiatica mosaic virus* from Sakhakin and Primor'e regions, PVX, TMV on plantain and BYMV on white clover from Sakhalin region, etc. In biocenoses, some viruses of legumes, namely Vicia pseudorobus mosaic virus, Vicia unijuga mosaic virus and Trifolium montanum (clover) mosaic virus, were first studied (22). *Alfalfa mosaic virus* was detected in red clover. Recently the BYMV frequently occurs in different clover species. For BYMV isolates derived from Trifolium montanum of Primirskii krai and Khabarovsk region, the narrow rang of host plants was revealed comparing to that of standard strain.

Immunochemical study of capsid proteins, together with biological features of the viruses, were the key for identification of the Far East isolates (1). The methods for preparative isolation were developed and precise immunochemical tests used to examine the antigenic relationships among the species of a genus. For virus-free plant cultivation the immunodiagnostics was used. Furthermore, the biological features of vectors, the ahids and leafhoppers, and the mode of natural and experimental tramsmission of viruses by vectors and via seeds were studied, etc. The viroses of wild plants and weeds were revealed, and the biophysical methods used to obtain the seeds which were free of viruses, etc. (23).

Due to molecular methods, the results of identification were confirmed, and a genetic variability of some Far East isolates (e.g. *Cauliflower mosaic virus* Far-East Russian isolates, CMV, TMV, etc.) studied. Also a phylogenetic analysis of the isolates has been carried out using data from GenBank. At the end of 1990s, almost 40 viruses were identified, while much more isolates and strains were studied. Described viruses were assigned to 16 genera and 5 families (1, 24). A decade later, the number of described viruses increased, and they were assigned to 17 genera and 8 families (8). The isolates derived from the Asian Russia are the member of 18 genera and 10 families (see Table.) of 87 genera and 20 families of plant viruses currently known (25). In the table there are some viruses the properties of which are not yet understood, namely *Grapevine plum line virus*, *Pea streak virus*, *Potato yellow dwarf virus* (26), *Carnation ringspot dianthovirus*, *Carnation mottle virus* и др.

In virology for the last 20 years the changes in taxonomy are much more fundamental comparing to other fields of biology. Unlike the viruses of vertebral animals and microorganisms, plant viruses were not associated with specific taxons, being mostly assigned to groups but not the genera. Recently developed taxonomy of viruses includes the hierarchical levels, i.e. the order, family, genus, species (virus). Due to extra data about viral genomes together with common tests used, more correct identification of viruses is possible, and new genera and families of plant viruses appear. Comparison of our data and the results reported by ICTV (27, 28), allowed to suggest the taxonomy of plant viruses of the Russian Far East region according to the concepts accepted in modern virology. Nevertheless, in the next ICTV report (25) there were sufficient amendments. In particular, new genera and their families were reported. These changes have also affected the Russian Asian isolates (see Table). Thus, the members of *Tobamovirus* genus are included in new family *Virgaviridae*. *Flexiviridae* family is turned into three new ones, of which two, *Alphaflexiviridae* and *Betaflexiviridae*, were

replenished with new genera, etc. The most impressive data concerning a relationship among viruses of a species are obtained by molecular methods. Nevertheless, there are some mismatches between systematics based on classical and molecular approaches, therefore the criteria must be suggested to solve the contradictions. Certainly, during the coming years, in the taxonomy of viruses dramatic changes have to occur since the characteristic of many viruses is still based on incomplete information about their genome.

Thus, the accumulated phytovirological data reflect the level of investigation in the Asian Russia. Doubtlessly the number of plant viruses detected in this region will increase.

Contemporary approaches to estimation of virological situation and development of effective practical technologies were the goals of fundamental and applied research conducted since 2004 in Far East Institute of Biology and Soils (RAS) and a number of regional agricultural institutes (Primirskii institute, Far East institute, Sakhalin institute. Kamchatskii institute). Taking into account the regional peculiarities, such as remoteness from the central regions of Russia and the instability of the weather conditions, the regional strategy for agricultural development was formulated. According to the strategy, the potato and vegetable crops could be grown throughout, the soybean production was located in Primorskii krai and Khabarovskii krai, grain crops production was located in Amurskaya Province, Primorskii krai was the area of rice production, etc. Long-term investigations showed the role of treatment with chemicals to control the number of insects that serve as virus transmitters, but breeding tolerant varieties and hybrids is the most effective. During evolution the plants formed different defense mechanisms to prevent invasion and development of infection. There are host plants not infected by viruses, and also host plants with different tolerance ot infection. Non host resistance, being not individual, can be manifested in a species, if all the members of a species loss the factor necessary for successful infection development, or this factor is changed. Due to mutation the virus can obtain the ability to infect plants beyond the initial host rang, but loss the ability to infect its common hosts. Non host resistance mechanisms are not still found out (29). All these and many other aspects are important to create tolerant varieties.

So, in coenoses of the Russian Far East and Siberian regions, the wide-ranged experiments have been conducted for about 50 years to detect and identify viruses, to study their reservoirs and ways of transmission, a relationships in associations, plant tolerance to viral infection, etc. Till recent time, such comprehensive observations have been carried out in the Far East only. The route survey for the length, the territory, and the diversity of climatic zones could be considered unique. Nevertheless, the Asian Russia, in particular Siberia, Magadanskaya Province, Sakhalin island, Kamchatka, still remains insufficiently known according to species composition, prevalence and severity of viruses. These investigations seem to be relevant, as the taxonomy of viruses is one of the most dynamically developing and permanently changing field of virology. Due to molecular methods of sequencing genome and phylogenetic study of viral isolated derived from different sources, both fundamental and applied research became more effective.

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STUDY OF THE INITIAL STEPS OF POTATO VIRUS X ASSEMBLY

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Abstract

Potato virus X (*Potexvirus*) is an important pathogen of number of economically significant agricultural plants of the *Solanaceae* family. Potato virus X (PVX) virions are flexible filamentous particles 515 nm in length and 13.5 nm in diameter with a helical structure. PVX genome consists of a single-stranded RNA (6435 nucleotides) which is capped and polyadenylated. The study of various stages of the infection process of plant viruses during infection, including the assembly of viral particles is of great practical interest. Identification of these mechanisms can be the basis for developing new approaches in virus-free crop production. Herein, the initial stages of potato virus X (PVX) virion assembly were examined on the example of virus-like particles (VLPs) produced by incubation of PVX RNA with PVX coat protein (CP) in vitro. The formation of identical set of VLP with discrete size under different incubation conditions (ionic strength, pH) was shown. However, the amount of VLP of a different size changes depending on incubation conditions. Most efficient VLP similar to the native virion size assembly has been achieved in a buffer of low ionic strength at pH 8.5. PVX RNA fragments from 800 to 5700 nt in length which were protected by coat protein within VLPs were isolated. Their individual analysis confirmed that all of them represent the 5′-terminal fragments of the genomic PVX RNA of different lengths. Thus, it was revealed that RNAs within VLP are genomic RNA 5′-terminal fragments of different lengths. PVX virions assembly initiation at the RNA 5′-end which is cooperatively extending in the 5′- to 3′-end direction was confirmed. Nucleotide sequence analysis of RNA fragments isolated from VLP of different sizes showed that sites capable to form RNA hairpins were discovered near the RNA 3′-end. They could act as «stop-signals» that prevent CP and RNA interaction and continuous cooperative assembling PVX VLP and virions. Probably, CP could «melt» RNA hairpins more or less efficiently and overcome «stop-signals» during the viral particles formation, depending on the conditions of incubation with the RNA in vitro.

Keywords: plant viruses, potato virus X, virus-like particles, RNA, coat protein, virion assembly.

The study of various stages of the infection process of plant viruses including the assembly of viral particles is of great practical interest. Identification of these mechanisms can be the basis for developing new approaches in virus-free crop production. Potato virus X (PVX), a typical *Potexvirus* (*Alphaflexiviridae*), is a common pathogen of many plant species of *Solanaceae* family, including number of economically significant agricultural plants, particularly potato plants. It should be noted that potato, together with rice, wheat and corn, are the main food stuffs in the world.

PVX virions are flexible filamentous particles 515 nm in length and 13.5 nm in diameter with a helical structure. PVX genome consists of a single-stranded RNA (6435 nucleotides) which is capped and polyadenylated. About 1300 subunits of viral coat protein (CP) are polymerized to form polar helix with a pitch of 3.6 nm, each coil consist of 8-9 CP subunits, with viral RNA being encapsulated into the CP helix (1).

PVX is the first filamentous plant virus reconstructed in vitro from RNA and CP (2). No differences between native virions and the reconstructed parti-

cles (i.e. virus-like particles, or VLP, and viral ribonucleoproteins, or vRNP) have been found. Furthermore, it was shown that the CP of PVX is unable to polymerize without RNA (3), and in vitro can form vRNP not only with RNA of PVX but also with heterologous nucleic acids (2). In further study of structure and characteristics of these vRNP or VLP, their homology to native virions has been identified (4, 5).

K.H. Kim and C. Hemenway (6) suggested that 5′-end of PVX RNA can be involved into coordination of viral part assembly. The region responsible for initiation of PVX assembling is not completely characterized, though in different publications there are evidences for its location at the 5′-end of RNA molecule (7-9). Recently published data suggest a key role of a cap-structure at the 5′-end of RNA in assembling vRNP of PVX through changing conformation of the 5′-end of RNA and, as a result, the CP recognition at initiation of the particle assembly (10).

Herein, the initial stages of potato virus X (PVX) virion assembly were examined on the example of virus-like particles (VLPs) produced by incubation of PVX RNA with PVX coat protein (CP) in vitro.

Technique. PVX virions, the Russian strain, were isolated from *Datura stramonium* leaves (11), PVX coat protein was obtained by treatment of viral suspension with LiCl (12), and RNA was purified using phenolic method (13).

To obtain VLPs, PVX RNA and CP (1:700) were incubated for 10 min at room temperature in 0.01-0.001 M Tris-HCl buffer at pH 7.5, 8.0, or 8.5. The VLPs were treated with micrococcal nuclease (MN) («Fermentas», Lithuania), activated before use by 100 mM $CaCl_2$. After 10 min incubation at room temperature the reaction was stopped by adding 250 mM EGTA. RNA fragments were isolated from MN treated VLPs using tRNA as coprecipitator.

RT-PCR was carried out according to manufacturer's protocol («Promega», USA) with forward and reverse primers, complementary to PVX RNA sequences of 21-40 nt and 950-981 nt positions, respectively.

Nucleic acid study and analysis of RNA encapsulated in VLPs were conducted by gel retardation assay using 1 % agarose gel made with TAE (40 mM Tris acetate, 2 mM EDTA, pH 8.0; 0.025 μg ethidium bromide). After incubation, the RNA and CP mixtures were diluted in electrophoretic buffer containing 10 % glycerin and 0.2 % bromophenol blue, and then the samples were placed into the wells. Quick screen horizontal electrophoresis system QS-710 («IBI Scientific», USA) was used for the separation at constant voltage 70 V, with chemiluminiscent detection system ChemDOC XRS+ («Bio-Rad», USA; λ = 254 nm) applied for documentation.

For transmission electron microscopy of VLPs, the preparations were contrasted by 2 % uranyl acetate and examined in JEOL JEM-1011 (JEOL Ltd., Japan) at an accelerating voltage of 80 kV. The images were obtained with a digital camera (ES500W, Erlangshen, Gatan, USA) using DigitalMicrograph (Gatan, USA). To calculate VLPs lengths and diameters, micrographs were analyzed by scientific image manipulation software ImageJ (National Institutes of Health, USA).

The nucleotide sequences were analyzed using data and tools of The mfold Web Server (14).

Results. To investigate initial steps of assembly of PVX virions, the PVX RNA was incubated with PVX CP at the rate of 1:700 in different conditions. In a native PVX particle, the RNA to protein rate of 1:1300 is reported (1). So, the amount of CP used in our experiment was not enough for full RNA encapsulation, resulting in the VLPs in which the RNA molecule remains partly uncoated by protein. Single tailed particles (STP) with free 3′-end of RNA and rod-like

heads due to helical encapsulation of the 5′-end fragment of the viral RNA with CP, were reported earlier (9). STP probably is a transport form together with PVX virions (9). It should be noted that even if the molar rate of RNA to CP was the same as in the native virus, in most of the reconstructed preparations the VLPs were not full-sized (3).

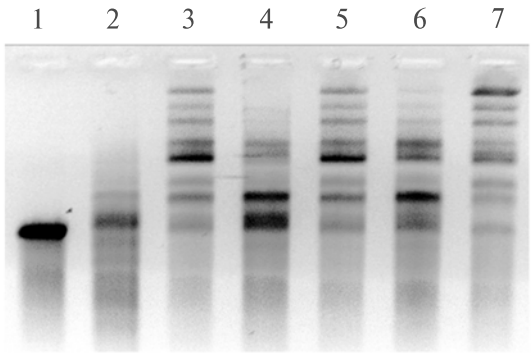

Fig. 1. Electrophoresis of virus-like particles reconstructed in vitro by reassembly from potato virus X (PVX) **RNA and coat protein** (CP) **at different pH and ionic strength:** 1 — control (RNA of PVX); 2, 4, 6 — 0.01 M Tris-HCl, pH 7.5, 8.0 and 8.5, respectively; 3, 5, 7 — 0.001 M Tris-HCl, pH 7.5, 8.0 and 8.5, respectively. RNA to CP molar rate is 1:700; 1 % agarose, staining with ethidium bromide.

The electorphoregrams (Fig. 1) show that the VLPs reassembly resulted in less intensity of the RNA band (see Fig. 1, track 1), while the slower moving products appeared due to particles with different level of encapsidation (see Fig. 1, tracks 2-7). The bands of the highest molecular weight corresponded to the VLPs with high extent of RNA encapsidation with CP. In 0.01 M Tris-HCl buffer, as pH increased from 7.5 to 8.5, the efficacy of reassembly notably rose and the number of products with higher molecular weight increased (see Fig. 1, tracks 2, 4, 6), while in 0.001 M Tris-HCl, there were no notable changes (see Fig. 1, tracks 3, 5, 7), except the maximum intensity of the band with the highest molecular weight (see Fig. 1, track 7). Thus, these conditions contribute to effective RNA encapsulation and formation of VLPs in which the PVX genome RNA is mostly coated by CP. It is also important that the intensity of the bands formed by VLPs of different sizes varied depending on the conditions of reassembling, while their positions in 1 % agarose remained unchanged. So, the stop signals seem to occur in the course of reassembly of CP on RNA from 5′- to 3′-end, and their overcoming is possible due to change of ionic strength and pH of incubation medium.

Electron microscopy of the VLPs which were obtained in the favorable conditions (0.001 M Tris-HCl, pH 8.5) confirmed that in the preparations there are particles of different size (Fig. 2, B) with the diameter and structure as those in PVX virions (see Fig. 2, A). Nevertheless, the VLPs were shorter than PVX virions, so, the RNA molecules were not completely packed into CP with «tails» remained free.

In the histogram, there are 8 discrete peaks corresponding to particle sizes of 60, 90, 160, 220, 300, 360, 420 and 460 nm (see Fig. 2, C). These data are consistent with results of gel retardation assay (see Fig. 1, track 7) according to which the obtained VLPs are a discrete set of particles heterogeneous in size. Considering that the native PVX virion of 515 nm in length contains the PVX genome RNA molecule of 6435 nt, the VLPs of specified sizes can presumably contain 800, 1100, 2000, 2700, 3700, 4500, 5200 and 5700 nt fragments of PVX genome RNA, respectively.

To characterize the RNA regions packed into VLPs, the particles were treated with MN in conditions when the RNA bound to CP in the particle is protected from the nuclease. Under electrophoresis of the samples obtained after the MN treatment, the fragments from 800 to 5700 nt were found (Fig. 3, A, track 1). To elucidate whether they are 5′-end regions, each individual fragment

(see Fig. 3, A, tracks 2-9) was isolated and used in RT-PCR.

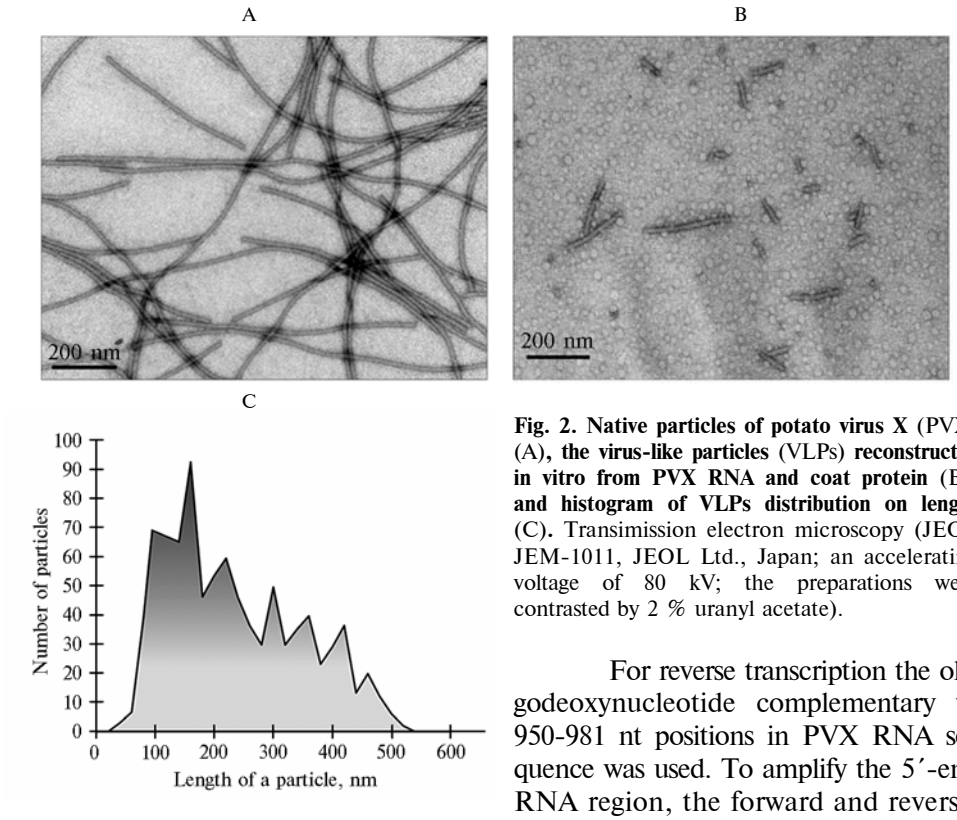

Fig. 2. Native particles of potato virus X (PVX) (A)**, the virus-like particles** (VLPs) **reconstructed in vitro from PVX RNA and coat protein** (B)**, and histogram of VLPs distribution on length** (C)**.** Transimission electron microscopy (JEOL JEM-1011, JEOL Ltd., Japan; an accelerating voltage of 80 kV; the preparations were contrasted by 2 % uranyl acetate).

For reverse transcription the oligodeoxynucleotide complementary to 950-981 nt positions in PVX RNA sequence was used. To amplify the 5′-end RNA region, the forward and reverse primers, complementary to the PVX RNA sequences at 21-40 nt and 950-981 nt positions, respectively, were used. The expected amplification product (see Fig. 3, B) was obtained for most fragments (see Fig. 3, tracks 2-8), except the fragment the length of which was less than 876 nt (see Fig. 3, A, track 9). So, it could not have the site for binding reverse primer complementary to RNA sequence at 950-981 nt positions. Thus, the RNAs within VLP are genomic RNA 5′-terminal fragments of different lengths, and PVX virions assembly initiation at the RNA 5′-end which is cooperatively extending in the 5′- to 3′-end direction is confirmed.

Using SELEX method, S.J. Kwon et al. (7) earlier found a number of RNA sequences having high affinity to PVX CP. By prediction of their secondary structure using computer modeling, a capability to form RNA hairpins was demonstrated. According to our suggestion, the similar hairpins could be distributed along the genome RNA of PVX affecting interaction between RNA and CP.

Indeed, the computer analysis of predicted secondary structure of the 3′-ends in individual RNA fragment from VLPs of different sizes confirmed a probability of hairpins formation.

The figure 4 illustrates the predicted hairpin structures for 3′-ends in individual fragments (see Fig. 3) of 1100, 2700, 5200 and 5700 nt in length calculated on the base of histogram of VLPs distribution according to their size (see Fig. 2, C). A capability to form similar RNA hairpins was also demonstrated for other individual fragments observed in our experiments (800, 2000, 3700 and 4500 nt, data not shown).

It can be assumed, that these hairpins act as stop signals preventing cooperative assembly of VLPs. In early experiments it was shown that coat protein

of potexviruses is capable of melting secondary structure in RNA (15).

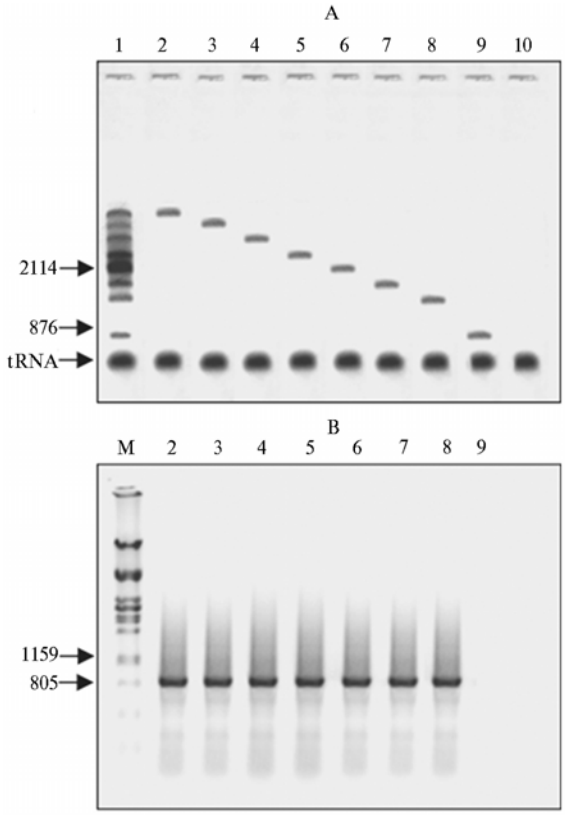

Fig. 3. Analysis of potato virus X (PVX) **RNA fragments from in vitro reconstructed virus-like particles** (VLPs) **of different sizes treated with micrococcal nuclease** (MN)**:** A — electrophoretic pattern, B — RT-PCR analysis of individual fragments; 1 — RNA fragments isolated from VLPs (A), 2-9 — individual RNA fragments, 10 — tRNA as coprecipitator (A); M — DNA molecular weight marker (B). The arrows indicate the markers of RNA length, nt (A), and DNA length, bp (B); 1 % agarose, staining with ethidium bromide. In RT-PCR the forward and reverse primers, complementary to the PVX RNA sequences at 21-40 nt and 950-981 nt positions, respectively, were used.

To overcome stop signals, the coat protein should be detained at such a region, melt the hairpin, and only after that the process of assembling virion could be continued.

Thus we have shown that incubation of potato virus X (PVX) RNA and coat protein (CP) under different conditions (ionic strength, pH) formed one and the same pattern of virus-like particles (VLPs) of specific size. Nevertheless, the amount of individual VLP

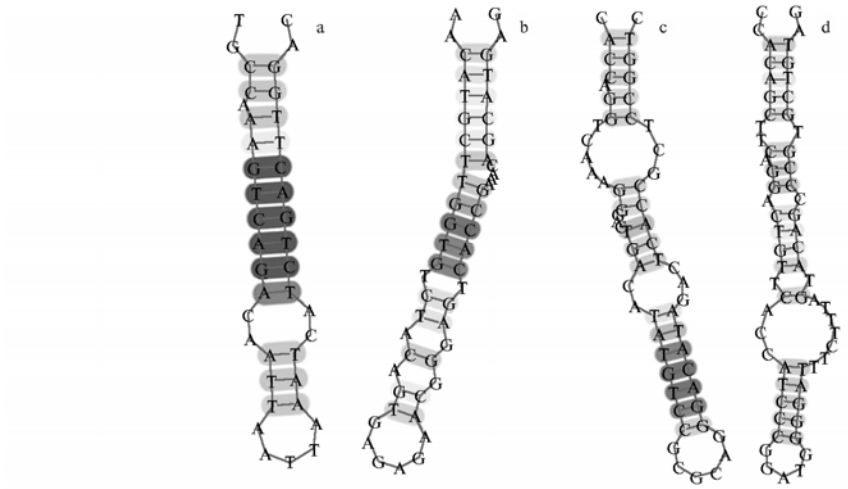

Fig. 4. Schemes of a predicted secondary structure in the 3′-ends of RNA fragments isolated from virus-like particles (VLPs) **of different sizes reconstructed in vitro from potato virus X** (PVX) **RNA and coat protein** (CP)**:** a — 1078-1117 nt, b — 2705-2757 nt, c — 5125-5183 nt, d — 5730-5792 nt. Grayscale colors reflect the strength of interaction between nucleotides. The images were obtained with the use of The mfold Web Server (http://mfold.rit.albany.edu/).

depends on the conditions during incubation. Most efficient VLP similar to the native virion size assembly has been achieved in a buffer of low ionic strength at

pH 8.5. From VLPs the RNA fragments protected by coat protein helix were isolated. All of them were shown to be the 5′-proximal fragments of PVX genome RNA and have different length. More evidences are obtained that the PVX assembly is initiated at the 5′-end of the RNA molecule, being cooperatively extended to its 3′-end. Due to analysis of nucleotide sequences of 3′-regions of RNA fragments isolated from the reconstructed VLPs, the sites were discovered where the RNA-hairpins could be probably formed. These RNA-hairpins may play a role of stop signals and prevent cooperative assembling VLPs. The changes of incubation conditions probably affect the CP capability of melting and overcoming these hairpins.

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THE FRAGMENTS OF HOMOLOGIES OF ENDOGENOUS RETROVIRUSES IN THE GENOMES OF PLANTS AND ANIMALS

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Abstract

Using sequences of mobile genetic elements in order to mark polymorphic sites in their insertion might be the most effective approach to identify specific features of gene pools of different groups of organisms and control their dynamics, which is especially important in dealing with agricultural species. *Sireviruses* (*Pseudoviridae*) one of the oldest LTR-retrotransposons are widespread throughout the plant kingdom. SIRE-1 is one of the largest and most detailed studied retrotransposon. Its analysis showed that insertions of the retroelement (particularly in the genome of maize) have occurred recently. We studied the polymorphism of DNA fragments flanked by LTR-retrotransposon SIRE-1 (IRAP-PCR markers) in the genomes of different taxonomic groups. The objects of our study were *Triticum aestivum* (Moscovskaya 39, Mironovskaya 808, Omskaya 36 varieties), *Glycine soja* (five wild populations of Primorskii region of Russia) and *G. max* (China), as well as representatives of the factory and indigenous breeds of cattle — Black-and-White cattle improved by Holstein cattle, Ayrshire, Yakut and Red Estonian cattle (97 animals in total). A terminal site of SIRE-1 was chosen as primer in IRAP-PCR (GCA-GTT-ATG-CAA-GTG-GGA-TCA-GCA). The data indicate that the multiloci genotyping by IRAP-PCR using retrotransposon LTR SIRE-1 as a marker reliably differentiates not only representatives of the monocotyledonous and dicotyledonous plants, but also their varieties. Spectrum of DNA fragments (13 to 16 depending on the breed) obtained in studies different breeds of cattle using IRAP-PCR markers was in the length range of 330-1470 bp. The highest polymorphism of DNA fragments was observed in the middle part of the spectrum (760-980 bp) in Ayrshire and Black-and-White cows. According to the obtained dendrogram one of the groups of Black-and-White cows and Ayrshire cattle were closer to each other, while the other with Red Estonian and Yakut cattle became isolated in a separate subcluster. Identified differences in heterogeneity in the two studied groups of Black-and-White from different farms possibly could be explained by the peculiarities of breeding work carried out or with other factors of artificial and/or natural selection. The possibility of using LTR-retrotransposons as molecular genetic markers for polyloci genotyping plants and animals is discussed.

Keywords: inverted terminal sites of LTR-retrotransposons, *Glycine*, *Triticum*, *Bos taurus*, polymorphic information content, the share of polymorphic loci.

A lot of molecular methods developed recently to mark different polymorphic region of genomes (e.g. RFLP, a restriction fragment length polymorphism, SNP, a single nucleotide polymorphism, AFLP, an amplified fragment length polymorphism, SSR-PCR, simple sequence repeats PCR, etc.) contribute to successful genotyping in both cultivated plant varieties (1-4), animal breeds (5-7). Of these markers, the mobile genetic elements have special status comparing to other structural and functional genetic elements due to their capability of transposition, and the high rate of transposition allows suggesting their essential role in generation of genetic variability (8).

In plants the LTR (long terminal repeat) retrotransposons make up a significant portion of genomes, particularly slightly more than 7 % in *Arabidopsis thaliana* (9), 50 and 90 % in rice and wheat, respectively (10), 75 % in corn (11).

Sireviruses is one of the oldest LTR-retrotransposons, which became

widespread in the plant kingdom and the only member of *Pseudoviridae* family that supposed to have gene similar to that encoding coat proteins of viral particles (i.e. *env*-like gene). SIRE-1 of about 11 000 bp is one of the largest and well studied member of *Sireviruses*. SIRE-1 sequence analysis evidences that SIRE-1 insertions of the retroelement (particularly in the genome of maize) have occurred recently.

The analysis of the sequence of SIRE-1 shows that the insertion of this element in the genome of corn occurred recently (12). The using of the sequences of mobile genetic elements in order to mark polymorphic sites of their insertion might be the most effective approach to identify specific features of gene pools of different groups of organisms and control their dynamics, which is especially important in dealing with agricultural species.

Herein, we studied the possibility to use terminal regions of endogenous retroviruses to characterize specific parameters of cultivated plant varieties and genetic features of cattle breeds.

Technique. Monocots, the *Triticum aestivum* plants, and dicots, the *Glycine soja* and *G. max* plants, were investigated. The examined wheat varieties were Moskovskaya 39 (soft winter wheat), Mironovskaya 808 (soft winter wheat derived from a spring wheat), and Omskaya 36 (soft spring wheat). Soybean was represented by 5 wild populations of *G. soja* derived from Primorskii krai, and a weedy, or semi-wild, form of *G. max* from China. Of tested animals, there were Black-and-White holsteinized cattle from commercial herds (Moscow Province) and the vivarium of the Russian State Agrarian University (Moscow) (groups I and II, respectively), Ayrshire cattle (Moscow Province), Yakut cattle (Sakha Republic) and Red Estonian cattle (Pskov Province) (a total of 97 animals).

The IRAP-PCR markers were applied in polyloci genotyping (13). Genome DNA was extracted by means of DNA-extran 1 Commercial Kit (Syntol, Russia). The Tercik amplifier (Russia) and PCR-RT mixture (Syntol, Russia) were used for PCR. The PCR protocol was as follows: initial denaturation at 94 °C for 2 min; denaturation at 94 °C for 30 sec; annealing (hybridization) at 55 °C for 30 sec; elongation at 72 °C for 2 min; final elongation at 72 °C for 10 min (35 cycles). The terminal regions of LTR-retrotransposon SIRE-1 (GCA-GTT-ATG-CAA-GTG-GGA-TCA-GCA) served as primers. Amplification products were analyzed by electrophoresis in 1.5 % agarose with DNA molecular marker GeneRuler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas, USA). The bands were stained by ethidium bromide and visualized in UV light. Only the fragments less than 1500 bp in size were examined to avoid inaccuracy.

TFPGA program was applied for calculation of the genetic distances. PIC (polymorphic information content) index was calculated according to the equation for diallel loci: $PIC = 2f(1 - f)$ where f is a frequency of one of two alleles (14).

The GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find out the homologous sequences.

Results. Because of prevalence of LTR-retrotransposons, including SIRE-1, in plant genomes, we used its terminal regions as primers in IRAP-PCR. As a result, both in soybean and wheat the clearly reproducible spectra of DNA fragments of the same size rang were obtained, particularly up to 22 loci of 350-1240 bp and 26 loci of 220-1450 bp in total (Fig. 1, 2).

The examined *Glycine* groups were high polymorphic ($PIC_{avarage} = 0.414$, $P = 91$ %) comparing to *T. aestivum* ($PIC_{avarage} = 0.120$, $P = 65$ %). The $PIC_{avarage}$ was calculated as the average value of the PIC index across the spectrum

of amplicons.

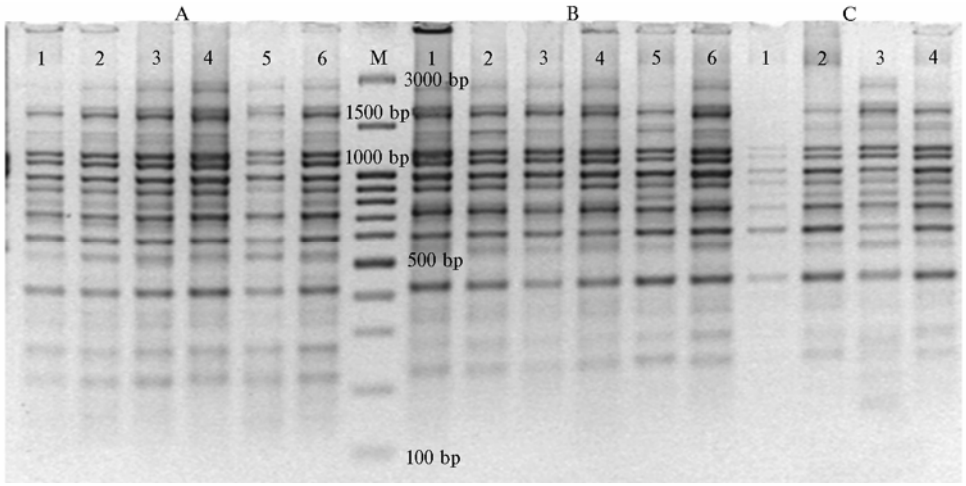

Fig. 1. Electrophoretic spectra of fragments obtained in IRAP-PCR on DNA from winter (A — Moscovskaya 39, B — Mironovskaya 808) **and spring** (Omskaya 36) **varieties of wheat *Triticum aestivum*:** M — molecular marker GeneRuler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas, USA). Terminal region of retrotransposon LTR SIRE-1 was used as primers.

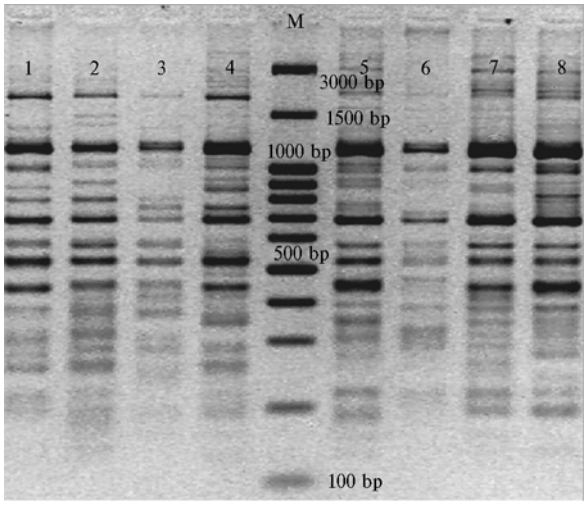

Fig. 2. Electrophoretic spectra of fragments obtained in IRAP-PCR on DNA from different *Glycine soja* groups: 1-4 — group I, 5-8 — group II; M — molecular marker GeneRuler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas, USA). Terminal region of retrotransposon LTR SIRE-1 was used as primers.

The sequences homologous to the fragment of LTR SIRE-1 were found using databases of the GenBank, so 122 and 102 fragments were found in the genomes of *T. aestivum* and *G. max*, respectively (Table 1). In *Glycine*, 350 to 490 bp fragments and 1010 to 1240 bp fragments appeared the most polymorphic. For them, $PIC_{average}$ values were 0.449 and 0.364, respectively. Conversely, in *T. aestivum* middle-sized fragments were high polymorphic, in particular, for 520 до 720 bp fragments and 760 to 990 bp fragments the $PIC_{average}$ values were 0.196 and 0.155, respectively. All large fragments were monomorphic and found in 100 % of tested samples of wheat (Table 2).

1. Number of fragments homologous to terminal sites of retrotransposon LTR SIRE-1 in genomes of soybean *Glycine max* and wheat *Triticum aestivum* and some genetic parameters based on IRAP-PCR data

Parameter	*G. max*	*T. aestivum*
Genome length, $\times 10^9$ bp[1]	1.10	1.78
Number of:		
homologuos region found[1]	122	102
DNA fragments in amplified spectrum	15-22	26
PIC averaged on the primer	0.414	0.120
P, %	91	65

Comments. 1 — according to http://blast.ncbi.nlm.nih.gov/Blast.cgi; IRAP-PCR — inter-retrotrans-poson amplified polymorphism PCR, PIC — polymorphic information content, P — share of polymorphic loci.

2. Frequency of amplicons (FA) **in patterns obtained in IRAP-PCR with terminal region of retrotransposon LTR SIRE-1 as primers, and PIC per locus in wheat *Triticum aestivum* varieties**

Fragment length, bp	Moskovskaya 39		Mironovskaya 808		Omskaya 36	
	FA	PIC_{locus}	FA	PIC_{locus}	FA	PIC_{locus}
1450	0	0	0.2	0.159	1	0
1420	1	0	1	0	1	0
1370	1	0	1	0	0.4	0.349
1340	0.8	0.483	0	0	0.4	0.349
1210	1	0	1	0	1	0
1130	1	0	1	0	1	0
990	1	0	1	0	1	0
900	1	0	1	0	1	0
810	1	0	0.3	0.300	1	0
790	1	0	0	0	0	0
760	0	0	0.8	0.483	0	0
720	1	0	1	0	1	0
680	1	0	1	0	1	0
630	0.500	0.414	0.800	0.483	1	0
590	1	0	1	0	1	0
570	0	0	0.3	0.3	0	0
550	0	0	1	0	1	0
540	1	0	0	0	0.2	0.189
520	0.300	0.300	0	0	0	0
460	1	0	0.700	0.488	0	0
420	1	0	1	0	1	0
360	1	0	1	0	1	0
320	1	0	1	0	1	0
270	1	0	1	0	1	0
250	0.500	0.414	0.300	0.300	0.200	0.189
220	1	0	1	0	1	0
P, %	15		27		15	

Comments. IRAP-PCR — inter-retrotransposon amplified polymorphism PCR, PIC — polymorphic information content, P — share of polymorphic loci.

In *Glycine* there was the only monomorphic locus of 700 bp, at 93 % as an average level of polymorphic loci for all amplicons and $PIC_{avarage} = 0.414$. That evidences about relatively high genetic diversity in the investigated groups on both levels (i.e. within the genus *Glycine*, and within the species *G. soja*). The locus of 680 bp was not observed in *G. max* but found in *G. soja*.

A specific polymorphic spectrum was obtained for each wheat variety in IRAP-PCR with LTR SIRE-1 as primers. Thus, in Moskovskaya 39 wheat the DNA fragment of 790 bp was observed in tested samples while in Mironovskaya 808 and Omskaya 36 varieties it was not found. Moreover, the locus corresponding to DNA fragment of 550 bp was not found in Moskovskaya 39 wheat but in other varieties this locus was detected with PIC = 1.

It was shown that inbreeding in plants leads to phisiological and phonotypical changes, presumably due to epigenetic effects resulted from a pairwise interaction of chromosomes. Such epigenetic changes can cause activation of mobile genetic elements and, as a result, the intensification of karyotype modification, the increase of genetic diversity and epigenetic rearrangements, being an important source of phenotypic variability (15).

Basing on this suggestion, we can partly explain the high polymorphism within loci in *T. aestivum*, especially considering the wheat as a self pollinating plant. It is also known that the number of plants with cross-pollination in this autogamous culture reaches to 3 % and, sometimes, up to 10 % of the population. Besides, there are some forms for which an increased frequency of cross pollination, from 2.6 to 5.0 % depending on the variety, is peculiar (in particular, the line of soft wheat Mironovskaya winter varieties) (16). Hence, the high intervarietal genetic heterogeneity, e.g. the large share of polymorphic loci and an increase of PIC value (see Table 2), in the Mironovskaya

808 if compared with two other varieties is probably due to this fact.

The loci of 790 and 550 bp fragments in wheat, and the loci of 790, 680 and 550 bp fragments in soybean seem to be important because of their unequal prevalence in different varieties.

The genetic distances (DN) were calculated for the plants according to M. Nej (1972) (Fig. 3) basing on the frequency of amplicons with different sizes flanked with inverted regions of LTR SIRE-1. There are two distinct big clusters of monocots and dicots with a specific cluster of wheat varieties Mironovskaya 808 and Omskaya 36 within monocots in the obtained dendrogram (see Fig. 3).

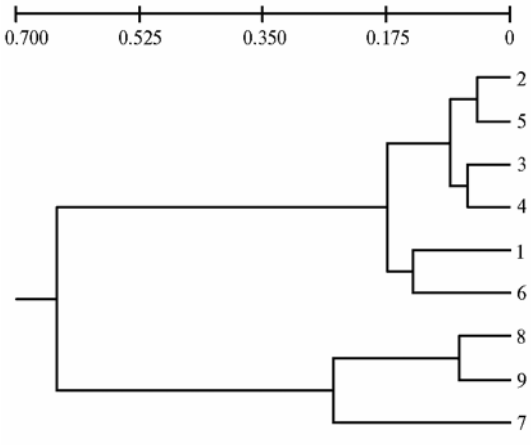

Fig. 3. Dendrogram of studied plants based on the frequency of amplicons flanked by inverted regions of LTR SIRE-1: 1 — *Glycine max* (China); 2-6 — *G. soja* (Primorskii krai); 7-9 — *Triticum aestivum*, varieties Moskovskaya 39, Mironovskaya 808 and Omskaya 36, respectively.

The differentiation reflects the phenotypic features and the origin of plants. Particularly, the white awnless and beardless ears are characteristic for both Mironovskaya 808 and Omskaya 36 varieties (17), while Moskovskaya 39 has the white awn and beardless ears (http://www.nemchinovka.ru/sorta/yar). It should be noted that the winter variety Mironovskaya 808 has been obtained from spring soft wheat by means of group and mass selection of morphologically homogenous plants (18).

The obtained data evidence that polyloci genotyping by means of IRAP-PCR markers, when LTR SIRE-1 fragment is used as a primer, allows to differentiate reliably not only the monocots and dicots, but also the varieties..

More than 165 homological regions have been found for cattle in the course of our screening data of GenBank by BLASTn algorithm to show the homology to LTR SIRE-1 among animals.

We investigated Ayrshire, Red Estonian, Yakut and Black-and-White cattle from different livestock farms and also from a vivarium with regard to the IRAP-PCR markers detected due to amplification with LTR SIRE-1 as a primer in order to estimate possible use of plant transposons in farm animal polyloci genotyping and gene pool control. The patterns of DNA amplicons, from 13 to 16 fragments within 330-1470 bp, were observed (Fig. 4).

The highest polymorphism occurs among the middle-sized DNA fragments of 760-980 bp in the Black-and-White cattle from group I and in Ayrshire cows. The locus of 1470 bp fragment was the most polymorphic in other breeds. In Black-and-White cattle from group II and Yakut cattle no loci of 380 bp and 760 bp fragments were revealed, while they were found in other breeds (Table 3).

The amplicon patterns generated in PCR with LTR SIRE-1 as a primer across all the loci demonstrated no significant differences in Yakut, Red Estonian and Black-and-White cattle of group II with respect to the level of polymorphic loci and the $PIC_{average}$ values, being 14, 19 and 23 %, and 0.062, 0.066 and 0.094, respectively. The Ayrshire cattle population was the most heterogeneous ($PIC_{average}$ = 0.212, P = 56 %) (Table 4).

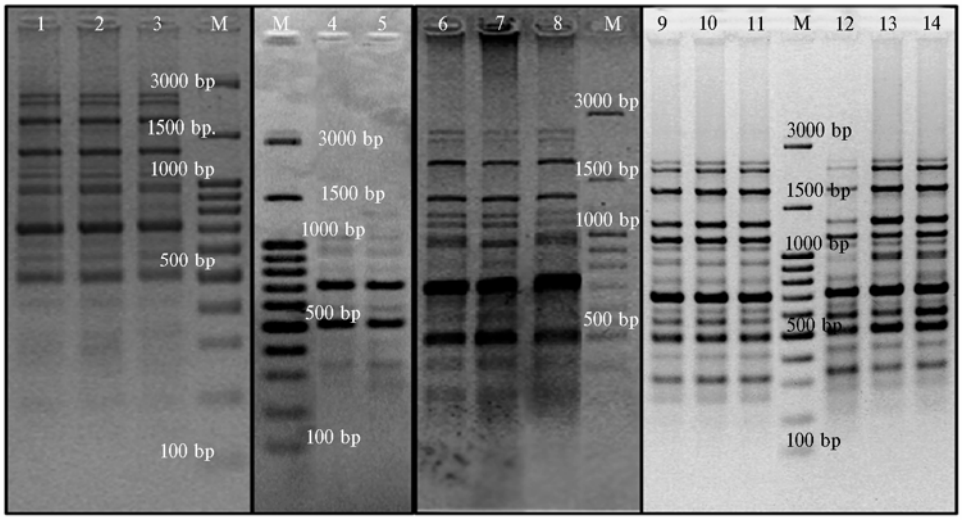

Fig. 4. Electrophoretic spectra of amplicons obtained in IRAP-PCR with DNA of *Bos taurus* cattle breeds using LTR SIRE-1 as a primer: 1-3 — Ayrshire cattle, 4, 5 — Red Estonian cattle, 6-8 — Black-and-White cattle (group I), 9-11 — Black-and-White cattle (group II), 12-14 — Yakut cattle; M — molecular marker GeneRuler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas, USA).

3. PIC_{locus} for amplicons obtained in IRAP-PCR with DNA of *Bos taurus* cattle breeds using LTR SIRE-1 as a primer

Fragment length, bp	Ayrshire	Black-and-White cattle		Yakut	Red Estonian
		group II	group I		
1470	0.325	0.486	0	0.488	0.452
1300	0.500	0	0.465	0	0
1200	0	0	0	0	0
1100	0	0	0	0	0
980	0.325	0.416	0.494	0	0.470
950	0.325	0	0	0	0
820	0.483	0	0.349	0	0
800	0.496	–	0	0.380	0
760	0.121	–	0.349	–	0
720	0	0	0	0	0
640	0	0.330	0.432	0	0
590	0	0	0	0	0
510	0	0	0	0	0
440	0	0	0	0	0
380	0.361	–	0.097	–	0.137
330	0.457	0	0	0	0

Comments. IRAP-PCR — inter-retrotransposon amplified polymorphism PCR, PIC — polymorphic information content. Dashes indicate absence of the locus.

4. Polymorphysm of the loci identified in IRAP-PCR with LTR SIRE-1 as a primer in *Bos taurus* cattle breeds

Breed, group	PIC_{loci}	P, %
Ayrshire	0.212	56
Bkack-and-White:		
group I	0.137	38
group II	0.094	23
Yakut	0.062	14
Red Estonian	0.066	19

Comments. IRAP-PCR — inter-retrotransposon amplified polymorphism PCR, PIC — polymorphic information content, P — share of polymorphic loci.

A phylogenetic tree between the studied groups of animals was constructed based on the estimation of amplicon polymorphism (Fig. 5). Black-and-White cattle from group I and Ayrshire cattle were clustered the most tightly, while Black-and-White cattle from group II, Red Estonian cattle and Yakut cattle formed a segregated subcluster. It should be noted that the genetic distance between Black-and-White and Yakut cows was extremely small (DN = 0.0076). Two groups of Black-and-White cattle clearly differed according to their genetic characteristics (see Table 4). The animals from group I were more heterogeneous (P = 38 %, $PIC_{average}$ = 0.137), probable due to different strategy of selection or

other artificial and natural factors of selection.

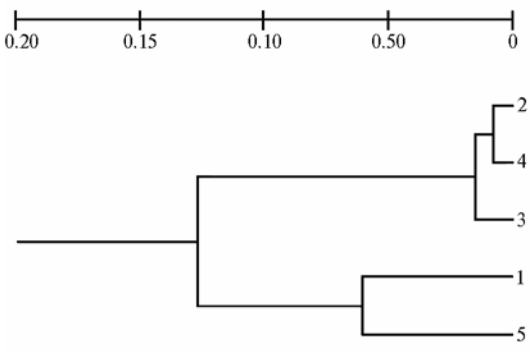

Fig. 5. Dendrogram of studied *Bos taurus* cattle based on the frequency of amplicons flanked by inverted regions of LTR SIRE-1: 1 — Ayrshire cattele, 2 — Black-and-White cattle from group I, 3 — Yakut cattle, 4 — Red Estonian cattle, 5 — Black-and-White cattle from group II.

Thus, the obrained data show that the fragments of mobile element, particullarly LTR SIRE-1, could be used for investigations of genetic structure of the groups and varieties of both dicotyledonous (*Glycine*) and monocotyledonous (*Triticum*) plants. Moreover, such polyloci genotyping of DNA fragments generated in IRAP-PCR with LTR SIRE-1 as a primer allows to identify the genetic characteristics of cattle breeds.

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Scientific events

IV INTERNATIONAL WORKSHOP «GENETICS FOR AGRICULTURE: GENOME BIOTECHNOLOGIES FOR AGROINDUSTRIAL COMPLEX»

(October 15 октября, 2014, Institute of Genetic and Cytology of NAS of Belarus, Minsk

The aim of the workshop is to inform scientists and experts about recent achievements in the genetics of agricultural plants, acceleration of practical application of modern genetics in plant breed, development of the relationship between scientists and experts.

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A RELATIONSHIP BETWEEN PLOIDY LEVEL AND THE NUMBER OF CHLOROPLASTS IN STOMATAL GUARD CELLS IN DIPLOID AND AMPHIDIPLOID *Brassica* SPECIES

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Abstract

Doubled haploid lines production through isolated anthers and isolated microspore cultures has been used widely for genetic studies and plant breeding of *Brassica* crops. The ploidy level of microspore derived plants varies, and normally haploid, diploid and mixoploid plants could be obtained in vitro. The determination of ploidy level is essential in doubled haploid pure line production. The determination of ploidy level by counting the number of chloroplast in stomatal guard cells (NCSGC) is less time consuming, laborious and expensive comparing to chromosome counting in root tip cells or mother pollen cells and flow cytometry methods. Several studies have been reported concerning relationship between ploidy level and number of chloroplast in stomatal guard cells of *Brassica rapa*, *B. napus* and *B. oleracea* species, however a small number of genotypes had been analyzed. In our study, the NCSGCs of haploid (*n*) and diploid (2*n*) Chinese cabbage (*B. rapa* ssp. *pekinensis*), winter oilseed rape (*B. napus* var. *napus*) and haploid, di- and tetraploid white cabbage (*B. oleracea* var. *capitata*) microspore derived plants were estimated, and also the influence of plant growth temperature (6±2 °C и 24±2 °C) and development stage (vegetative or generative) was investigated. High correlation between the ploidy level of microspore-derived plants and NCSGC is found for white cabbage (*Brassica oleracea*, $r = 0.94$), Chinese cabbage (*B. rapa*, $r = 0.90$) and oilseed rape (*B. napus*, $r = 0.94$). The chloroplast average number in stomatal guard cells was very similar among the same ploidy genotypes of Chinese cabbage as well as rapeseed, while the variation of chloroplast number in diploid and tetraploid white cabbage plants was significant. In a range of early, middle and late maturing diploid white cabbage inbred lines there was established the tendency to form more chloroplasts in the early lines (Pl, Sus and others) and less in the late lines (AM2, Sa1, Ges2 and others), with the difference up to 1.7 times, that is comparable to the difference between haploid and diploid plants of Chinese cabbage or rapeseed. The chloroplast number in stomatal guard cells is 4.2-7.8 and 7.9-13.6 for Chinese cabbage (*B. rapa*) haploids and diploids, respectively, 7.5-12.4 and 14.1-20.3 for rapeseed (*B. napus*) amphihaploids and amphidiploid, respectively, and 7.7-9.9, 11.7-17.9 and 18.0-26.5 for white cabbage (*B. oleracea*) haploids, diploids and tetraploids, respectively. No significant influence of vegetative or generative stage of plant development or growth temperature on NCSGC was found.

Keywords: haploid, diploid, white cabbage, Chinese cabbage, stomatal guard cells, number of chloroplasts, ploidy, rapeseed, tetraploid, *Brassica oleracea*, *Brassica napus*, *Brassica rapa*.

The biotechnology of producing pure breeding lines, the doubled haploids, via anthers and isolated microspore cultures is recently widely applicable in genetic studies and breeding cole crops (1-3). Due to this approach, the in vitro generated plants have different ploidy, and, together with doubled haploids, also haploid, tetraploid and mixoploid forms can occur (4). Estimation of ploidy is essential step in doubled haploid production (5, 6).

Various methods different in accuracy, the complexity and cost are used for ploidy estimation. They are counting the number of chromosomes under microscopy of cytological preparations (7); qualitative assay of chromatin content in cell nuclei by flow cytometry (8); analysis of complex indirect signs in plants, namely their morphological features, size of guard cells, the guard cell number per unit leaf area, number of chloroplasts in the guard cells of stomata (NCGCS),

size of pollen grains and the number of pores on its exine, fertility and frequency of seed formation (9).

Counting the number of chromosomes in the mitotic cells of root meristems is laborious and time-consuming, as the *Brassica* chromosomes are small, and the number of metaphase plates depends on root growth. The counting can not be performed for a large number of plants, therefore, it remains a laboratory method (5). Flow cytometry seems to be one of the most effective, accurate and convenient. Easy methods for preparation allow scanning few hundred samples daily, besides, a minimal amount of leaf tissue is enough. Nevertheless, it application is limited by high price of the device, resulting in high cost per analysis (10). Phenotypic identification on distinguishing features of haploid plants, e.g. male sterility, the smaller size of the vegetative organs, narrow leaves, etc., is uncomfortable and long because the plants must be cultivated for a few months to reach flowering (11). NCGCS estimation is easy to perform, cheap and used in practical plant breeding for a long time.

In anther derived winter rape (*B. napus*) plants the varying number of chloroplasts, 12.0-14.0 in haploids and 19.5-20.9 in diploids, was detected in stomatal guard cells (12). In *B. campestris* ssp. *pekinensis* the reported NCGCS values were 2-4 for haploids, 4-6 for diploids and 8-10 for tetraploids (5). Another study found that the number of chloroplasts in a pair of stomatal guard cells in Chinese cabbage varies from 6.1 to 8.6 in haploids, from 10.1 to 12.7 in diploids and from 15.9 to 17.8 in tetraploids (13).

In the report of S.J.C. Dias the NCGCS values in haploid, diploid and tetraploid *B. oleracea* ssp. were 6-9, 10-15 and 20-25, respectively (11). In experiments carried out by S. Yuan et al. (14), in *B. oleracea* var. *capitata*, *B. oleracea* var. *italica* and *B. oleracea* var. *alboglabra* derived from isolated microspore the NCGCS were less than 10 for haploids (at an average value of 6.96 to 7.67 per plant), 11-15 for diploids (at an average value of 12.36 to 13.89 per plant), and more than 15 for polyploids (at an average value of 16.96 to 17.61 in a triploid plant and 22.61 to 24.97 in a tetraploid plant). In the experiment the accuracy of the method for determining the ploidy reached 93.93 % and did not depend on the growth conditions, in particular cultivation in the greenhouse or cold nursery (14).

An average numbers of chloroplasts in stomatal guard cells in *B. oleracea* var. are very similar in top, middle and bottom part of a leaf, and in 3^{d}, 5th and 7th true leaves of the same ploidy plants (14). Also no significant differences were noted in NCGCS within a single plant and between regenerated plants of the same ploidy in *B. campestris* ssp. *pekinensis* indicating stability of this trait independently of plant age, nevertheless, the stages of plant development when the study was conducted are not specified (5).

In our study, the NCSGCs of haploid (n) and diploid ($2n$) Chinese cabbage (*B. rapa* ssp. *pekinensis*), winter oilseed rape (*B. napus* var. *napus*) and haploid, di- and tetraploid white cabbage (*B. oleracea* var. *capitata*) microspore derived plants were estimated, and also the influence of plant growth temperature (6±2 °C и 24±2 °C) and development stage (vegetative or generative) was investigated.

Technique. We studied the members of three *Brassica* species, i.e. the *Brassica rapa* ssp. *pekinensis* populations of haploid and diploid regenerants MEDH, (MChE)DH, XMDH, Xa642DH, MlchDH, Kit1-3DH, TPV36DH (a total of 219 plants), derived from isolated microspore culture; the winter oilseed rape *B. napus* var. *napus* Severyanin variety (originated by V.R. Vil'yams All-Russian Research Institute of Forage), breeding lines Gal1, Lim1 and RS23 (N.N. Timofeev Breeding Station) and haploid and diploid regenerants SevDH, GalDH,

LimDH and RS23DH derived via isolated microspore culture of the abovementioned accessions; white cabbage *B. oleracea* var. *capitata* populations of haploid, diploid and tetraploid regenerants FarDH, SurDH, EtDH, ParDH, NazDH (a total of 100 plants), derived through isolated microspore culture, and 22 inbred lines of white cabbage (genetic collection of N.N. Timofeev Breeding Station) including early maturing lines (80-100 days; Pl, Sus1, Et1, Sf, Sh5a, Dpp2, Dt), middle maturing lines (110-140 days, Ak3, Meg1, B25, Uf1, S110) and late maturing lines (150-180 days, Fl4, Pr3, Vb4, Xt5, Pm4, Fu44, Bu1, AM2, Sa1, Ges2.

The influence of temperature on NCSGCs was assessed using the diploid Chinese cabbage *B. rapa* (F_1 hybrids Hydra and Nezhnost), the white cabbage *B. oleracea* (F_1 hybrids SB-3 and Valentina) originated by N.N. Timofeev Breeding Station, and the amphidiploid winter oilseed rape *B. napus* (Severyanin variety). Three plants of each sample were grown in greenhouse at 24±2 °C and in climatic chamber at 6±2 °C according to standard agrotechnology. NCSGCs were counted after 75 days of cultivation.

To elucidate variability of NCSGCs due to the stage of plant development, the Chinese cabbage inbred line Xa642 and white cabbage line Bu1b originated by N.N. Timofeev Breeding Station, and also winter oilseed rape variety Severyanin were studied. NCSGCs were counted in 20-day seedlings with 4-5 true leaves and in the leaves of flowering shoots after vernalization for the time necessary for the respective culture (the flowering stage).

The regenerated plants derived from isolated microspores, after their adaptation, and the plants derived from seeds were grown at controlled conditions in greenhouse at 24-26 °C/20-22 °C (day/night) in spring and summer according to common procedure. The sowing was carried out into 64-cell containers, 5×5 cm per cell, and 25-30 day seedlings were further transferred into plastic 8-liter pots. Milled peat Klasmann TS-1 («Klasmann-Deilmann GmbH», Germany) with mineral fertilizers (N — 100-120 mg/l, P_2O_5 — 120-220 mg/l, K_2O — 140-240 mg/l, 14:16:18) served as the substrate. Watering and mineral fertilizing were held if necessary.

Chloroplasts were counted in leaves as described (15) with slight modifications. The leaf segments were rinsed with running tap water to remove waxy layer and dust, then, with forceps, an epidermal layer was manually removed from the underside of a leaf and placed on a microscope slide into drop of 1 % $AgNO_3$ with cover glass. The preparations were viewed under a microscope (Axioskop 40, Carl Zeiss, Germany). In each sample chloroplasts were counted in 10 pairs of stomatal guard cells at magnification ×400. The chromosomes were counted in meiotic anthers cells of young flower buds. The specimens were collected in the morning and fixed in 3:1 96 % ethanol to glacial acetic acid fixative. Permanent cytological preparations were made by spreading method (16) with some modifications. Before preparing permanent slides, the buds with cell division were chosen. The stage of cell cycle and meiotic phase for each bud was classified by preparing anther squashes with drop of acetocarmine. The buds with anaphase I and II cells were used for preparing permanent slides.

Fixed specimens were rinsed by running tap water for 15 min, then 5-10 removed anthers were placed into 1.5 ml Eeppendorf tubes containing of *Aspergillus niger* pectinase (13.5 U/ml) and *Trichoderma reesi* cellulase (80.0 U/ml) (Serva, Germany) in citric buffer (pH 4.8) and incubated for 50-70 min at 37 °C in water bath. With pipette, anthers were gently removed, placed on a glass slide, carefully crushed with a dissecting needle in a drop of 60 % acetic acid and waited for 1 min. The obtained suspension was traced around by fixative with followed addition of 1-2 drops of fixative into the center of it. Preparations were

rinsed in 96 % ethanol, dried, stained in 1 % Giemsa's solution in phosphate buffer (pH 6.9-7.0) for 10-15 min, then rinsed in distilled water and air dried. The preparations were observed using Axioskop 40 immersion system (Carl Zeiss, Germany). Chromosomes were counted in 10-15 metaphase and/or anaphase plates at magnification ×630.

Correlation coefficients, the reliability of differences and the confidence interval were calculated using Microsoft Excel 2010 on the base of *t*-Student's distribution at 0.05 level of significance.

Results. We estimated an average number of chloroplasts in stomatal guard cells in Chinese cabbage *B. rapa* and winter rape *B. napus* haploid and doubled haploid regenerants derived through isolated microspore culture. In each of 7 tested populations of Chinese cabbage and 4 population of rape, there were two groups. In Chinese cabbage, an average NCSGC values in these groups were 5.50 and 9.95, respectively (Table 1, 2), and in rape they were 9.85 and 17.03, respectively (see Table 2, 3).

1. Average number of chloroplasts in a pair of stomatal guard cell (ANCSGC) **in haploid and diploid regenerants of Chinese cabbage (*Brassica rapa*), derived through isolated microspore culture**

Population	Haploids			Diploids			Diploids to haploids ratio
	plant number	ANCSGC		plant number	ANCSGC		
		$X \pm x$[1, 2]	limits		$X \pm x$[1, 2]	limits	
MEDH	16	5.29±0.46[a]	4.2-6.9	47	9.71±0.30[a]	7.9-12.0	1.84
(MChe)DH	20	5.42±0.45[a]	4.3-7.8	21	9.51±0.44[a]	7.9-11.6	1.75
XMDH	24	5.28±0.30[a]	4.3-7.4	25	9.66±0.24[a]	8.5-10.8	1.83
Xa642DH	6	5.16±0.27[a]	4.5-5.8	6	9.05±0.50[ac]	8.4-11.1	1.75
MlchDH	9	6.23±0.41[b]	4.8-7.7	19	11.29±0.42[b]	8.6-13.6	1.81
Kit1-3DH	8	5.76±0.57[ab]	4.9-6.8	6	10.55±1.35[ab]	8.4-12.2	1.83
TPB36DH	2	4.90±1.27[ab]	4.8-5.0	10	8.45±0.41[c]	7.9-9.5	1.72
Total	85	5.50±0.17	4.2-7.8	134	9.95±0.20	7.9-13.6	1.81

C o m m e n t s. 1 — confidence interval according to *t*-Student's distribution at 0.05 level of significance; 2 — values marked with the same letters (a, b, c) do not differ at P ≤ 0.05 according to *t*-Student's test; 3 — seed progeny of initial donor plant used in isolated microspore culture.

2. Average number of chloroplasts in a pair of stomatal guard cells in *Brassica* plants of different ploidy

Crop	Species	Haploids	Diloids	Tetraploids
Chinese cabbage	*B. rapa*	5.50[a]	9.95[b]	–
Rape	*B. napus*	9.85[a]	17.03[b]	–
White cabbage	*B. oleracea*	8.53[a]	13.46[b]	21.28[c]

C o m m e n t s. Values marked with the same letters (a, b, c) do not differ at P ≤ 0.05 according to *t*-Student's test. Dashes mean the data were not obtained.

3. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in haploid and diploid rape *Brassica napus***

Population	Haploids			Diploids			Diploids to haploids ratio
	plant number	ANCSGC		plant number	ANCSGC		
		$X \pm x$[1, 2]	limits		$X \pm x$[1, 2]	limits	
RS23[3]	–	–	–	3	17,97±0,76[a]	17,7-18,3	–
RS23DH	37	9.55±0.35[a]	7.5-11.9	12	16.48±0.88[a]	14.5-18.4	1.73
Severyanin	–	–	–	3	16.27±1.46[a]	15.6-16.7	–
SevDH	19	10.19±0.44[b]	8.5-11.8	5	17.94±2.49[a]	14.9-20.3	1.76
Gal1[3]	–	–	–	3	16.23±1.86[a]	15.5-17.0	–
GalDH	23	9.95±0.31[ab]	8.7-11.5	6	17.58±1.29[a]	16.0-19.4	1.77
Lim1[3]	–	–	–	2	17.40±1.05[a]	17.1-17.7	–
LimDH	28	9.95±0.40[ab]	8.0-12.4	5	16.36±2.65[a]	14.1-19.7	1.64
Total	107	9.85±0.19	7.5-12.4	39	17.03±0.46	14.1-20.3	1.73

C o m m e n t s. 1 — confidence interval according to *t*-Student's distribution at 0.05 level of significance; 2 — values marked with the same letters (a, b, c) do not differ at P ≤ 0.05 according to *t*-Student's test; 3 — seed progeny of initial donor plant used in isolated microspore culture. Dashes mean the data were not obtained.

A comparison of complex morphological traits in regenerated plants (i.e.

the thickness of the stem, leaf size, size and fertility/sterility of flowers) indicated their haploid and diploid characters in different groups, being also confirmed by cytological analysis of pollen mother cells in several typical plants from each group (Fig. 1).

Both in Chinese cabbage and rape, the lines of the same ploidy, except for one or two populations, were rather homogenous according to NCSGC. That allows to suggest no significant effect of the genotype to NCSGC within tested accessions and probably subspecies. An absence of significant differences between diploid rape regenerants derived from microspore culture and the plants from seeds of initial donor plants should also be noted.

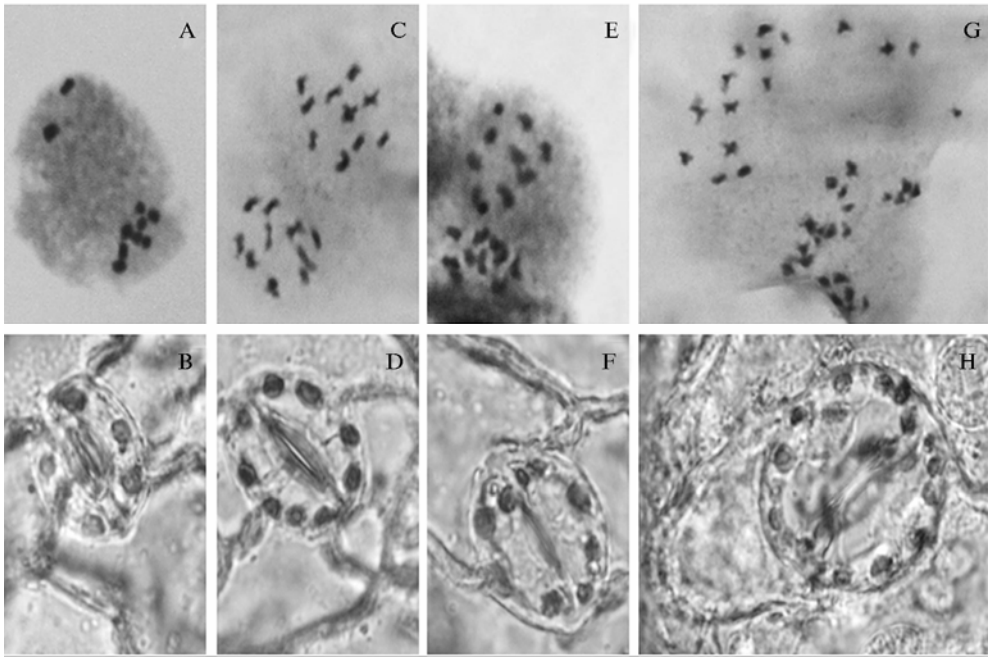

Fig. 1. Chromosomes in dividing pollen mother cells (anaphase I) (A-D) **and stomatal guard cells with chloroplasts** (E-H) **in *Brassica* plants of different ploidy:** A, B — haploids (n = 10, the number of chloroplasts in a pair of stomatal guard cells/NCSGC = 5), C, D — diploids ($2n$ = 20, NCSGC = 9) of Chinese cabbage (*B. rapa*); E, F — amphidiploids (n = 19, NCSGC = 9), G, H — amphidiploids ($2n$ = 38, NCSGC = 19) of rape (*B. napus*). Silver staining, magnification ×630.

Minimum and maximum average NCSGC values in a pair of stomatal guard cells in Chinese cabbage plants were 4.2 and 7.8 in haploids and 7.9 and 13.6 in diploids, respectively. In rapes, these values were 7.5 and 12.4 in haploids and 14.1 and 20.3 in diploids, respectively. In the absence of overlapping NCSGC maximum values in haploids and minimum values in diploids, in Chinese cabbage and rape the NCSGC in diploids was 1.7-1.8 times higher than in haploids. The Pearson's correlation coefficient (r) for NCSGC and the chromosome number (ploidy) was 0.90±0.03 in 219 haploid and diploid Chinese cabbage plants and 0.94±0.03 in 146 rape plants.

Based on NCSGC values analysis, each population of FarDH, ParDH and NazDH white cabbage (*B. oleracea*) regenerated plants was divided into three groups, and two groups were identified in each of SurDH and EtDH populations (Table 4). By means of cytological analysis of dividing cells within a bud and the pollen fertility, the ploidy was identified for all the groups. In the haploid, diploid and tetraploid groups the NCSGC was the lowest (8.53), inter-

mediate (13.46), and the highest (21.28), respectively (Fig. 2).

In FarDH, ParDH and NazDH regenerants the haloid group was rather homogeneous on NCSGCs which varied slightly (7.7-9.9), while in the diploids and tetraploids a very wide range of variation was observed, 11.7-17.9 and 18.0-26.5, respectively, leading to essential differences between the populations. Moreover, there were few plants with significantly higher NCSGCs comparing to the tetraploids. Such plants usually were tetraploid with one or two additional chromosomes (data not shown).

4. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in haploid, diploid and tetraploid white cabbage (*Brassica oleracea*) regenerants derived through isolated microspore culture**

Population	Haploids			Diploids			Tetraploids		
	plant number	ANCSGC		plant number	ANCSGC		plant number	ANCSGC	
		$X \pm x$[1, 2]	limits		$X \pm x$[1, 2]	limits		$X \pm x$[1, 2]	limits
FarDH	6	8.27±0.63[a]	7.7-8.9	36	12.76±0.24[a]	11.7-14.2	17	20.31±0.66[a]	18.0-22.7
SurDH	–	–	–	9	12.72±0.37[a]	11.7-13.4	5	20.70±2.59[ac]	19.0-23.3
EtDH	–	–	–	5	15.36±0.56[b]	13.0-16.8	2	22.60±0.93[bc]	21.6-23.6
ParDH	3	9.30±0.53[a]	8.5-9.9	5	15.24±0.44[b]	13.4-16.3	2	23.60±2.44[bc]	20.7-26.5
NazDH	1	7.80±0.30[a]	7-8	6	15.65±0.72[b]	14.5-17.9	3	25.30±0.48[b]	24.9-26.1
Total	10	8.53±0.56	7.7-9.9	61	13.46±0.38	11.7-17.9	29	21.28±0.87	18.0-26.5

Comments. 1 — confidence interval according to *t*-Student's distribution at 0.05 level of significance; 2 — values marked with the same letters (a, b, c) do not differ at $P \leq 0.05$ according to *t*-Student's test; 3 — seed progeny of initial donor plant used in isolated microspore culture. Dashes mean the data were not obtained.

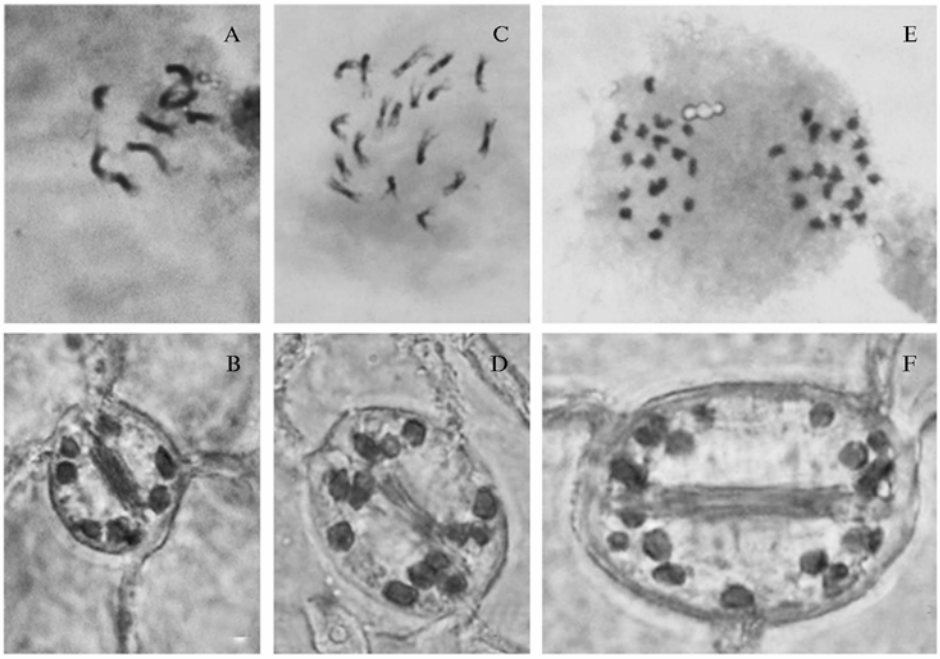

Fig. 2. Chromosomes in dividing pollen mother cells (metaphase I) (A-C) **and stomatal guard cells with chloroplasts** (D-F) **in white cabbage (*Brassica oleracea*) plants of different ploidy:** A, B — haploids ($n = 9$, the number of chloroplasts in a pair of stomatal guard cells/NCSGC = 8); C, D — diploids ($2n = 18$, NCSGC = 13); E, F — tetraploids ($4n = 36$, NCSGC = 20). Silver staining, magnification ×630.

In 22 inbred diploid lines of white cabbage with different maturing time the NCSGCs varied widely, from 11.1 to 19.2, depending on a genotype at the genotype-specific maximum to minimum average NCSGCs ratio of 1.73 (Fig. 3). Such difference is comparable to the average NCSGCs ratios in the diploid and haploid Chinese cabbage or rape plants. In early, middle and late maturing lines the NCSGCs were 15.7-19.2, 14.4-16.3 and 11.1-14.7, respectively.

Thus, we first noted a trend towards the formation of a larger number of stomatal guard cell chloroplasts in the white cabbage early maturing lines comparing to late maturing lines, probably due to biological peculiarities of their growth and development not taken into consideration by the other investigators (15) when dividing into groups with respect to ploidy.

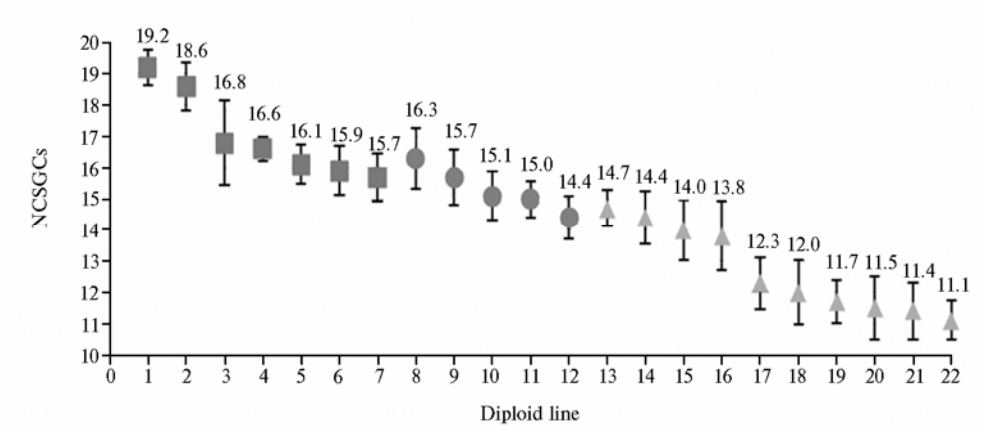

Fig. 3. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in diploid** (2*n*) **white cabbage (*Brssica oleracea*) lines with different maturing:** 1 — Pl, 2 — Sus1, 3 — Et1, 4 — Sf, 5 — Sh5a, 6 — Dpp2, 7 — Dt (■, early maturing, 8-100 days); 8 — Ak3, 9 — Meg1, 10 — B25, 11— Uf1, 12 — S110 (●, middle maturing, 110-140 days); 13 — Fp4, 14 — Pr3, 15 — Vb4, 16 — Xt5, 17 — Pm4, 18 — Fu44, 19 — Bu1, 20 — AM2, 21 — Sa1, 22 — Ges2 (▲, late maturing, 150-180 days). The confidence intervals are shown according to *t*-Student's test at $P \leq 0.05$.

Because of NCSGCs dependence on maturing and the wide range of NCSGCs variation found in the regenerants with the same ploidy, there are some difficulties in setting precise variation limits that could be reliably used under estimation of white cabbage plant ploidy. Herewith, the essential differences in NCSGCs between haploid, diploid and tetraploid white cabbage (see Table 2) and the high correlation between NCSGCs and the level of ploidy ($r = 0.94 \pm 0.03$) indicate the possibility for accurate differentiation of the regenerants derived from microspore culture in genotypes not studied earlier, providing that diploid donor plant is used as a control.

Success in using NCSGC as an index depends on its stability during plant growth and development and at different conditions. We estimated the influence of temperature on NCSGC in diploid Chinese cabbage (*B. rapa*), white cabbage (*B. oleracea*) and amphidiploid rape (*B. napus*) (Table 5). The NCSGC variability in the plants of different age was also studied (Table 6).

5. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in dioloid and amphidiploid *Brassica* species depending on the growth temperature**

Population, variety	Species	Temperature, °C	
		6	22
F_1 Hidra	*B. rapa*	9.97[a]	10.30[a]
F_1 Nezhnost'	*B. rapa*	10.45[a]	11.00[a]
F_1 SB-3	*B. oleracea*	12.00[a]	12.23[a]
F_1 Valentina	*B. oleracea*	12.45[a]	12.20[a]
Severyanin	*B. napus*	17.50[a]	17.45[a]

Comments. Values in the line marked with the same letters (a) do not differ at $P \leq 0.05$ according to *t*-Student's test.

In all three studied *Brassica* species, the obtained data confirmed stability of NCSGCs slightly differing during ontogenesis at the phase of 4-5 true leaves and at the beginning of flowering. The growth temperature also did not affect significantly the NCSGCs in five genotypes of these *Brassica* species. It should be

noted that the obtained results were the same as in doubled haploid regenerants of these diploid species.

6. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in diploid and amphidiploids *Brassica* species depending on the stage of plant development**

Population, variety	Species	Stage	
		4-5 true leaves	flowering
Xa642	*B. rapa*	10.75[a]	10.5[a]
Bu1б	*B. oleracea*	11.70[a]	12.0[a]
Severyanin	*B. napus*	16.90[a]	17.0[a]

Comments. Values in the line marked with the same letters (a) do not differ at $P \leq 0.05$ according to *t*-Student's test.

Hence, the essential differences in NCSGCs between haploid and doubled haploid Chinese cabbage, white cabbage and rape allow accurately differentiate the plants according to their ploidy. Nevertheless, it is incorrect to state that the NCSGCs discovered in this investigation are absolute and unchangeable for these species. For instance, despite of almost complete conformity of an average NCSGC to that observed in *B. rapa* (5), there some inconsistencies with other data for *B. rapa* (13), *B. napus* (12) and *B. oleracea* (14), probably due to more diversity of genotypes we used in our investigation and because of some biological peculiarities of plants, such as the length of growing period, or some external factors.

So, an average NCSGCs (number of chloroplasts in stomatal guard cells) are 4.2-7.8 and 7.9-13.6 in haploid and diploid Chinese cabbage (*Brassica rapa*), respectively, 7.5-12.4 and 14.1-20.3 in amphihaploid and amphidiploid rape (*B. napus*), respectively, and 7.7-9.9, 11.7-17.9 and 18.0-26.5 in haploid, diploid and tetraploid white cabbage (*B. oleracea*), respectively. In Chinese cabbage and rape the NCSGC is not genotype specific, since in the plants with the same ploidy the same NCSGCs are observed independently of the crop variety. In white cabbage, the NCSGC depends on the length of growing period, and there is a trend towards NCSGC increase in the early-maturing lines and decrease in late-maturing lines. In diploid early-maturing inbred lines the NCSGC is 1.7 times higher than in late-maturing lines, that is comparable to the differences between haploid and diploid Chinese cabbage or rape plants. Temperature and plant development do not affect significantly the expression and stability of NCSGCs. According to our data, the chloroplast counting in stomatal guard cells is a reliable method to estimate the ploidy level in *B. rapa*, *B. napus*, and, with some assumption, in *B. oleracea*, thus being a routine procedure applicable in the analysis of regenerated plants derived through isolated microspore culture, or in other plant material.

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THE MODELS FOR ESTIMATION OF A COMBINING ABILITY OF VARIETIES AND ROOTSTOCKS TO FORECAST YIELDING IN APPLE TREES

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Abstract

Vegetative reproduction of the best varieties of fruit crops by grafting on clonal rootstocks provides uniform trees, early onset of fruiting, it allows to create dense plantings. In order to identify rootstock, which gives maximum crop yield of the grafted varieties, a large number of expensive tests of cultivar-rootstock combinations, lasting dozens of years, are carried out, and the results of such empirical selection were reported in numerous publications. Therefore, so far there is no theory or methods of forecasting yields for trees grafted on indicators of variety and rootstocks. To increase efficiency of such searches and to lower expenses and time for their carrying out, there is the only possible way based on theoretical researches targeted to development of the principles of prediction of signs of grafted trees according to characteristics of varieties and rootstocks. Our researches summarized herein, are curried out to clarify the features of use of the principles and mathematical models of biometrical genetics for knowledge of communications in a system «grafted components—grafted plant». The implementation of this program started with studying possible applications of the formulas for calculation of the combinational ability of varieties and rootstocks as measures of their influence on the quantitative signs of the resulting combinations. The data on yielding in 28 such combinations (4 grades of apple, namely Jonathan, Golden Delicious, Idared, and Korah, and 7 rootstock, 1-48-1, 1-47-55, 1-48-46, M2, M3, M4 and M7), averaged up for 21 years fruiting, were used as the experimental material. It is established that the genotypes of varieties differ on both the productivity averaged for all rootstocks and the degree of dependence of this sign from the rootstocks. Besides, the rootstocks unequally differentiate varieties according to phenotypic manifestation of productivity. The widely used method and mathematical models for an assessment of combining ability of the parental lines of F_1 hybrids can be successfully applied to estimate the influence of common and specific combining ability of the varieties and rootstocks on crop yield in grafted trees. It is based on the fact that in variation of the total combining ability of grafting components the general combining ability (GCA) as a function of additive genes, similar to that observed in the parental forms of hybrids, is relatively prevalent. When formulas of biometrical genetics are used, the calculations show that the general combining ability (GCA) of grafting components is 6.4 times more than their specific combining ability (SCA). It caused high efficiency of the forecasts on productivity of grafted trees based on the GCA of varieties and rootstocks. The high coefficient of correlation between the actual and predicted estimates of productivity has been noted (r = 0.930). Close correlation between the predicted on the GCA and actual estimates of productivity of the variety—rootstock combinations shows the high degree of integration of grafting components in the manifestation of quantitative sings of a grafted tree. The preliminary analysis revealed the possibility for further improving accuracy of such forecasts due to separating the linear component from the dispersions of the specific combinational ability (SCA) estimates.

Keywords: models, variety, rootstock, combining ability, the forecast yield.

«Manage means to foresee»
Catherine II the Great
«To foresee means to manage»
Blaise Pascal

Modern industrial gardening is based on vegetative propagation of the best varieties of fruit trees grafted on clonal rootstocks (1, 2). This ensures uniformity of trees, compact planting and early onset of fruiting. The influence of rootstocks on yields is also important. Thus, due to favorable rootstocks the ap-

ple and pear yields increased 1.5-2.0 times (3-6).

To find the rootstock contributing to the maximum yield of the grafted cultivar, the expensive and long termed empirical investigations must be conducted using numerous combinations. The only way to intensify and accelerate the search is to develop the principles and models for predicting traits of grafted plants basing on characteristics of the grafted varieties and the rootstocks (7-9).

First attempts were made long ago. F. Kobel' (10) wrote that the causes of unequal effects of different rootstocks are difficult for elucidation, and the question is remains poorly studied. N.P. Krenke, a foremost authority in plant transplantation, stated the impossibility to select rootstocks basing on theoretical approach (11). Afterward, J. Shmadlak (1), with respect to complicated mutual effects between grafted varieties and rootstocks made a conclusion about little knowledge of their relationships. In next period the problem still remained unsolved. In numerous publications the results of empiric selection were most reported, so, nowadays, there is neither theoretical background, nor practical recommendation on forecasting yields in grafted plants on the base of characteristics of varieties and rootstocks.

Investigation and long practical experience show that the yield in grafted trees is influenced by both variety and rootstock genotypes. Thus, the study of their interaction should be based on the principles of genetics. The peculiarities of mutual effects in different combinations were being studied for long time (12-14), but these investigations have been undertaken to elucidate the inheritance of modification in one of the component caused by other component in the combination. As far as it had been found out no inheritance, and the modification were termed temporary, so the forecasting modifications due to grafting has been considered irrelevant for genetics (13). Nevertheless, it should be noted that these modifications are temporary in sense of occurring until one of grafting component affects the other component but not transferring by seed progeny. Moreover, S.Ya. Kraevoi (14) reported the modifications in the grafted variety are adequate to a specificity of the rootstock genotypes. In industrial gardening a specific effect of rootstock genotype on grafted fruit crop variety traits is extremely imported, being a genetic background for forecasting most effective combinations. Though high yield in the best of such combinations is a temporary modification in a genetic sense, it is the constant parameter for gardeners since they use only grafted plantations and do not propagate plants via seeds.

For studying modifications caused by mutual effect of grafted varieties and rootstocks, the samples with different qualitative traits should be used. S.Ya. Krevoi showed (14) that in tomato plants after grafting tobacco or datura plants, the nicotine or atropine were synthesized instead of solanine, and a potato variety used as a cion induced tuber formation in a wild species, the *Demissum*. But for practical gardening the quantitative polygenic traits, i.e. yield value, adaptability, etc., are most important (15). Variability of these traits is the subject of biometrical genetics, but even in a fundamental monograph of K. Mather and J. Jinks (16) the problems of grafting was not raised. According to their opinion, the correlations are mainly due to gene linage but not the pleiotropy. Though such a reaction of the variety to rootstocks can not been explained by gene linkage, the principles and mathematical models of biometrical genetics should be used to study an interaction between the grafting components, mostly formulas for estimation of combining ability of the variety and the rootstock because the combining ability reflects the extent of their impact on qualitative traits in a pair combination.

In trees, long time from seedling to flowering and fruiting restricts the reconnaissance crosses in genetic study of parental forms and selection of prospective donors for breeding (17). Despite that, these experiments are conducted

to study the structure of genotypic dispersions (18-20) and to estimate general and specific combining ability of crossed components (21-23). But the scientists who studied the inheritance of variability of polygenic traits in fruit crops did not try to use the principle and models of biometrical genetics when examined the effect of varieties and rootstocks on yields and other properties of grafted plants. There was even no attempt to use the formulas for calculation of combining ability at crosses to estimate the combining ability at grafting, though the grafted plant is a holistic organism (1, 2, 11, 12), in some way similar to that of F_1 plant containing hereditary factors of gametes of both parents.

We aimed to study the efficacy of mathematical models of biometrical genetics under estimation of general and specific combining ability in cions and rootstocks used for forecasting yield production in the grafting combinations, and also to elucidate the extent of integration of grafting component into the traits of grafted plants.

Technique. Four tested apple varieties, i.e. Jonathan, Golden Delicious, Idared and Korah, are winter ripening with branched spreading crown shape. Each variety was grafted on 7 rootstocks, the 1-48-1, 1-47-55, 1-48-46, M2, M3, M4 и M7 (Zonal Research Institute of Orcharding and Viniculture; the garden was planted in 1977). The rootstocks were mostly middle-height, except a semidwarf M7. M2, M3, M4 and M7 were originated from Great Britain, and 1-48-1, 1-47-55, 1-48-46 were selected in Zonal Research Institute of Orcharding and Viniculture.

For all tested combinations the same scheme for planting (7×5 m) was applied and the same agrotechnologies and protection methods were used. The weather was mostly typical for the region, but different stressors also occurred.

Yield (x_{ik}) of *i-th* variety on *k-th* rootstock was averaged for 21 year fruiting to minimize random errors under estimation.

Indicators of combining ability of grafting components and forecasted yield of grafted trees were calculated using the formulas of biometrical genetics (24-26). The results were subjected to correlation and regression analyses on the entire data set and by stratified sampling. To determine the reliability of correlation between forecasted and actual yield the calculated correlation coefficients were compared with their critical values at different levels of significance with respect to degrees of freedom.

Results. Fruit yield, as an integrated index of adaptability and productivity of perennial fruit plants, reflected the effects of weather stressors.

1. Average yields (x_{ik}, centner/ha) **in different grafted combinations of apple trees** (OPKh «Tsentral'noe», Krasnodar, 1982-2002)

Variety	Rootstock							Average, $x_{i.}$
	1-48-1	1-47-55	1-48-46	M2	M3	M4	M7	
Jonahtan	129,3	122,7	173,1	169,2	158,8	195,2	146,7	156,4
Golden Delocopus	187,4	202,9	204,4	213,9	202,0	211,3	187,4	201,3
Idared	196,4	186,7	232,7	203,6	187,5	217,4	185,3	201,4
Korah	189,9	166,5	235,0	232,5	234,5	241,6	212,2	216,0
Average, $x_{.k}$	175,8	169,7	211,3	204,8	195,7	216,4	182,9	$x_{..}$ = 193,8

Comments. $x_{i.}$ — average value for individual variety on each rootstocks, $x_{.k}$ — average value for each variety on indicidual rootstock, $x_{..}$ — average value over all combinations.

Our tests revealed significant differences in yield production between the combinations, from x_{ik} = 122.7 centner/ha in Jonathan grafted on 1-47-55 to x_{ik} = 241.6 centner/ha in Korah on M4 (Table 1). General variability appeared to be influenced by the cions rather the than by rootstocks. A specific effect of individual variety and rootstocks was also found out. For instance, the 1-47-55 and M4 rootstocks were the best and the weakest differentiators, respectively, the differences between maximum and minimum actual yields were 80.2 and 46.4 centner/ha, respectively. Among the varieties, the reaction to rootstock

genotype was the highest in Jonathan varieties and the lowest in Golden Delicious, the same differences were 72.5 and 26.5 centner/ha, respectively. Unexpected capabilities of the rootstocks to differentiate the grafted varieties and different response of the varieties to rootstock specificity allow to suggest different combining ability of the tested grafting components.

To estimate the traits of parents via their hybrids, two parameters, i.e. general combining ability and specific combining ability, are proposed in biometrical genetics (24). As V.K. Savchenko described (25), general combining ability (GCA) means an average genotypic value of a parental line in hybridization, and specific combining ability (CCA) reflects the frequency of better or worse combinations than those predicted from an average quality of crossed lines. There is a basic mathematical model for estimation of a combining ability, and its modification was proposed by L.V. Khotyleva and LA. Tarutina (26) for full diallel crosses:

$$x_{ik} = x.. + g_i + g_k + S_{ik} + r_{ik} + e_{ik}, \quad [1]$$

with x_{ik} as the value of each hybrid from crossing *i*-th and *k*-th parents; x.. as an average value for the hybrids of all used combinations; g_i as GCA effect of *i*-th parent; g_k as GCA of *k*-th parent; S_{ik} as SCA effect of *i*-th and *k*-th parents; r_{ik} as a reciprocal effect; e_{ik} as random deviations for each hybrid.

Diallel analysis allows to elucidate structure of inherited variability for qualitative traits in plants subject to number of requirements, but these limitations are not essential under estimation of a combining ability, thus making this approach widely applicable (26). It also allows to suggest this method and mathematical modeling to be applied with a view to calculation of the combining ability in the variety and rootstock genotypes from yield values in their combinations.

Using the equation [1], it should be noted that the reciprocal grafting is meaningless, thus, r_{ik} should be excluded. Since the data in Table 1 are averaged for 21 years, e_{ik} random deviations are of the minimum, allowing their conditional neglecting to simplify calculations. Then the following formula could be used to analyze the combining ability in t he grafted combinations:

$$x_{ik} = x.. + g_i + g_k + S_{ik}, \quad [2]$$

g_i being the GCA_i effect of the variety and g_k being the GCA_k effect of the rootstock. For g_i and g_k the data from Table 1 and the formulas are used:

$$g_i = x_{i.} - x.., \quad [3]$$

$$g_k = x._k - x.., \quad [4]$$

meaning the GCA_i effect of *i*-th variety is equal to its average yield on all rootstocks minus an average yield of all tested combinations. Similar calculations should be also carried out for GCA_k of each *k*-th rootstock.

2. GCA effects of apple tree varieties (g_i, centner/ha) **and rootstocks** (g_k, centner/ha)**, and the forecasted yields** ($\widehat{x}_{ik}$, centner/ha) **in their combinations** (OPKh «Tsentral'noe», Krasnodar, 1982-2002)

Variety	Rootstocks							g_i
	1-48-1	1-47-55	1-48-46	M2	M3	M4	M7	
Jonathan	138,4	132,3	173,9	167,4	158,3	179,0	145,5	–37,4
Golden Delicious	183,3	177,2	218,8	212,3	203,2	223,9	190,4	7,5
Idared	183,4	177,3	218,9	212,4	203,3	224,0	190,5	7,6
Korah	198,0	191,9	233,5	227,0	217,9	238,6	205,1	22,5
g_k	–18,0	–24,1	17,5	11,0	1,9	22,6	–10,9	

To elucidate whether the mathematical methods and models commonly used for estimation of combining ability in F_1 parents are applicable at grafting, and to verify the possibility for forecasting yield in the grafted trees from GCA and SCA of the variety and the rootstock, it is necessary to carry out the calculations and estimate correlations between the forecasted and actual values. Ac-

cording to experimental data (see Table 1), we calculated g_i and g_k for each variety and rootstock (Table 2).

Different formulas are used to calculate the yield in grafted trees from GNA together with SCA generated deviations. Contribution of g_i and g_k effects to GCA based yield x_{ik} could be calculated as

$$\widehat{x}_{ik} = x.. + g_i + g_k, \quad [5]$$

or

$$\widehat{x}_{ik} = x_{i.} + g_k, \quad [6]$$

as far as from [3] $x_{i.} = g_i + x..$.

Formula [6] shows that the yield generated by GCAs of *i*-th variety and *k*-th rootstock without SCA effects could be denoted as an average yield $x_{i.}$ of the *i*-th variety adjusted for g_k as a GCA_k effect of *k*-th rootstock. In fact, the $\widehat{x}_{ik}$ values calculated from [5] and [6] are the same (see Table 2).

Comparing $\widehat{x}_{ik}$ (see Table 2) to x_{ik} (see Table. 1), reliability could be estimated for forecasting yield in a variety-to-rootstock combination from the GCA effects of its components. The correlation was high ($r = 0.930$) exceeding 0.1 % significance level, with determination coefficient $Cd = 86.5$ %. This indicates a relatively high efficiency of these forecasts, which allow to use only the most promising combinations of grafted varieties and rootstocks, without analyzing combination with reliably low predictive yields. According to assessment of the significance of combining ability of genotypes for breeding, P.F. Rokitskii wrote (24) that these methods were first used on corn, but also are applicable on other plants or animals due to being much more informative if compared to empiric analysis of crossed race, lines, or animal breeds. For forecasting yields under grafting, the importance of this method is as much higher, as the tree testing is longer then annual crop F_1 testing.

Data in the Tables 1 and 2 indicate that no forecasted value was accurately the same as the actual value, though in 7 of 28 forecasts the deviations were less then 2 centner/ha (e.g. Jonathan on 1-48-46, M2, M3 and M7 rootstocks, Golden Delicious on M2 and M3, and Korah on 1-48-46). An explanation arises when comparing [2] and [5]. Thus, the forecast, if estimated on GCA for the variety and rootstocks (see Table 2), ignores effects S_{ik} of their interaction, i.e. the SCA (22). S_{ik} is calculated as

$$S_{ik} = x_{ik} + x.. - x_{i.} - x._{.k}; \quad [7]$$

data for these calculations are taken from Table 1 and the results are presented in Table 3.

3. Effects of specific combining ability (S_{ik}, centner/ha) **in variety and rootstock combinations in apple trees** (OPKh «Tsentral'noe», Krasnodar, 1982-2002)

Variety	Rootstock						
	1-48-1	1-47-55	1-48-46	M2	M3	M4	M7
Jonatha	–9,1	–9,6	–0,8	1,8	0,5	16,2	1,2
Golden Delicious	4,1	25,7	–14,4	1,6	–1,2	–12,6	–3,0
Idared	13,0	9,4	13,8	–8,8	–15,8	–6,6	–5,2
Korah	–8,1	–25,4	1,5	5,5	16,6	3,0	7,1

The same as at estimation of SCA in parents of F_1, the characteristic feature of grafted varieties and rootstock SCAs is that the sum of these effects is equal to zero for each variety and for columns in the table, the deviations due to rounding being less than 0±1) (see Table 3):

$$(\sum_k S_{ik} = 0).$$

When comparing the SCAs (see Table 3), an average effects due to interaction of Jonathan with rootstocks were found to be minimal, the 1-47-55 affects the varieties most significantly, and the effects of M2 and M7 were minimal. We

failed to reveal clearer patterns of S_{ik} variability fro all the data from Table 3. Only in Jonathan and Korah a trend was observed of S_{ik} increase as x_{ik} rises.

In general, for the entire dataset (see Tables 1 and 3) the correlation between x_{ik} to S_{ik} was although significant at the 5% level, but low (r = 0,368 at Cd = 13,5 %). It means yield forecasting in grafted trees basing on SCAs, as the effects of variety and rootstock interaction, in unreliable. In this connection it should be noted that in biometrical genetics an interaction means the deviation from additivity. In our case the $\widehat{x}_{ik}$ values in Table 2 were calculated for an additive contribution of the components. Withal their values could be calculated not only by [7], but also as:

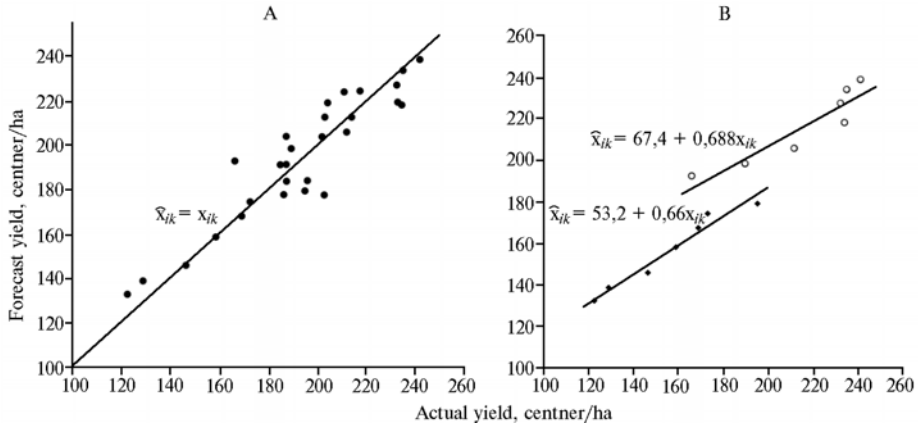
$$S_{ik} = x_{ik} - \widehat{x}_{ik}, \qquad [8]$$

since an interaction hereby implies a nonadditivity. Hereinabove, there was Cd = 86.5 % shown for the yield variations in grafted trees predicted from GCAs of the variety and the rootstock, and Cd = 13.5 % due to unpredictable effects of their SCAs, thus the predictable variation for the entire set of studied combinations being 6.4 times higher comparing to unpredictable.

Obtained results indicate that the further improvement of yield forecasting in grafted trees requires the in-depth analysis of this trait variation caused by the unpredictable effects of SCSs of the components by means of both biological and statistical approaches. Of biological traits, the morphophysiological features of varieties, rootstocks and grafted trees with different SCA effects should be investigated. At statistical analysis a dispersion of the variety and rootstock interaction should be separated into the predictable linear and unpredictable nonlinear components.

Some bases for underestimating S_{ik} values in individual combinations of i-th varieties and k-th rootstocks could already be seen from data of Tables 1 and 2. So far as the same averaged g_i values for i-th variety were used in calculation, the differences of $\widehat{x}_{ik}$ between any two varieties were the same for all rootstocks. Accordingly, a specific response of i-th variety to the rootstock was not considered. For instance, in Golden Delicious and Idared the average yield values $x_{i\cdot}$, as well as g_i, differed by 0.1 centner/ha, resulting in the same difference of 0.1 centner/ha between their $\widehat{x}_{ik}$. In Korah and Jonathan, the differences between average $x_{i\cdot}$ values and also between forecasted $\widehat{x}_{ik}$ values were identical and equal to 59.6 centner/ha. Because of this calculation method, k-th rootstock contributed the same g_k value to x_{ik}, predicted from GCA, therefore, for example, the differences of $\widehat{x}_{ik}$ values for each variety on 1-48-1 and 1-47-55 were the same and equal to 6.1 centner/ha. As a result of such estimation of $\widehat{x}_{ik}$ on GCAs of k-th and i-th components, the forecasts are overestimated in low-yielding combinations, and underestimated in high-yielding combinations, leading to an overestimation of variety and rootstock SCAs and less effective prediction. This disparity could be assessed by regression (Fig.) of plotted $\widehat{x}_{ik}$ on x_{ik} from Tables 1 and 2 .

According to [3], [4] and [5] used for calculation of $\widehat{x}_{ik}$ from OCAs of the variety and the rootstock, a regression coefficient (b) for $\widehat{x}_{ik}$ on x_{ik} should be equal 1, so the drown line should cross the axes at 0. It means that in case the actual yield is higher than an average x.. by 1 centner/ha, the forecasted yield value should also increase by 1 centner/ha on average with a regression line defined by $\widehat{x}_{ik} = x_{ik}$. The line on a graph (see Fig., A) fits the plotted points quite enough. Obviously, not all of the points fall on the line due to calculated GCA effects of grafting components, and these deviations reflect both SCAs impact and the differences between actual and forecast estimations.



A forecast yield calculated from general combining ability of the grafting components ($\hat{x}_{ik}$, centner/ha)**, the actual yield** (x_{ik}, centner/ha) **summarized for all grafted apple trees (Jonathan, Golden Delicious, Idared and Korah)** (●) (A)**, and those for Jonathan** (♦) **or Korah** (○) **varieties** (B) (OPKh «Tsentral'noe», Krasnodar, 1982-2002).

On the left (see Fig., A), all the points for 28 combinations of four varieties on 7 rootstocks are visualized as a single aggregate group, while two groups can be seen if each individual variety on different rootstocks is analyzed (see Fig., B). Particularly, for Jonathan and Korah there were free terms in $\hat{x}_{ik}$ on x_{ik} regression equations, and the regression coefficients decreased to b = 0,674 on average resulting in $^2/_3$ centner/ha increase of forecast yield predicted from GCAs, when the actual yield increased by 1 centner/ha. It is due to smaller differences between forecast yield estimations calculated as [3], [4] and [5]. Similar system errors cause the point deviations from $\hat{x}_{ik} = x_{ik}$ line (see Fig., A) and SCAs overestimation, and decrease efficacy of yield prediction from GCAs of grafting components. Therefore, the improved models free from these limitations allow us to forecast yield of grafted trees more accurate. Basing on them, a new method and formulas could be allowed to separate SCA effect into two components, the predictable linear and unpredictable non-linear.

In existing manuals on GCA and SCA assessment in F_1 parents (22-24) the system errors that arise at SCA calculations and yield prediction for the hybrids from GCA of the parents, are not considered. Moreover, there is no attempt to divide SCA effects into linear and non-linear. An estimation of combining ability of the parental plants is empirical in itself and based on numerous crosses and tests (25-27). L.V. Khotyleva and L.A. Tarutina ascertain all these complications being a result of poor understanding the genetic basis of a combining ability (26). The same is the reason of poor understanding whether an entire organism of grafted tree is functionally similar to F_1 hybrid. However, rather high accuracy of yield forecast from GCAs of the variety and the rootstocks shows a significant integration of the traits at grafting.

So, genotypes of apple varieties differ on both the productivity averaged for all rootstocks and the degree of dependence of this sign from the rootstocks. Besides, the rootstocks unequally differentiate varieties according to phenotypic manifestation of productivity. This variability leads to yield variations in grafting combinations from 122.7 to 241.6 centner/ha observed in the course of 21 year examination. parental lines of F_1 hybrids can be successfully applied to estimate the influence of common and specific combining ability of the varieties and rootstocks on crop yield in grafted trees. It is based on the fact that in variation of the total combining ability of grafting components the general combining ability (GCA) as a function of additive genes, similar to that observed in the parental forms of hybrids, is relatively prevalent. When formulas of biometrical genetics are

used, the calculations show that the general combining ability (GCA) of grafting components is 6.4 times more than their specific combining ability (SCA). It caused high efficiency of the forecasts on productivity of grafted trees based on the GCA of varieties and rootstocks. The high coefficient of correlation between the actual and predicted estimates of productivity has been noted (r = 0.930). Close correlation between the predicted on the GCA and actual estimates of productivity of the variety—rootstock combinations shows the high degree of integration of grafting components in the manifestation of quantitative sings of a grafted tree. The preliminary analysis revealed the possibility for further improving accuracy of such forecasts due to separating the linear component from the dispersions of the specific combinational ability (SCA) estimates.

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ANALYSIS OF SPRING BARLEY INTRASPECIFIC POLYMORPHISM IN CONNECTION WITH TOLERANCE TO LEAD

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Abstract

Under technogenic pollution, phytotoxicity of heavy metals (HM) becomes a factor limiting yield and quality of crop production. In breeding, an intraspecific polymorphism of resistance to technogenic factors should be estimated with the analysis of its formation and maintenance. Using spring barley (*Hordeum vulgare* L.) Zazerskii 85, Gorinskii and Chelyabinskii 1 varieties, we studied the influence of different $Pb(NO_3)_2$ concentrations (1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5; 5.0 mg/ml) on the growth of roots and offsprings in seedlings. Then, a testing concentration of $Pb(NO_3)_2$ found out was applied to investigate an intraspecific polymorphism of barley plant tolerance to the toxicant. The cultivars from the VIR World Collection (N.I. Vavilov Research Institute of Plant Industry, St. Petersburg) were tested using seeds reproduced in 2008, 2009 and 2010 (36, 100 and 24 varieties, respectively). The varieties were divided into classes according to Sturges' rule. The lead sensitive and lead tolerant forms were separated basing on a depression coefficient. According to root growth, the highest tolerance was observed in the Gorinskii variety, and the Zazerskii 85 variety was the most sensitive. The influence of lead resulted in a shift of distribution of 100 cultivars to less offspring length, but according to Kolmogorov-Smirnov criterion there were no significant differences between the empiric distributions ($D = 0.17 < D_{0.05} = 0.26$). The tolerance to lead in varieties from the first and the last classes differed 2.0-4.5 times (i.e. from total suppression to growth stimulation). According to the length of the offsprings from the seeds reproduced in 2008 and 2010, there were no reliable differences from control ($D = 0.167 < D_{0.05} = 0.434$ and $D = 0.125 < D_{0.05} = 0.531$, respectively). A statistically unreliable stimulation of the seed germination also occurred ($D = 0.306 < D_{0.05} = 0.320$ and $D = 0.208 < D_{0.05} = 0.392$, respectively), probably because of less number of the tested samples. Lead caused multiple changes of the root morphology. Basing on morphological parameters, the varieties with a contrast tolerance to lead was revealed. Possible mechanisms of polymorphic tolerance of barley cultivars and other plants to HM are discussed. These data can be used under creation of agricultural plants tolerant to heavy metals.

Keywords: lead, barley, intraspecific polymorphism, contrast cultivars.

Technogenic pollutions destruct both natural and agricultural ecosystems. The polluted territory covers about 18 million hectares, or 1 % of the total area of the Russian Federation. The area of heavy metal pollution of soil is 3.6 million ha. More than 1 million hectares of agricultural land are contaminated with high toxic elements (I hazard class), 2.3 million hectares with toxic elements (II hazard class) (1). Therefore, the HM phytotoxicity becomes one of the factors limiting the yield and quality in crop production.

For efficient agriculture, it is essential to use plant varieties tolerant to technogenic pollution provided high quality of crop production (2, 3). Thus, an estimation of intraspecific polymorphism of the main crops with respect to their tolerance to technogenic factors and the elucidation of how this polymorphism is generated and maintained are extremely important. Barley is a valuable food grain and forage crops. There are enough scholarly publications on different aspects of HM effects in barley. For instance, cytogenetic violations (4, 5), antioxidant activity (6), apoptosis (7), physiological processes (8), etc., are being studied. However, the mechanisms of barley tolerance to HM, in particular lead as a main pollutant of aquatic and terrestrial ecosystems, still remain unclear (9-13).

In this paper there are summarized the data on intraspecific polymorphism analysis of lead tolerance in spring barley.

Technique. In the preliminary examinations, 100 seeds of barley (*Hordeum vulgare* L.) Zazerskii 85 variety were exposed to $Pb(NO_3)_2$ solutions (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 mg/ml). In extra experiment the seeds of Gorinslii and Chelyabinskii 1 variety were treated with $Pb(NO_3)_2$ at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/ml to verify the results obtained. For each variety EC_{50} was assessed to find out the HM concentration depressing 50 % growth of roots or shoots. This parameter was used to compare lead tolerance in the varieties.

For further experiments, the seeds reproduced in 2009 were tested in 100 barley varieties from the VIR World Collection (N.I. Vavilov Research Institute of Plant Industry, St. Petersburg). Additionally the seeds of 36 and 24 varieties reproduced in 2008 and 2010, respectively, were used. For each variant the samples, i.e. 100 control seeds and 100 tested seeds, were germinated at 20 °C for 7 days using rolled paper (14). Effect of $Pb(NO_3)_2$ 1.5 mg/ml concentration was compared to deionized water as a control. We determined the parameters of germination, shoot and root length, the percent of strong seedlings with embryonic leaf of more that half the lengths of coleoptile, and morphological violations were examined. In case the embryonic leaf was less than that half the length of coleoptile or the roots were less than 5 mm in length and had no specific triple fork, the seedlings were considered weak.

To analyze barley tolerance to lead, the varieties were divided into classes according to Sturges' rule (see 15):

$$k = 1 + 3{,}3\lg n,$$

$$i = \frac{X_{\max} - X_{\min}}{k},$$

with n as sample number, k as class number, $X_{\max/\min}$ as maximal and minimal parameter, i as the width of the class intervals. Class number increased as variety tolerance increased.

Lead sensitive and lead tolerant varieties were chosen with respect to depression coefficient (*DC*), calculated as:

$$DC = \frac{MV_c - MV_d}{MV_c} \times 100\,\%,$$

with MV_c as a parameter value in control and MV_d as that at 1.5 mg/ml lead concentration.

DC was calculated for the length of shoot and root, and for the percent of strong and germinated seedlings, then summarizing the indices. The seedlings were considered tolerant or sensitive if the sum was less than 50 of more than 100, respectively. In case the variety was classified as sensitive for 2 years, but the aforementioned sum for the year 3 was 50-60, so approaching a variation rage of the opposite group, or the variety was classified as tolerant for 2 years, but the aforementioned sum for the year 3 was 90-100, these varieties were discarded.

To compare results, the Kolmogorov-Smirnov test (16, 17) and Mann-Whitney test (18) were used. Calculations were carried out by means of Statistica v. 10.0 and MS Excel 2003.

Results. Root length decreased sharply (Fig. 1, A) at minimal lead nitrate concentration (1.0 mg/ml). In Zazerskii 85, Chelyabinskii 1 and Gorinskii varieties the EC_{50} values were 2.0 mg/ml (shoots) and 1.0 mg/ml (roots), 2.5 mg/ml (shoots) and 1.0 mg/ml (roots), and 3.5 mg/ml (shoots) and 1.5 mg/ml (roots),

respectively, so Gorinskii variety was the most tolerant, while Zazerskii 85 variety was the most sensitive.

From 4 mg/ml concentration there was a complete repression of root growth in all variants. However, the length reduction in shoots was much less noticeable (see Fig. 1, B). At 1 mg/ml lead nitrate there was a reliable stimulation ($p = 0.01$) of shoot development in Chelyabinskii 1 and Gorinskii varieties. P. Soudek et al. (19) described similar effect of lead to flax. At 2 mg/ml and more an shoot growth repression was observed. There also are the similar results in V.V. Talanova's et al. (20) report.

Fig. 2 shows the distribution of 100 barley varieties on shoot length and seed germination influenced by $Pb(NO_3)_2$ at 1.5 mg/ml. The samples are grouped from 1 to 8-9 classes of the most sensitive and most tolerant samples, respectively.

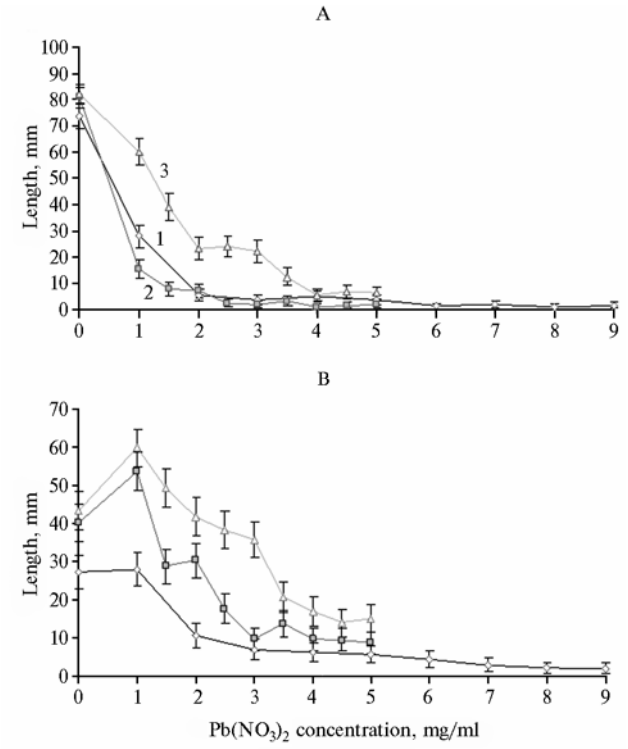

Fig. 1. Root (A) **and shoot** (B) **length in spring barley (*Hordeum vulgare* L.) varieties as influenced by different $Pb(NO_3)_2$ concentrations:** 1 — Zazerskii 85, 2 — Chelyabinskii 1, 3 — Gorinskii.

The distributions in control and at the lead nitrate influence were relatively smooth. At lead presence there was a shift to less shoot lengths (see Fig. 2, A), however, the Kolmogorov-Smirnov test did not show the significance of difference between empirical distributions ($D = 0.17 < D_{0.05} = 0.26$). The shoot length curves under lead influence reflect a more even variety distribution between the classes.

Germination curve at lead nitrate stress was right shifted (see Fig. 2, B). Besides, a reliable stimulation was shown for seed germination ($D = 0.29 > D_{0.05} = 0.26$). Moreover, the varieties in the first and last class differed on lead tolerance 2.0-4.5 times, from total growth repression to stimulation.

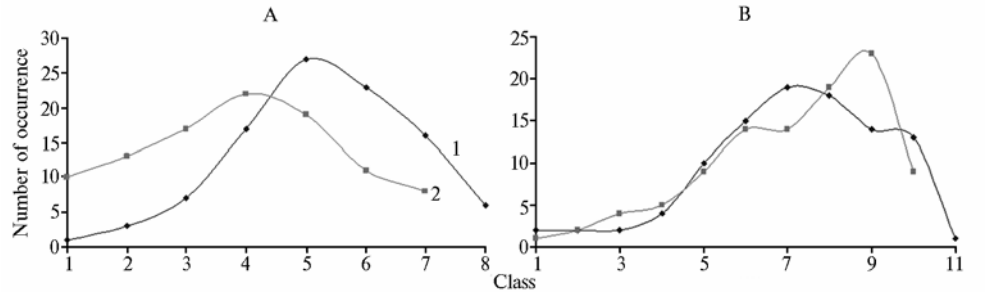

Fig. 2. Spring barley (*Hordeum vulgare* L.) variety distribution on $Pb(NO_3)_2$ tolerance with regard to shoot length (A) **and seed germination** (B) **as influenced by $Pb(NO_3)$:** 1 — control, 2 — $Pb(NO_3)_2$ concentration 1.5 mg/ml (the seeds reproduced in 2009).

As to length of the shoots from seeds harvested in 2008 and 2010 (Fig. 3, A, B), no reliable differences from control were found ($D = 0.167 < D_{0.05} = 0.434$ and $D = 0.125 < D_{0.05} = 0.531$, respectively). Stimulation of seed germination was statistically unreliable ($D = 0.306 < D_{0.05} = 0.320$ and $D = 0.208 < D_{0.05} = 0.392$, respectively) (see Fig. 3, C, D). Unreliable differences from the control values could be due to less number of varieties tested.

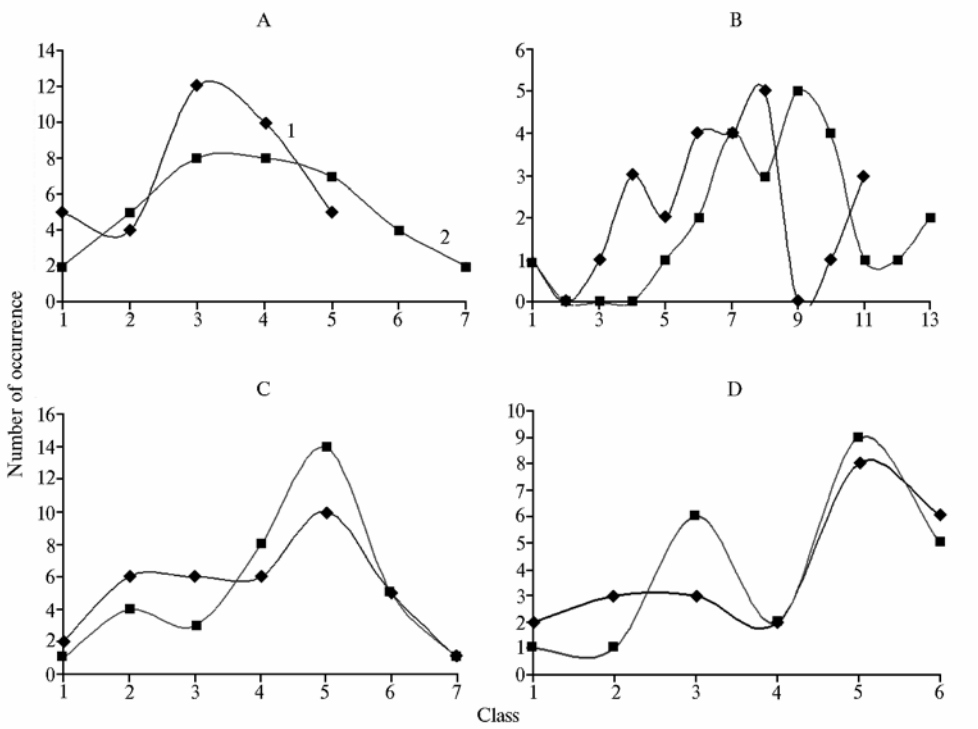

Fig. 3. Spring barley (*Hordeum vulgare* L.) variety distribution on $Pb(NO_3)_2$ tolerance with regard to shoot length (A, B) **and seed germination** (C, D) **depending on the year of seed reproduction:** A, C — 2008, B, D — 2010; 1 — control, 2 — $Pb(NO_3)_2$ concentration 1.5 mg/ml.

From the obtained date we calculated the depression coefficients (*DC*) to choose lead sensitive and lead tolerant varieties (Table). The same index was used in other studies to investigate HM tolerance in flax seeds (19) and detect the wheat varieties with contrasting resistance to ionizing radiation (21). In the Table there are listed varieties with the status confirmed at least for 2 years.

Sum of depression coefficients (*DC*) in the spring barley (*Hordeum vulgare* L.) varieties with contrasting tolerance to $Pb(NO_3)_2$ depending on the year of seed reproduction

Variety and its origin	2008	2009	2010
Tolerant			
Vyatskii (Kirovskaya Province)	25.9 (+)	53.2	32.4 (+)
Teo (Great Britain)	35.1 (+)	24.6 (+)	32.4 (+)
Zarya (Kirovskaya Province.)	15.8 (+)	11.9 (+)	–34.2 (+)
Donum (Czech Republic)	72.3	–78.2 (+)	–35.0 (+)
Simfoniya (Khar'kovskaya Province)	21.6 (+)	56.3	42.9 (+)
Pongo (Sweden)	26.3 (+)	–9.2 (+)	53.2
Sensitive			
Melikum 336 (Samarskaya Province)	244.0 (+)	116.0 (+)	70.6
Myt' (Ukraine)	106.3 (+)	119.5 (+)	99.2
Jelen (Yugoslavia)	105.7 (+)	114.1 (+)	113.1 (+)
NSGL 1 (Yugoslavia)	82.7	215.9 (+)	166.3 (+)
Заветный (Rostovskaya Province)	115.9 (+)	245.4 (+)	241.6 (+)
Rubezh (Belarus)	155.5 (+)	100.5 (+)	89.6

Comments. The varieties with lead tolerance or sensitiveness confirmed for seeds of the relevant year are marked as «+».

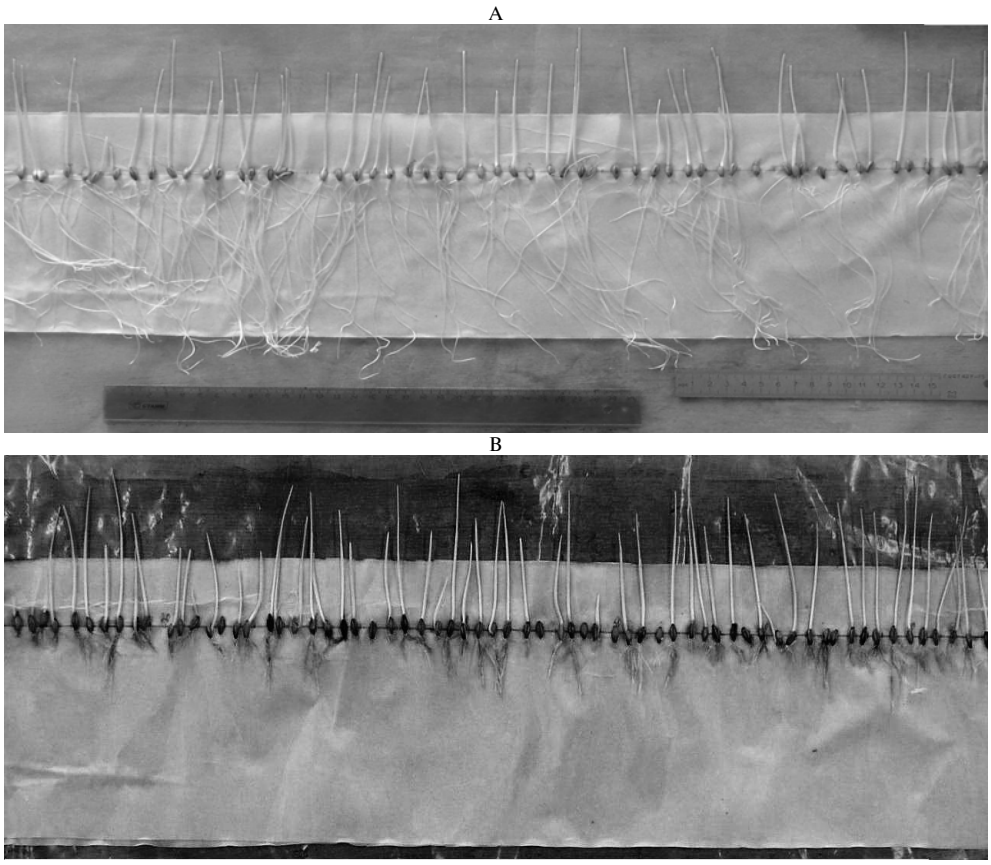

Fig. 4. Changes of root morphology in 7-day old seedlings of spring barley (*Hordeum vulgare* L.) variety Zavetnii sensitive to $Pb(NO_3)_2$: A — control, B — $Pb(NO_3)_2$ concentration 1.5 mg/ml.

In addition to growth depression, lead exposure caused numerous changes in root morphology (Fig. 4), such as apex curvature (the violation of geotropism), swelling, induration, discoloration. Similar lead effects were reported by S.V. Murzaeva (8).

There are the basic strategies of plant adaptation to the environment containing redundant concentrations of metals, namely a decrease in their input, activation of the excretion, and metabolic change to minimize the harmful effects. In case the plants accumulate HM at the levels higher than or the same as the external metal concentration, they are referred as accumulators and indicators, respectively (22). Most plants are indicators. Different varieties of the same species can demonstrate different adaptation strategies (23), which are the base for polymorphism on metal tolerance.

At HM absorption from soil, the cation-binding uronic acids from the root mucus are the main barrier (24). There are some more barriers in plants to protect from harmful external substances. The first of them are the cells of the endoderm and stele responsible for the inception of lateral roots, so the excess HM disrupts the development of the root system and decreases the root number. Besides, plasmalemma, due to ion retaining ability, prevents HM involvement into metabolism (25). Our data (see Fig. 1, 4) also show higher sensitiveness to lead in roots.

Stimulation of germination and further growth depression observed herein can result from an increased intracellular concentration of reactive oxygen species due to HM (26), then leading to the activation of antioxidant enzymes. As a result, the germination ability improves (8). Nevertheless, as lead

concentration in plant increases up to toxic level, the growth repression occurs. In pre-tests at high HM concentrations no stimulated germination was observed.

Plasmalemma is a major HM target in cell. Lead ions change its permeability and ion balance (30), impact the H^+-АТФase activity and the lipid composition of membranes (31), probably because of disruption of the lipid synthesis and lipid oxidation by reactive oxygen species generated due to HM. In case lead still got into the cytoplasm, the synthesis of metal binding compounds, the phytochelatins and metallothioneins, is triggered via HM activated synthesis of their precursor, the glutathione. HM ions generate insoluble compounds which are deposited in the vacuoles (27-29).

Lead can indirectly affect the metabolism by binding with SH-groups and active sites of enzymes, thus inhibiting their activity. Such HM repression in plants results in photosynthetic violations (e.g. thylakoid membrane destruction and failures in the Calvin cycle), water stress (e.g. increased cutin synthesis and decreased transpiration), repression of cell division (e.g. DNA crosslinking, violation of cytokinesis because of delayed microtubule assembly), and an inhibition of respiratory enzymes occurs and the mitochondrial membranes are damaged (32). In our experiments, growth repression in barley could result from these processes. The seed germination observed herein has been also observed by other researchers, being firs described for X-rays (33, 34).

Therefore, lead has a general toxic effect on plants. In response to lead presence the defense systems are activated, in particular, superoxide dismutase, catalase and peroxidase functions enhance (35), osmolytes (proline) and polyamines (putrescine) are synthesized, the changes occurs in cell wall composition due to callose and suberin depositions (36) as well as in hormonal balance, including ethylene and abscisic acid (37), and the expression of metal binding proteins, the phytochelatins, is triggered (38).

It is shown (39-41), that species, varieties and even populations within a species differ on HM tolerance. Possible causes for this may be (38) the different tolerance of transport and absorption processes, the different intensity of HM binding and deposition into vacuoles, the different rate of ion transport from roots and HM deposition into root tissues, the synthesis of HM-resistant enzyme, the activation of HM excretion from the cell. Basing on these processes, the researchers can differentiate plant varieties and lines on tolerance to HM (11, 42-45), X-rays (46) and other agents (47-49).

T.V. Zhuikov et al. (44) studied the effect of HM (Cd, Zn, Pb) to dandelion seedlings of two lines grown from seeds that were collected at 8 contaminated sites around the city of Nizhnii Tagil. One of these lines was shown to produce more viable seeds, while the other one produced more tolerant seedlings. Depending on pollution gradient the tolerance in each line changes in different ways. Authors suggest a different strategy of response to pollution, so the first line is targeted to higher seed quality, and in the second one there is higher seed yield resulting in more seedlings which develop faster and form more roots and leaves. Both the lines co-exist in the same cenopopulation.

M.R. Broadley et al. (50) compared the species of angiosperms on their response to different HM. The rate of HM accumulation was shown to be due to phylogenesis. There are the evidences that specific reactions in plants were formed during the evolution, and they change according to taxonomy.

In general, in all these researches similar results are reported, namely the stress tolerance in plants of the same species can differ significantly, thus allowing selecting forms with a contrasting tolerance. This differentiation results from different detoxication efficacy among the varieties of the same species. Also there

are publications dedicated to the impact of the pollutant on one species, regardless to its varietal differentiation. P.M. Kopittke et al. (11) investigated the effect of different lead concentration to seedlings of cowpea *Vigna inguiculata*. These results are consistent with our data, in particular, they revealed morphological violations, especially in roots (bending, thickening, discoloration), and also the lead compound deposits were shown in the root tissues. Effect is enhanced as the concentration increases.

Thus, we have shown the negative effect of high lead concentrations, more than 4 mg/ml, on the morphological parameters of barley seedlings. The roots of seedlings were most sensitive to this agent. There was detected a significant stimulation of germination of seeds in certain varieties under the influence of lead at 1-2 mg/ml concentration. The spring barley polymorphism according to lead tolerance is described. Based on the morphological parameters of seedlings, the varieties contrasting in lead tolerance are revealed.

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Temperature: influence on photosynthesis and crop yield

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A RELATIONSHIP BETWEEN CHLOROPHYLL PHOTOSYNTHETIC POTENTIAL AND YIELD OF WINTER WHEAT (*Triticum aestivum* L.) AT ELEVATED TEMPERATURES

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Abstract

Development of the indices for selection of high-yielding crops and the models, forecasting crop yields, necessitate the analysis of the relationship between photosynthetic traits and productivity. Chlorophyll photosynthetic potential, characterizing the total amount of chlorophyll in the above-ground parts of plants (or in leaves) per unit of ground surface area during the growing season or a certain period, under optimal weather conditions correlated most closely with yield (T.M. Shadchina et al., 2007; E. Kutasy et al., 2005). Modern global climate change may primarily affect the heat-sensitive crops in particular winter wheat causing disruption in the relationship between photosynthetic traits and productivity. In field experiment, we examined the effects of increased air temperatures during the spring—summer growing season on indices of photosynthetic apparatus in the high-yielding varieties of common winter wheat Smuhlyanka and Pereyaslavka on the different levels of mineral nutrition (without fertilizers and fertilizing in the fall and at different phases of the spring—summer vegetation in doses $N_{90}P_{60}K_{60}S_{10}$ and $N_{120}P_{90}K_{90}S_{20}$). Investigations were carried out in 2009 and 2011 with elevated air temperatures during the growing season at the experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine (Kiev region). It was shown that such weather conditions led to a decrease in the chlorophyll content in the leaves, the size of crop surface and the duration of its operation, as well as grain yield. In all cases (both at high level of mineral nutrition and without fertilizers) leaf surface area in 2009 was higher than under the corresponding treatments in 2011 at all studied vegetation phases. Mineral fertilizers increased the leaf area index in both years by average 1.5-2.0 times (in Pereyaslavka at milk-wax ripeness phase it increased almost 5 times if compared to the control). Chlorophyll content in leaves of both varieties depended on year: it was 5.5-6.0 and 3.5-4.5 mg/dm^2, respectively, in 2009 and 2011 if fertilizers were used, and 3.2-4.3 and 2.8 mg/dm^2, respectively, in the control. In most cases, the maximum values of the pigments content in the averaged sample of all green leaves from plant were observed in the phase of milk ripeness, probably, due to the complete withering away of leaves of lower layers. Mineral fertilizers increased the amount of chlorophyll in the leaves of both varieties. According to the results of dispersion analysis (*F*-Fisher test), the greatest influence on changes in the indices of the photosynthetic capacity in our experiments have the conditions of the year, the second most important factor was the level of mineral nutrition. The value of chlorophyll photosynthetic potential was more dependent on a combination of factors «variety × year conditions» and less on the interaction of the factors «year conditions × mineral nutrition level» and «variety × mineral nutrition level». High and similar correlation coefficient values (0.93 to 0.99) between grain yield and chlorophyll photosynthetic potential were observed for data sets within single year, variety or mineral nutrition treatment, a strong positive dependence revealed for the combined data sets for both years. The data have shown that the close correlation between the leaf chlorophyll photosynthetic potential and yield of winter wheat is retained in the conditions of air high temperature and this dependence can be described by the same regression equation for any varying factors.

Keywords: *Triticum aestivum* L., grain yield, leaf area index, chlorophyll, chlorophyll photosynthetic potential of leaves.

The analysis of the relationship between photosynthetic traits and productivity is essential for understanding regularities and patterns of yield formation with a view to further development of the breeding criteria of high-yielding crops and the models to forecast the crop yields. According to data reported, chlorophyll photosynthetic potential (ChPhP), which characterizes the total

amount of chlorophyll in the above-ground parts of plants (or in leaves) per unit of ground surface area during the growing season or a certain period, is most closely related to crop yield (1-3). In different winter wheat genotypes we have found a close correlation between ChPhP in leaves and crop productivity allowing forecasting the yield magnitude (4). High correlation between ChPhP and crop yield is due to the representativeness of the ChPhP as a parameter reflecting both dynamics of biomass production and the effect of absorbed photosynthetically active radiation (PAR) in crops during vegetation (5-7).

In cereals the high correlation between ChPhP and productivity is usually obseved in the years when the weather conditions are optimal (4, 8, 9). It is considered that the high leaf area and leaf chlorophyll indexes in crops, especially at high doses of nitrogen fertilizes, could be counterproductive because of more losses of soil moisture and more consumption of assimilated carbon for the respiration under water deficit and high temperatures (10). Due to global climate changes a frequency and duration of periods with higher temperatures increased significantly (11, 12), and in Ukraine from 2007 to 2012, the record maximum daily temperatures during the spring-summer growing season were not registered in 2008 only.

Common winter wheat (*Triticum aestivum* L.) is heat sensitive, and the highest yield losses are caused by the impact of high temperature during the reproductive phase (13-15). High temperature stress inhibits chlorophyll synthesis and photosynthetic activity, accelerates leaf aging, decreases leaf life span, oppresses and interferes with the formation of the elements of the ear and the pollen fertility, suppresses seeds formation and filling, ultimately causing a decrease in the number and weight of grains per ear (16, 17). It is still unclear whether the changes in reproductive processes affect yield production alone or they are related to deficit of photoassimilates (14, 18), nevertheless, an increase in temperature during wheat vegetation evidently causes a disruption in the relationship between photosynthetic traits and productivity. Therefore, in the years with extremely high temperatures the use of the relationship between photosynthetic parameters and productivity for breeding and the crop yield forecasting could be problematic.

In this study we examined a relationship between the performance of photosynthetic apparatus and grain productivity in winter wheat crops at different levels of mineral nutrition in the years with temperature excesses during plant vegetation.

Methods. Investigations were carried out in 2009 and 2011 with elevated air temperatures during the growing season at the experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine (Kiev region). Two high-yielding varieties of common winter wheat, Smuhlyanka and Pereyaslavka, were estimated. Smuhlyanka is a early to middle ripening variety with 278-281 day vegetation period and the crop yield ranging from 6,0 to 11,5 t/ha, and Pereyaslavka is a middle ripening variety with 280-287 day vegetation period and the crop yield ranging from 6,0 to 10,3 t/ha (19). A sowing rate was 5.0-5.5 million per ha. The shoot number at harvesting varied from 500 to 750 per m^2. A plot size was 40 m^2 at a 4-fold repetition, and 20 plants from each plot were analyzed. Experiments were carried out on the sod slightly to medium podzolized gleyed sandy soils with 1.8 % humus and pH (KCl) 6.2. Mineral fertilizers ($N_{90}P_{60}K_{60}S_{10}$ and $N_{120}P_{90}K_{90}S_{20}$) were used in autumn and in certain phases of plant growth in spring and summer. In control no fertilizers were used.

To determine the content of photosynthetic pigments and assimilation surface area in crops the samples were collected during the period of flowering

to milky-wax ripeness. Chlorophyll content in average probe of all leaves from a shoot was determined spectrophotometrically after dimethylsulfoxide (DMSO) extraction (20). Green leaves area per shoot was calculated as their length multiplied by the maximum width with a correction factor 0.76, and the obtained value multiplied by shoot density was taken as the leaf area index (LAI). Chlorophyll index (ChI) was calculated as LAI multiplied by chlorophyll content in leaves. ChPhP was determined by summarizing daily CHIs for certain period (21). For its calculation the graphs of chlorophyll indexes in green leaves for the time from flowering to milky-wax ripeness phase were plotted.

Experimental data were statistically processed using Microsoft Excel.

Results. Both in 2009 and 2011 an average temperature during the reproductive development of winter wheat plants was significantly higher than historical averages. In 2009 daily average temperatures exceeded the climatic norm by 0.8 °C for 2 weeks from plant earing to flowering and then by 1.5 °C from milk ripeness (MR) to milk-wax ripeness (MWR) phase. From flowering to MWR the difference was 1.2 °C on average. In 2011 an average temperatures exceeded significantly, by 5.1 °C, the climatic norm for all time from flowering to MWR, moreover, in some days it was more than 8 °C higher. Besides, in 2011 thermal abnormalities occurred 1.5 week prior to flowering and coincided with the lack of rainfall. Hydrothermal coefficient in Mays 2009 and 2011 was 0.8 and 0.6, respectively, while its long-term average value for the Kiev region in May is 1.2.

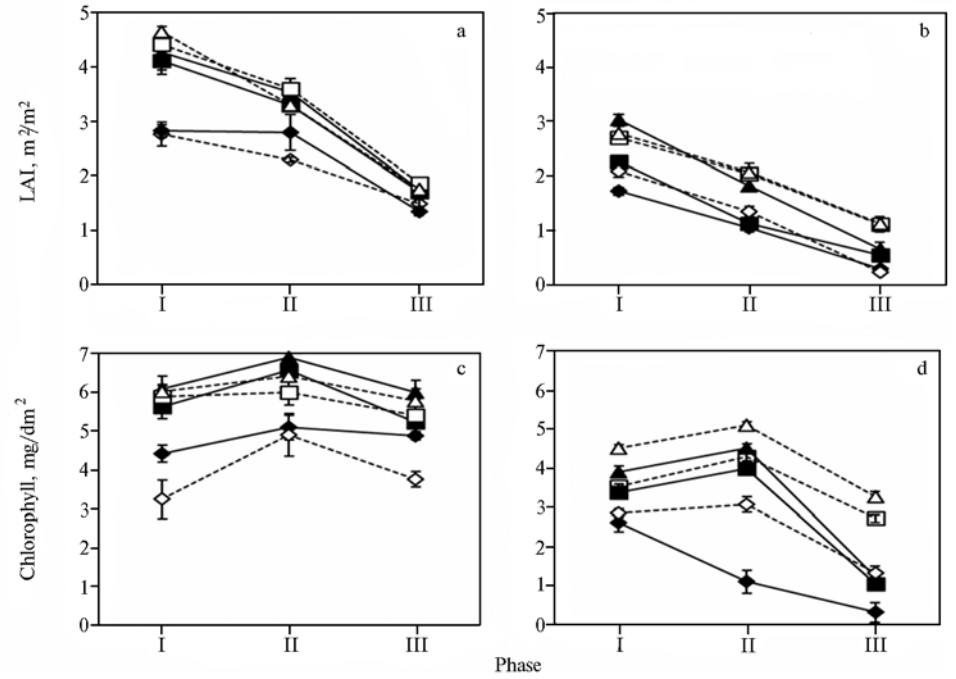

Fig. 1. Dynamics of leaf area index (LAI) **of crops** (a, b) **and chlorophyll content** (c, d) **in the years with high air temperature during vegetation** (a, c — 2009, b, d — 2011) **in common winter wheat** (full line referred to Smuhlyanka variety, dotted line referred to Pereyaslavka variety) **from flowering** (I) **to milk-wax ripeness** (III) **at different levels of mineral nutrition:** ◆ and ◇ — control; ■ и ⊡ — $N_{90}P_{60}K_{60}S_{10}$; ▲ и △ — $N_{120}P_{90}K_{90}S_{20}$ (experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kiev region).

These weather conditions significantly affect plant development. In 2011 the flowering occurred 12 days earlier compared to 2009, while the time from flowering to MWR differed slightly, being about 4 days less in 2009.

Weather conditions influenced considerably the assimilation apparatus of

crops and LAI values during wheat vegetation (Fig. 1). In all cases (both at high level of mineral nutrition and without fertilizers) leaf surface area in 2009 was higher than under the corresponding treatments in 2011 at all studied vegetation phases. Mineral fertilizers increased 1.5-2.0 times the leaf area index in both years, while in the Pereyaslavka plants at milk-wax ripeness phase the leaf area index was almost 5 times as much as in the control.

Chlorophyll content in leaves of both varieties depended on year, being in 2011 lower that in 2009. It was 5.5-6.0 and 3.5-4.5 mg/dm^2, respectively, in 2009 and 2011 if fertilizers were used, and 3.2-4.3 and 2.8 mg/dm^2, respectively, in the control (see Fig. 1). Similar patterns were observed for next two phases. However, in 2011 a reduction in chlorophyll content was more severe, being particularly noticeable in Smuhlyanka plants, especially under deficit of mineral nutrients, probably due to acceleration of the leaf aging and the nitrogen reutilization induced by elevated temperatures. In Pereyaslavka plants the chlorophyll content was less influenced by weather during vegetation, and in warmer 2011 this parameter was in general higher compared to Smuhlyanka plants.

Mineral fertilizer increased chlorophyll content in leaves of both varieties. In 2009 and 2011 it was 1.3-1.5 and 1.7-2.2 times higher, respectively, if compared to control. As the dose of fertilizer increased, the level of photosynthetic pigments in plants reliably went up in both varieties.

It should be noted that in most cases the maximum values of the pigments content in the averaged sample of all green leaves from a plant were observed during the phase of milk ripeness, probably due to the complete withering away of leaves of lower layers.

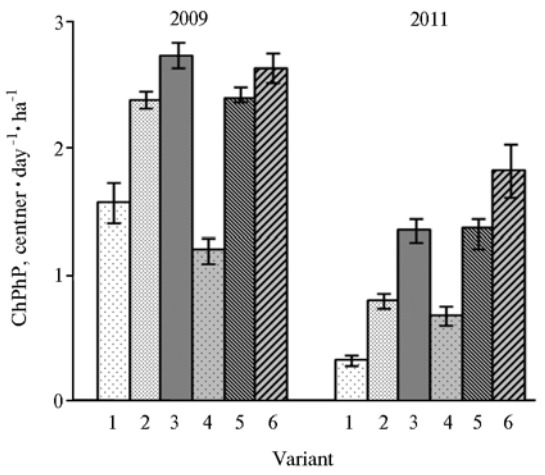

Fig. 2. Chlorophyll photosynthetic potential (ChPhP) **in common winter wheat** (1-3 — Smuhlyanka variety, 4-6 — Pereyaslavka variety) **from flowering to milk-wax ripeness at different levels of mineral nutrition in the years with high air temperature during vegetation**: 1 and 4 — control, 2 and 5 — $N_{90}P_{60}K_{60}S_{10}$, 3 and 6 — $N_{120}P_{90}K_{90}S_{20}$ (experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kyiv region).

In Pereyaslavka plants the changes in chlorophyll content during ontogenesis were similar for both years and did not depend of the mineral nutrition level, thus providing in late ontogenesis approximately the same relative differences when the fertilizers were or were not used. In Smuhlyanka plants the most influence of the fertilizers were detected at MR, in 2011 especially, and at MWR phase the differences decreased due to rapid reduction of chlorophyll content under fertilization.

ChPhP as an integrated crop index reflecting plant assimilation area, chlorophyll content and the changes in two these parameters during ontogenesis (Fig. 2) in 2011 were much lower if compared to 2009. Nevertheless, the effect of weather conditions differed for the varieties and depended on mineral nutrition. In 2009 as compared to 2011 Smuhlyanka plants had the ChPhP value 4.8 times higher in control, and 3.0 and 2.0 times higher at $N_{90}P_{60}K_{60}S_{10}$ and $N_{120}P_{90}K_{90}S_{20}$, respectively. In Pereyaslavka plants the differences were significantly less, being 1.8-fold in control and 1.5-fold at $N_{120}P_{90}K_{90}S_{20}$. In turn, the influence of

fertilizers on the ChPhP also depended on the varietal specificity and the year conditions. In 2009 fertilizers effects were less and similar, i.e. 1.7-fold and 2.2-fold for Smuhlyanka and Pereyaslavka varieties, respectively. In 2011 fertilizing $N_{120}P_{90}K_{90}S_{20}$ increased ChPhP 4.0 times and 2.5 times in Smuhlyanka and Pereyaslavka varieties, respectively.

1. Estimation of factors affecting variations of photosynthetic parameters in Smuhlyanka and Pereyaslavka common winter wheat varieties (Fisher *F*-test; experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kyiv region)

Factors and their combination	F_{actual}				$F_{calculated}$	
	chlorophyll index			ChPhP		
	flowering	MR	MWR		05	01
Year (a)	374	633	431	3913	4.84	9.85
Variety (b)	2.30	1.08	6.84	54.30		
Mineral nutrition (c)	123	103	30.1	1516	3.98	7.20
ab	0.40	28.40	7.30	264	4.84	9.85
ac	28.00	8.53	3.73	46.80		
bc	2.57	2.47	3.61	33.10	3.98	7.20
abc	1.28	0.13	0.42	2.57		

Comments. ChPhP — chlorophyll photosynthetic potential of leaves, MR and MWR — milk ripeness and milk-wax ripeness, respectively.

2. Grain yield (centner/ha) **in Smuhlyanka and Pereyaslavka common winter wheat varieties at different levels of mineral nutrition in the years with high air temperature during vegetation** ($X \pm x$; experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kiev region

Variant	Smuhlyanka		Pereyaslavka	
	2009	2011	2009	2011
Control	60.0±3.1	27.7±1.9	55.1±2.6	24.5±1.4
$N_{90}P_{60}K_{60}S_{10}$	102.3±2.9	39.6±4.5	98.4±2.7	53.0±3.8
$N_{120}P_{90}K_{90}S_{20}$	105.8±2.6	45.3±5.6	99.0±3.8	56.4±2.8

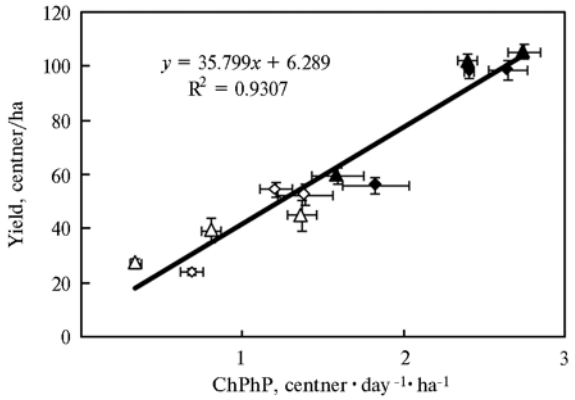

Fig. 3. Crop yield and chlorophyll photosynthetic potential (ChPhP) **in common winter wheat varieties in the years with high air temperature during vegetation:** ▲, Δ — Smuhlyanka variety, 2009 and 2011, respectively; ♦, ◊ — Pereyaslavka variety, 2009 and 2011, respectively (experimental agricultural station of the Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kiev region).

According to dispersion analysis, in our experiments the ChI and ChPhP values were mostly influenced by the year conditions (Table 1). Mineral nutrition was the second factor affecting variation of these indexes, and the least effect was found for genotype. Nevertheless, ChPhP was much dependent on combination «variety × year conditions», while the influence in combinations «year conditions × mineral nutrition» and «variety × mineral nutrition» was lower.

In plants of two investigated varieties grain productivity changed together with the photosynthetic traits. In unfavorable 2011 as compared to 2009 the crop yield decreased sharply (Table 2). In Pereyaslavka and Smuhlyanka plants grain productivity decreased 1.8-2.2 times and 2.0-2.5 times, respectively, depending on the level of mineral nutrition.

In a pooled data set for both years a close positive relationship between the crop grain productivity and the ChPhP during the period from flowering to milk-wax ripeness was confirmed by correlation analysis (Fig. 3). It should also be noted that the high and close *r* values (0.93-0.99) were found for the data sets for each year, each variety or each level of mineral nutrition.

Thus, the data herein confirm that in winter wheat under air high temperature the correlation between the leaf chlorophyll photosynthetic potential (ChPhP) as integrated parameter of photosynthetic capacity of plants and the crop yield remains close. This dependence can be described by the same regression equation for any varying factors, meaning year conditions, mineral nutrition level and the variety genotype. As the temperature during plant growth and reproductive development increases, the assimilation area, chlorophyll content and leaf life span decrease resulting in decline of crop yield. For both varieties, an increase in assimilation area and chlorophyll content due to higher mineral nutrition leads to a corresponding yield raise both at moderate and drastic temperature rise, despite a considerably different sensitiveness of the varieties to the growth conditions. At a high level of mineral nutrients the productivity of winter wheat crops is higher, probably due to higher chlorophyll content in leaves and a longer period during which the leaf index in crops remains optimal.

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LIGHT AND TEMPERATURE PARAMETERS TO OPTIMIZE PHOTOSYNTHESIS IN TWO *Amaranthus* L. SPECIES

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Abstract

Among the numerous taxonomic group of *Amaranthus* L., 12 species are cultivated as vegetable, cereal and fodder crops, medicinal and ornamental plants. A limitation for their introduction in Russia results particularly from lack of data on the physiological and ecological parameters of plant growth and development, which prevents the effective breeding investigations, especially with a view to create varieties for cultivation in northern regions, where the temperatures during spring vegetation are low. In controlled conditions of two factorial preplanned experiment we studied the effect of light intensity and temperature to net photosynthesis in intact *Amaranthus cruenthus* L. plants (Sultan variety) and *A. hypochondriacus* L. plants (Krepysh variety). At 6-7 leaf phase, the experimental plants were subjected to hardening for 3 days at 8 °C and 10 °C (for *A. hypochondriacus* and *A. cruenthus*, respectively). To evaluate the CO_2 concentration, the infrared gas analyzer Infralyt-IV (SAXON Junkalor GmbH, Germany) was used according to differential scheme. To describe the relationship between CO_2 exchange and external factors, the nonlinear equations (a model) were obtained: $NP = a_0 + a_1E + a_2T + a_3ET + a_4E^2 + a_5T^2$, with NP as the intensity of observed photosynthesis, mg CO_2/(g·h); E as illumination, W/m^2; T as air temperature, °C; a_0-a_5 as the coefficients calculated basing on the experimental data. A reliability of the equations was verified by the multiple determination index (R^2 = 85-94) and F-Fisher test (F = 4.1-5.6) at p = 0.05. This model can be applied for estimating photosynthetic activity at definite vegetation phase for each cultivar under different conditions and predicting parameters necessary to achieve definite net photosynthesis even under limiting factors. Thus, using multiple regression analysis, the equations were obtained, which allow estimating favorable combinations of the light intensity and temperature for maximal and optimal net photosynthesis at natural CO_2 concentration in the air. In both examined varieties, the net photosynthesis value of 37-38 мг CO_2/(g·h) was registered as a potential maximum. Sultan variety was more light- and heat-loving, while Krepysh variety demonstrated more cold-resistance. The photosynthetic optimum was provided within the limits of 26.7-47.0 °C and 335-580 W/m^2 for Sultan variety and at 23.5-39.6 °C and 284-501 W/m^2 for Krepysh variety. Plant hardening increased their tolerance to low temperature, decreased significantly the peak of net photosynthesis and led to a narrowing the range of light intensity and temperature, necessary to achieve the optimal net photosynthetic parameter.

Keywords: *Amaranthus* L., preplanned multifactor experiment, net photosynthesis, light and temperature parameters, CO_2-exchange.

Amaranthus, the ancient cultivated plant, was first used as food stuff 8 000 years ago by the peoples of the American continent. Products made of the grain of amaranth are very nutritious, with taste and flavor similar to those of nuts. Among the numerous taxonomic group of *Amaranthus* L. 12 species are cultivated as vegetable, cereal and fodder crops, medicinal and ornamental plants.

In Russia N.I. Vavilov initiated study of amaranth in 1930s, but soon this study was ceased and then resumed since 1950s by I.M. Magomedov. Recently this crop is wide spread in North and South America and Asia (1-3). In Russia its introduction is limited by poor ecological and physiological knowledge that prevent breeding, especially in the northern regions with low spring temperatures (4).

As ecological parameters are recently extremely important for practical goals, their indexes should be used instead of qualitative descriptions (5, 6). A labile integrated parameter that allows characterizing CO_2-exchnge as a primary productivity should be used to estimate plant response to the environment under controlled conditions of multyfactorial preplanned experiment. Such a parameter should be recorded remotely and continuously with no contact with the plant and also respond quickly to changes in environment. An assessment of the main external factors which provide optimal net photosynthesis is considered as a method to estimate the ecophysiological parameters in plants (7, 8). A zonal impact on poikilothermic organisms also should be under consideration, since under the transition to zone, where the quantitative characteristics of the factor differs, its specific influence on the genome can occur leading to metabolic changes (9).

Herein we studied the net photosynthesis in amaranth as influenced by lighting and temperature in the course of multifactor preplanned experiment.

Technique. *Amaranthus cruentus* L. Sultan cultivar and vegetable amaranth (*A. hypochondriacus* L.) Krepysh cultivar with different cold resistance and specialization were studied.

The plants were grown in plastic containers (0.5 l) filled with sandy substrate. From 12 to 15 calibrated germinated seeds were planted into each container. Luminescent lamps (110-120 W/m^2) with 14-hour photoperiod at 20/18 °C (day/night) were applied. The Knop's nutrient solution supplemented with microelements (pH 6.2-6.5) was daily used. The plants with low growth and visible abnormalities were removed, remaining 10 plants in each container.

At the phase of 6-7 true leaves the plants were undergone the hardening for 3 days at 8 °C and 10 °C, the specific temperatures preliminary identified for vegetable amaranth and *A. cruentus*, respectively. Changes in cold resistance during the hardening were detected as the temperature causing 50 % death of the cells in leaf fragments frozen for 40 min (micro thermostat TZhR-02/02, Interm Co., Russia) and then incubated at gradually increased temperature with 0.4 °C increment (10). Cell viability was assessed by the light microscope LOMO MIKMED-2 (Russia). Containers with the control and hardened plants were alternatively placed into the device for CO_2-echange evaluation (11) to study the influence of both illumination (150, 250 and 350 W/m^2) and temperature (10, 20 и 30 °C) in thrice-repeated two factorial experiment which was carried out according to three level plan (12) at 9 steps.

CO_2 concentration was assessed on a photoacoustical infrared gas analyzer Infralyt-IV (SAXON Junkalor GmbH, Germany) at differential scheme. After 40-60 min incubation at each step the CO_2 exchange in the plant was calculated as the difference between CO_2 input and output with respect to the rate of atmospheric air input, being normalized per plant dry weight.

Data was processed by multiple regression analysis using software package KyPlotStatistics v. 2.0 and Microsoft Excel.

Results. The nonlinear equation was obtained as a model to describe the interrelations between CO_2 exchange and the environmental parameters:

$$NP = a_0 + a_1E + a_2T + a_3ET + a_4E^2 + a_5T^2,$$

with NP as apparent photosynthesis, mg $CO_2/(g \cdot h)$; E as illumination, W/m^2; T as air temperature, °C; a_0-a_5 as coefficients calculated from the experimental data obtained.

The equation reliability was tested by $R^2 = 85$-94 and *F*-criterion $F = 4.1$-5.6 at a significance level $p = 0.05$.

The obtained regression model allows evaluating apparent photosynthesis as related to the specific phase of plant development for each cultivar under

varying conditions and calculating combinations of the factors providing a specified photosynthetic activity even under external limitations.

In intact and hardened plants we estimated the maximum photosynthesis value at natural CO_2 concentration in air, and also the optimal zone for 90 % of the maximum and the corresponding light and temperature parameters. It should be noted that in nature the combination of factors providing for maximum apparent photosynthesis is very rare. Active growth and development of plants mainly take place within the optimal zone (13, 14), the ecological parameters of which are specified as an ecological niche for the ecotype (7, 8). However in some regions the night temperature is significantly lower having a hardening effect, especially on heat loving plants, and affecting the ecological parameters of plant CO_2 exchange.

The highest potential net photosynthesis values in intact Sultan and Krepysh plants were 37.5 and 37.8 mg $CO_2/(g \cdot h)$, respectively, the Sultan plants being more heat and light loving with more wide optimal zone, especially within elevated temperatures, if compared to Krepysh plants (Table).

Experimental net photosynthesis and corresponding illumination and temperature conditions in two amaranth species at 6-7 true leaves under cold hardening

Species, cultivar	Variant	Net photosynthesis					
		maximum			optimum		
		NP	T	E	NP	T	E
Amaranthus cruentus L., Sultan	Control	37,5	36,8	459	> 33,8	26,7-47,0	335-580
	Hardening	15,8	24,1	303	> 14,3	17,7-30,5	218-388
A. hypochondriacus L.,	Control	37,8	31,5	393	> 34,0	23,5-39,6	284-501
Krepysh	Hardening	10,4	21,1	323	> 9,6	15,9-26,2	226-420

Примечание. NP — apparent net photosynthesis, mg $CO_2/(g \cdot h)$; T — air temperature, °C; E — illumination, W/m^2.

In Sultan cultivar the maximal net photosynthesis was at 36.8 °C and 459 W/m^2, and in Krepysh cultivar it was at 31.5 °C and 393 W/m^2. Both cultivars were frost resistant and survived after short freezing at ≤ –4 °C. Krepysh cultivar was more tolerant because its hardening occurred at lower temperature.

Cold hardening decreased sharply both the net photosynthesis and the impact of the temperature, especially low positive temperatures, on the photosynthetic indexes (Fig. 1). Earlier it was shown (15, 16) that lower net photosynthesis in hardened plants may be due to more active respiration, in particular to the maintenance respiration as a key component.

After hardening, the exposure of less heat loving Krepysh plants to 35 °C led to the negative balance in CO_2-exchange independently of the illumination parameters. The curves for not hardened plants of both cultivars were clearly dome shaped, with the peaks shifted to higher illumination, 400 W/m^2, when the temperature rose.

The curves reflecting relationship between net photosynthesis and temperature in two-factor experiment under the controlled conditions also approached the dome shape (Fig. 2). Consequently, in one factor experiments, the net photosynthesis plateau at an increased illumination is due not to light saturation as it was suggested (13, 17), but to a limiting factor, presumably temperature. One more contradiction arises from the fact that an excessive lighting leads to singlet oxygen generation having destructive effect on the photosynthetic system (18). At the same time the curves for both cultivars have confirmed the need to increase the illumination at high air temperatures (see Fig. 2). So, the cold hardening increased cold resistance in plants and changed the effect of lighting, especially at low temperatures.

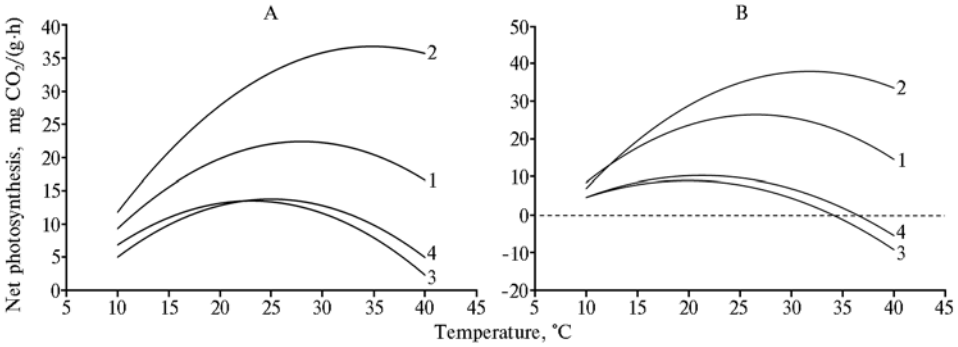

Fig. 1. Net photosynthesis in plants of amaranth *Amaranthus cruenthus* L. Sultan cultivar (A) **and *A. hypochondriacus* L. Krepysh cultivar** (Б) **at 6-7 true leaves depending on air temperature:** 1 and 2 — intact plants at 200 and 400 W/m^2, respectively; 3 and 4 — hardened plants at 200 and 400 W/m^2, respectively.

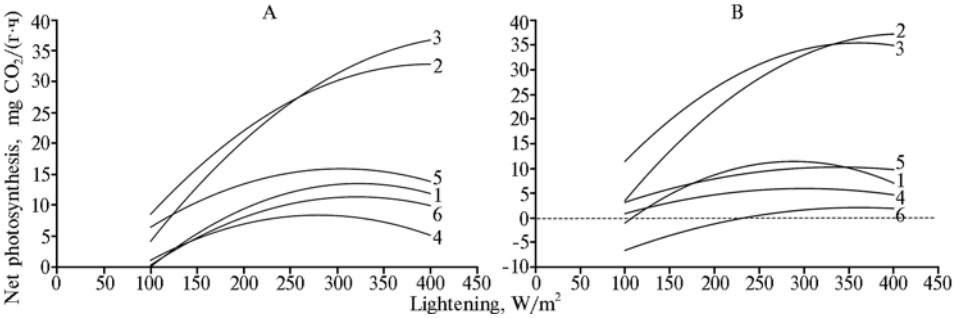

Fig. 2. Net photosynthesis in plants of amaranth *Amaranthus cruenthus* L. Sultan cultivar (A) **and *A. hypochondriacus* L. Krepysh cultivar** (Б) **at 6-7 true leaves depending on ligtening:** 1, 2 and 3 — intact plants at 10, 25 and 35 °C, respectively; 4, 5 and 6 — hardened plants at 10, 25 and 35 °C, respectively.

So, potential net photosynthetic rates in *Amaranthus cruenthus* L. Sultan cultivar plants and *A. hypochondriacus* L. Krepysh cultivar plants under natural CO_2 concentration are about the same in value, 37-38 mg CO_2/(g·h), but could be reached under different lightening and air temperature. The Sultan plants are much more light and heat loving, with the 335-580 W/m^2 and 26.7-47.0 °C optimal zones during 6-7 true leaf period. The same parameters in Krepysh plants are 284-501 W/m^2 and 23.5-39.6 °C, respectively. In both cultivars the hardening at low positive temperatures (8-10 °C) increases the thermal tolerance, decreases significantly maximum net photosynthetic rates and restricts the temperature and lightening ranges affecting optimal net photosynthesis.

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Bacillus megaterium 501^{rif} INTRODUCED INTO THE SOIL: FACTORS AFFECTING THE RATE OF SURVIVAL, SPORULATION AND DECOMPOSITION OF THE HERBICIDE PROMETRYN

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Abstract

Introduction of microorganisms into the soil is a fundamental problem in application of microbial preparations in agriculture, crop production and ecology. Its effectiveness depends on many factors (i.e. the type and strain of microorganism, the physical and chemical properties of the soil, plant cover, climate and so on), which are not well understood, leading to a high variability of the results of application of microbial preparations. In this article, there is reported the first investigation of survival and sporulation dynamics of *Bacillus megaterium* 501^{rif} introduced into sod-podzolic soil, depending on the temperature of the soil, organic matter content and herbicide prometryn, the 2-methylthio-4,6-bis (iso-propylamino)-sim-triazine, application. In laboratory experiments it was found that the optimal conditions for the *B. megaterium* 501^{rif} introduced into the soil are achieved at a temperature of 20 °C. After the third day after inoculation the titer of bacteria in the soil increased almost 10 times and remained at that level until the end of the experiment (32 days*)*. *B. megaterium* 501^{rif} throughout the experiment was mainly in the form of a physiologically active cells and fits well into the soil microbiom. Under these conditions an additional incorporation into the soil of the organic matter, such as cereal straw and corn flour, leads to 10-100-fold increase of the amount of *B. megaterium* 501^{rif}. At 37 °C the total number of *B. megaterium* 501^{rif} reduced several times, and stabilized at this level until the end of the experiment. In this case, 100 % of *B. megaterium* 501^{rif} revert into spores and are physiologically inactive. The temperature below the minimum for physiological reproduction of *B. megaterium* (4 °C) inhibits spore formation and leads to a rapid loss of physiologically active bacterial cells introduced into the soil. The total number of bacteria decreases 1000 times within a month. These data lead to the conclusion that the «low-temperature shock» reduces the competitiveness of *B. megaterium* to the indigenous microflora and its resistance to bactericidal metabolic products of soil biome. It is shown that at the optimum temperature (20 °C) of soil the *B. megaterium* inoculation and the use of additional organic matter such as straw accelerate the decomposition of herbicide prometryn, can potentially be used in a biotechnology of remediation of the soil, contaminated by herbicide.

Keywords: *Bacillus megaterium*, sporulation, introduction, soil, temperature, organic matter, prometryn herbicide.

Bacterial preparations are used in agriculture to stimulate plant growth and development, to protect plant against phytopathogens and pests, and as well for cleaning the soil from xenobiotics (1-4). Aerobic sporulating bacteria of the genus *Bacillus*, i.e. *B. thuringiensis* (5, 6), *B. subtilis* (7), *B. megaterium* (8-10) are widely applied as they are well manufacturable, long stored and suitable for use in the field. However, the efficiency of these preparations varies in a wide range due to poor knowledge of the behavior of the bacilli introduced into the soil.

Spore forming bacteria are common inhabitants in different soils. Their number depends on the soil and climatic conditions and vegetation, ranging from 10^3 to 10^6 CFU/g (11). Some researchers (11, 12) consider them to be free living soil microorganisms, while the others (13), on the contrary, designate these bacteria as a common inhabitant of the rhizosphere, at least in some plants, and *B. subtilis* and *B. megaterim* are referred to as the facultative endo-

phytes (14, 15). According to A.I. Melent'ev (16), a survival rate of spore forming bacteria in the rhizosphere of cereals depended on the number, species and strain of introduced microorganisms. Their number on the roots of plants varied from 10^4 to 10^7 CFU per 1 g of the roots, being lower by the end of vegetation compared to initial period. Most bacterial cells were transformed to spores which were not physiologically active and thus unable to affect plant growth and development.

As to spore forming aerobic bacteria inoculated into the soil, it is little known about their survival. Poor knowledge results in lack of the approaches to optimization of the inoculation procedure to make the conditions favorable for the bacteria and allow effective application of their preparations for plant growth stimulation and soil bioremediation in environmentally friendly agrotechnologies.

We studied the survival of *Bacillus megaterium* introduced into the soil and herbicide Promentryn degradation as influenced by different conditions.

Technique. Rifampicine resistant *B. megaterium* 501^{rif} mutant obtained by gradient selection of resistant forms (17) of parental *B. megaterium* 501 strain isolated from medium loamy ordinary chernozem (Kokchetav region, Kazakhstan). *B. megaterium* 501 is deposited in Collection of Agricultural Microorganisms (VKSHM, All Russian Research Institute of Agricultural Microbiology). *B. megaterium* 501^{rif} was cultivated in Erlenmeyer flasks on a rotary shaker at 140 rpm (UMVT-12-250, Ellion, Russia) using nutrient medium that consists of (g/l): K_2HPO_4 — 1.6, KH_2PO_4 — 0.4, NH_4NO_3 — 0.5, $MgSO_4$ — 0.2, $CaCO_3$ — 0.025, $FeSO_4$ — 0.025, yeast extract — 0,2, sucrose — 10, pH 6.8-7.0. Sod-podzolic sandy loam soil (pH 6.7) was filled into plastic containers and inoculated 2-day liquid culture of *B. megaterium* 501rif, containing not less than 90 % of the living cells. Soil moisture was maintained between 50-60% of the total capacity.

Effect of temperature was studied at 4, 20 и 37 °C. To estimate the influence of organic substances, the chopped oat straw (0.2 mm) and corn flour were added in an amount of 20 g per 1 kg of soil. To examine the effect and degradation rate of Prometryne, the 2-methylthio-4,6-bis(isopropylamino)-sym-triazine, 50 % water emulsion of powder herbicide (Panama Agrochemicals Inc., Panama Republica) was added to the soil at 4 and 20 mg/kg.

Total number of *B. megaterium* 501^{rif} (CFU/g of soil) was evaluated by serial dilution of bacterial suspension (19) spread onto nutrient media consisting of (g/l): NH_4NO_3 — 0.5, K_2HPO_4 — 1.6, KH_2PO_4 — 0.4, $MgSO_4$ — 0.2, $CaCO_3$ — 0.025, $FeSO_4$ — 0.025, sucrose — 10, yeast extract — 0,2, agar — 20, rifampicine — 0,02. The spores number was assessed in suspensions after their pasteurization at 80 °C for 10 min.

Prometryn was extracted from soil using acetone method and analyzed by gas-liquid chromatography (GLC) (20).

Statistical analysis was performed at $P_{0.05}$ level (21).

Results. In nonsterile soil at low temperature (Fig. 1) a sharp decrease of *B. megaterium* 501rif number was observed after inoculation. The most of bacteria were in the form of vegetative cells. At 4 °C, being lower than growth minimum for *B. megaterium* 501^{rif}, the sporulation delayed and therefore most of vegetative cells died. After 1 month the total number of *B. megaterium* 501^{rif} was 1000 times less than at the beginning of the experiment. Activation of *Fusarium* and *Penicillium* growth was detected. Therefore, the law temperature shock reduces protective functions and competitiveness of *B. megaterium*.

Relatively high survival of *B. megaterium* 501^{rif} was found at 37 °C. After 32 days the total number of inoculated bacteria in soil decreased 3 times compared to initial level, and the sporulation frequency reached 100 %

after 12 days.

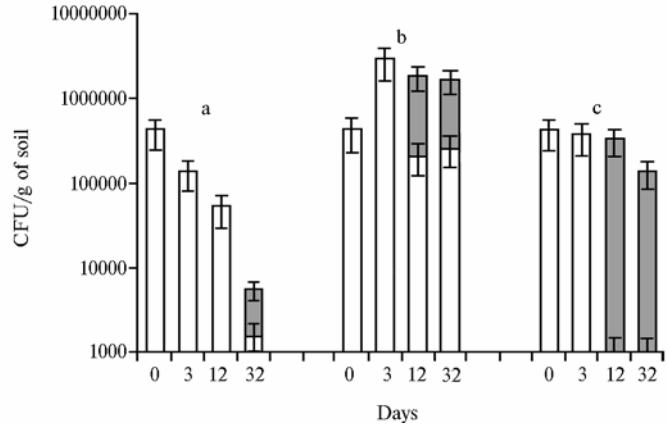

Fig. 1. Dynamics of survival and spore formation in *Bacillus megaterium* 501[rif] after inoculation as related to soil temperature: a, b, c — 4, 20 и 37 °C, respectively; grey and white color means spore number and cell number, respectively.

Most favorable conditions for *B. megaterium* 501[rif] in non-sterile soil were formed at 20 °C. Already 3 days after inoculation the bacteria increased in number almost 10 times and the level remained high until the end of the experiment after 32 days. Sporulation was observed on the day 12, but the proportion of the spores was less than 30 %.

Therefore, in the microbiome of sod-podzolic soil the *B. megaterium* 501[rif] remained active for all the time of observation.

When chopped straw added, the number of inoculated *B. megaterium* 501[rif] increased 10 times after 12 days incubation (Fig. 2). High titers were detected during a month, with the number of vegetative cells more than 10^5 CFU/g. The same was also found when corn flour added. However, in this case there was intensive sporulation, and after a month the number of spore reached 100 %.

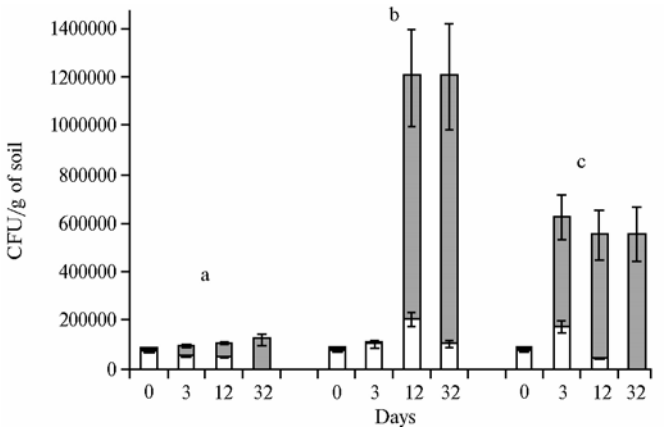

Fig. 2. Dynamics of survival and spore formation in *Bacillus megaterium* 501[rif] after inoculation as related to extra organic matter: a, b, c — control, oat straw and corn flour, respectively; grey and white color means spore number and cell number, respectively.

A positive effect of straw and corn flour on *B. megaterium* 501[rif] is obviously due to more nutrients in soil promoting more vital activity in the microorganism. Also it could result from positive effect of these substrates on formation of microbe association favorable for *B. megaterium*.

For several decades the interrelation between microorganisms and xenobiotics is being under consideration. Herbicides are such xenobiotics widely used in agriculture for weed control (18). These chemicals impact on soil microbiomes, and soil fertility in general, also detoxication and degradation of pesticides in nature are considered an urgent problem because many of them are dangerous for the environment, people and animals.

We studied the interrelation between *B. megaterium* 501[rif] inoculated into the soil and Prometryn, the herbicide widely used for weed control on potato, soybean, carrot and other crops. It was shown that Prometryn applied at the doses dozens times more than those used during commercial crop cultivation did not affect *B. megaterium* 501[rif], and its number even increased significantly in the

presence of oat straw (Table).

Dynamics of survival and spore formation (CFU, 10^3/g) **of *Bacillus megaterium* 501^{rif} after inoculation into soil as influenced by herbicide Prometryn and oat straw** ($X \pm x$)

Days	Control	Prometryn		
		4 mg/kg of soil	20 mg/kg of soil	20 mg/kg of soil + straw (2 %)
0	80±1.2	80±1.2	80±1.2	80±1.2
	13±0.5	13±0.5	13±0.5	13±0.5
3	200±3.4	135±24.2	90±6.0	1600±100.8
	150±12.6	65±0.9	40±6.2	500±8.0
12	135±5.3	114±3.5	100±10.0	1200±150.0
	115±1.5	112±4.0	54±2.7	1000±21.0
32	110±10.7	140±1.5	118±9.4	1200±63.6
	90±2.7	140±10.3	115±1.5	1100±49.5

Comments. Above line: the total number of bacteria, under line: number of spores.

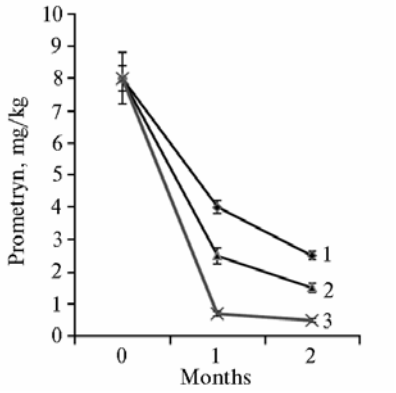

Fig. 3. Dynamics of Prometrine degradation in soil under *Bacillus megaterium* 501^{rif} inoculatiuon: 1 — control, 2 — inoculation, 3 — inoculation in the presence of oat straw (2 %).

In the soil inoculated with *B. megaterium* 501^{rif} the rate of Prometryn degradation increased significantly (Fig. 3). Most active degradation was observed when we added the oat straw. A month after inoculation the concentration of herbicide was 10 times lower if compared to control, thus correlating with an increase of the total *B. megaterium* 501^{rif} number.

These results lead to the conclusion that applying organic matter, particularly the plant residues enriched with cellulose, and the inoculation with microorganisms capable to degrade the herbicide, are essential and probably the main conditions for improving bioremediation under soil pollution with Prometryn.

So, it is shown that vegetative cells of *Bacillus megaterium* 501^{rif} inoculated into soil remain physiologically active for rather long time. When organic matter, such as cereal straw or corn flour, is added, it results in a rapid bacterial growth. Their number increases hundreds times, being kept stable. Active sporulation starts 12 days after the inoculation. The result of introduction and physiological state of the bacteria depend directly on soil temperature. The most favorable temperature for *B. megaterium* survival, provided optimal moisture, varies from 20 to 37 °C. Organic matter enriched with cellulose, such as the chopped straw, crop residues, etc., also contribute to the survival of bacteria. The isolated strain *B. megaterium* 501^{rif} significantly accelerates the Prometryn degradation and can be useful in biotechnological remediation of soils polluted with this herbicide.

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Abiotic stresses in strawberry

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FOLIAR FEEDING TO INCREASE YIELD VALUE AND QUALITY IN STRAWBERRY (*Fragaria ananassa*) UNDER METEOROLOGICAL STRESSES

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Abstract

In formation of fruit quality, an application of bio-stimulants that can protect plants against external unfavorable factors and regulate specifically the plant growth, development and metabolism, is prospective, enabling full realization of a varietal potency. In this paper, the data are summarized on studying influence of meteorological conditions of the growing season and the growing factors on the yield and quality of strawberries grown in the southern Russia in 2006-2010. There are reported the results of using GUMI 20K fertilizer and growth regulators Mival-Agro, Stimolante 66f, Alga mix B Mg at the phases of stem extension, early flowering and ripening of berries to control yield formation and chemical composition of strawberries under extreme weather conditions. Due to application of tested preparations, in the varieties Cleary, Arosa, Marmolada the weight of a berry was by 0.7-2.2 g higher as compared to the control. That improves trade quality and ultimately has a positive effect on productivity. Joint use of growth regulators Mival-Agro and fertilizer GUMI 20K improves the quality according to the content of dry matter and sugars (by 7-10 %), organic acids (by 10-15 %), vitamin C (by 9-14 %), and P-active substances (by 3-12 %). It was found out that the use of growth regulators Stimolante 66f and Alga mix B Mg on the Marmolada plants contribute to increased resistance to late spring frosts occurred in 2009. The number of flowers exceeded the control by 9.1 %, and the number of berries was higher by 18.5 % with an average weight of a berry increased by 1.7 g. The berries also accumulated more soluble dry matters (9.0 %) and sugars (6.8 %), their acid content increased (1.0 %), but the synthesis of vitamin C and P-active substances slowed. A response of varieties to the treatments differed. In Arosa variety the similar treatments resulted in decrease of sugar content and acidity, but the concentration of biologically active substances increased as compared to the control. Thus, the application of fertilizers together with growth stimulants can reduce the impact of stresses, improving productivity, fruit quality and content of biologically active substances in strawberries.

Keywords: strawberry varieties, foliar feeding, productivity, product quality, biologically active substances.

Strawberry (*Fragaria ananassa*) is one of the main berry crops in southern Russia due to its early ripening, little time before fruiting, high yield, excellent taste and medicinal properties (1-6). Quality attributes of the berries are the genetically controlled parameters but they can vary considerably under the influence of environmental factors. Weather stresses impact both yield production and quality, including chemical composition of the berries (7-10). Late spring frosts and lack of rainfall together with the elevated temperatures in late May—early June are the main weather extremums for the strawberry in Krasnodarskii region.

Plant metabolism activation is known to be influenced by the levels of lightening, water supply and the sum of active temperatures. Excess rainfall together with insufficient heat, the same as excessively high temperature combined with low humidity, adversely affect the vitamin content in berries. Under water deficit the synthetic processes are depressed, while the respiration energy and consumption of vitamins for the formation of enzymes increase (11-14). Therefore, strawberry cultivation without effective mineral nutrition is not

profitable (15-19).

Our goal herein was studying yield formation and quality in strawberry as influenced by foliar nutrition and the stimulators of a new generation.

Technique. Experiments were held as described (9) in 2006-2010 at the experimental field in Krasnodar region using differently ripening strawberry varieties, namely Cleary (early ripening), Marmolada (medium to early ripening) and Arosa (middle ripening). Mulching black perforated film and a drip irrigation were applied under 90-40×40 sm scheme of planting, the plot size was 3,5 m^2 with 30 plants in each random sample group. The soil (pH 6.5-7.0) was leached chernozem with 2.7-3.0 % humus, 140 mg/kg exchangeable potassium and 250-270 mg/kg mobile phosphorus in the plow layer. For foliar treatments during plant development we used the complex of humic salts GUMI 20K (BashIncom, Russia), the biostimulants Mival Agro (AgroSil, Russia) and Stimolante 66f (L. Gobbi, Italy), and also the growth activator Alga mix B Mg (L. Gobbi, Italy). Experiments were held in a 3-fold repetition, each lasting for 2 years. In the first experiment (2006 to 2007) the Cleary, Marmolada and Arosa plants were examined in control (no treatment) (i), under 3-fold GUMI 20K treatments (0,5 l/ha) at the phases of stem extension, early flowering and berry ripening (ii), and under 1-fold GUMI 20K treatment (0.5 l/ha) at the phase of stem extension together with 2-fold treatments with biostimulant Mival Agro (0.01 l/ha) at the phases of early flowering and berry ripening (iii). In the second experiment (2009 to 2010) the Marmolada and Arosa plants were investigated in control (no treatment) (i), under 3-fold foliar treatments with biostimulant Stimolante 66f (0.1 l/ha) at the phases of stem extension, early flowering and berry ripening (ii), and under 2-fold foliar treatments with biostimulant Stimolante 66f (0.1 l/ha) at the phases of stem extension and ovary formation together with 1-fold treatment with the growth activator Alga mix B Mg (0.2 l/ha) at mass flowering.

The efficacy of foliar treatments was estimated basing on yield value and merchantability and biochemical parameters in berries including a soluble dry matter content according to GOST 28561-91, sugars according to GOST 8756-13.87, acids according to GOST 25555.0-82, vitamin C according to A.Ya. Tribunskaya's method, and P-active substances according to L.I. Vigorov (20-21).

Data were processed (22) by means of dispersion analysis using Microsoft Office Excel 2003.

Results. In Kuban region the low winter temperatures are atypical, except the winter season of 2006 when the temperature dropped to –27 °C, while the spring frosts, which damage the flowers and cause a decrease in yield, are characteristic. The droughts were mostly observed in the plains and steppe zones of Kuban territory in spring and summer when the humidity decreases and temperature increases. In these parts the average humidity in summer was 35-40 %, ranging from 16 to 26 % in 2007 and from 14 to 39 % in 2010. Together with an average temperature of 35 °C, which reached up to 40 °C in 2007 and 2010, it results in yield and quality losses. In these areas the plants cultivated without drip irrigation are much suffering.

More favorable weather conditions are formed at humidity of 70-75 % in the Black Sea coast and in the foothill areas of Krasnodar region, where the plants are less exposed to overheating in the summer, when the temperature exceeds 30 °C and the surface of the soil warms up to 50-60 °C. Here, in the morning dew drops, reducing the effects of stress even after long drought.

A crucial time for berry formation begins from flowering. In 2006-2010 the weather conditions fluctuated considerably during April end to June beginning.

These fluctuations influenced considerably the content of biologically active substances in strawberry fruits (Fig. 1).

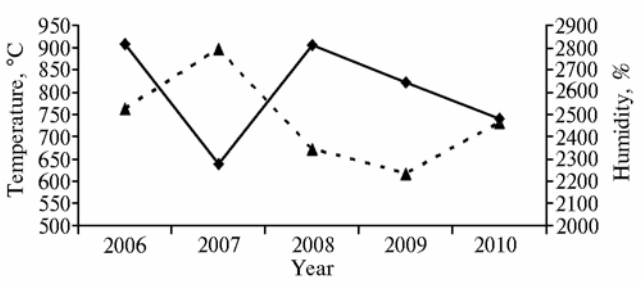

Fig. 1. Sum of average temperature (▲) and average humidity (♦) from the beginning of flowering to the end of berry ripening in strawberry (April 24-May 25) (Krasnodar).

Under the conditions of Krasnodarskii region 8.1-9.0 % soluble dry matters, 6.1-6.8 % sugars, 0.75-0.91 % organic acids, 64.3-69.0 mg/100 g vitamin C, 78.8-110.9 mg/100 g catechins and also 71.4-75.2 mg/100 g anthocyanins were accumulated in berries of the investigated varieties (23).

In 2006 the tested parameters were higher comparing to 2007 due to lower berry weigh and decreased yield resulted from the damage caused by winter frost. The content of soluble dry matters, sugars, vitamin C and P-active catechins ranged from 8.5 to 10.6 %, from 6.4-8.0 %, from 61.6 to 70.4 mg/100 g and from 76.0-109.8 mg/100 g, respectively. In 2007 by the end of flowering and at early ripening the abnormally hot and dry weather with a maximum average temperature up to 34.9 °C, or 3.6-7.7 °C above the norm, and an average relative humidity of 54 % did not allow to form the high quality strawberry fruits. In 2008, as yield increased and dry matter and sugars correspondingly decreased, a sufficient accumulation of vitamins was detected, in particular the vitamin C content reached up to 83.1 mg/100 g. It was due to more favorable conditions, 16.3-21.5 °C and 67 % humidity, during the ripening time. In 2010 similar to 2007, at May end to June beginning the weather was hot and dry with 30,6 °C maximum daily temperature and 58 % humidity, resulting in a decrease by 2-8 % in vitamin C and polyphenols content.

To increase stress tolerance, the plants were treated with adaptogens and immunostimulants. Foliar treatments with mineral fertilizers during plant development were shown to reduce negative effect of weather stress, contributing higher quality in berries (Table 1).

1. Quality parameters in strawberry varieties (*Fragaria ananassa*) as influenced by foliar treatment with mineral and biologically active substances (Krasnodar, 2006-2007)

Variant	1	2	3	4	5	6	7	8
Cleary variety								
Control	11.8	8.6	6.6	0.61	10.8	61.7	76.3	69.0
GUMI 20K	12.2	8.3	6.3	0.69	9.0	61.7	78.2	69.0
Mival Agro + GUMI 20K	12.5	9.4	7.1	0.70	10.1	64.0	84.3	77.6
Least mean difference$_{05}$	0.47	0.77	0.55	0.07	1.20	1.80	5.70	6.70
Arosa variety								
Control	11.0	10.2	7.7	0.59	13.0	62.4	90.6	77.6
GUMI 20K	11.4	10.6	8.0	0.68	11.8	63.8	100.8	72.2
Mival Agro + GUMI 20K	12.0	10.9	8.2	0.67	12.2	69.9	109.8	80.4
Least mean difference$_{05}$	0.68	0.08	0.34	0.08	0.83	5.40	13.00	5.60
Marmolada variety								
Control	15.8	7.7	5.8	0.62	9.4	53.3	70.6	73.1
GUMI 20K	16.4	7.8	5.9	0.62	9.4	55.7	79.0	77.6
Mival Agro + GUMI 20K	16.6	8.5	6.4	0.69	9.3	59.6	84.2	80.2
Least mean difference$_{05}$	0.57	0.59	0.43	0.06	0.20	4.30	9.30	4.90

Примечание. 1 — berry weight, g; 2 — dry matters, %; 3 — sugars, %; 4 — total acidity, %; 5 — sugar ti acidity index; 6 — vitamin C, mg/100 g; 7 — catechins , mg/100 g; 8 — anthocyanins, mg/100 g. For more detail see *Tenique*.

At 3-fold foliar treatments with GUMI 20K the humic salts had an ex-

pressed protective effect against stresses. According to the reports, it is due to optimal composition of biogenic microelements, which stimulate plant growth and immunity, and also enhance the biosynthesis of the substances determining fruit quality in strawberry. Due to good combination of biologically active components the biostimulant Mival Agro provides for activation of the antioxidation complex, and increases plant immunity and tolerance under biotic and abiotic stresses caused by weather conditions (24, 25).

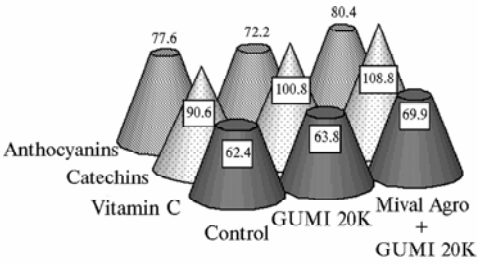

Fig. 2. Changes in content of biologically active components (mg/100 g) **in fruits in strawberry (*Fragaria ananassa*) variety Arosa as influenced by foliar treatment with mineral and biologically active substances** (Krasnodar, 2006-2007).

Under foliar application of all tested preparations on plantations of fruit-bearing strawberry varieties Cleary, Arosa, Marmolada, grown with mulching black perforated film and drip irrigation there was a general trend of increasing berry weight by 0.7-1.0 g comparing to the control. This improves fruit quality and eventually increases the yield. A combination of Mival Agro and GUMI 20K provides for 7-10 % increase in dry matter and sugar levels, up to 15 % increase in organic acid level and 9-14 % increase in vitamin C level when compared to control (Fig. 2).

The same trend was found in regard to P-active substances, particularly the level of catechins was higher in the treated plants of all the varieties tested. The peak value of 109.8 mg/100 g was found in the Arosa plants. Mival Agro together with GUMI 20K also intensified berry coloration, and this fact was confirmed by the data on the accumulation of anthocyanins exceeding the control by 3-12 %.

The varieties differed in their response to the treatment. In Arosa the greatest effect of dry matter, sugars, acids and P-active polyphenols assimilation was observed when the plants were treated with Mival Agro together with GUMI 20K.

2. Yield parameters in strawberry varieties (*Fragaria ananassa*) as influenced by foliar treatment with mineral and biologically active substances (Krasnodar, 2006-2007)

Variant	Number per 1 m of bed		Weight of berries		Yield	
	peduncles	berries	average, g/pcs	per 1 m of bed, kg	ton/ha	to control
Marmolada variety						
Control	131	365	7.3	2.648	17.0	
Stimolante 66f	152	452	8.3	3.728	24.0	+7.0
Stimolante 66f + Alga mix B Mg	144	448	9.0	4.036	26.1	+9.1
Least mean difference$_{05}$	14.4	66.9	1.3	0.99	6.6	
Arosa variety						
Control	59	230	9.0	2.020	13.0	
Stimolante 66f	72	212	11.2	2.175	14.0	+1.0
Stimolante 66f + Alga mix B Mg	102	347	9.5	3.296	20.4	+7.4
Least mean difference$_{05}$	30.0	99.9	1.8	0.95	5.5	

Comments. For more detail of applied preparations see *Technique* section.

Plant stimulant Stimolante 66f was reported to activate metabolism and facilitate stress overcoming caused by extremal temperture and water deficit, while the growth activator Alga mix B Mg accelerates the assimilation of nuntrients due to microements. This also was confirmed by our data of yield formation in the varieties (Table 2). It was found that a 3-fold foliar treatments

with Stimolante 66f or its combination with Alga mix B Mg contributed to the increase in plant tolerance to late spring frosts in 2009. In Marmolada variety at Stimolante 66f together with Alga mix B Mg treatment there were observed 9.1 % more peduncles, 18.5 % more berries and 1.7 g higher berry weight resulting in 9.1 ton/ha more yield compared to control.

Also the plants were more tolerant to heat and drought, especially in May to June 2010. This led to higher weight and better chemical composition (Table 3). The specific reaction to foliar treatment was also revealed. When Alga mix B Mg was used together with Stimolante 66f on middle ripening Arosa plants the sugar and acid contents decreased and the concentration of biologically active substances increased as the berry weight increased compared to control. In contrast, in higher yielding Marmolada variety with middle ripening under the same treatment the dry matter content, sugar content and acid concentration were 9.0 %, 6.8 % and 1.0 % higher, while the synthesis of vitamin C and P-active substances slowed down.

3. Main parameters of berry quality in strawberry varieties (*Fragaria ananassa*) as influenced by foliar treatment with biologically active substances (Krasnodar, 2009-2010 год)

Variant	1	2	3	4	5	6	7
			Arosa variety				
Control	7.6	5.7	0.91	6.3	59.0	106.0	60.0
Stimolante 66f	6.8	5.1	0.95	5.4	63.4	103.0	69.5
Alga mix B Mg + Stimolante 66f	6.4	4.8	0.84	5.7	65.4	109.8	66.8
Least mean difference$_{05}$	0.80	0.62	0.04	0.62	4.40	4.60	6.60
			Marmolada variety				
Control	8.8	6.6	0.98	6.9	72.2	97.8	68.9
Stimolante 66f	7.3	5.6	1.12	4.9	73.6	97.8	69.5
Alga mix B Mg + Stimolante 66f	9.0	6.8	1.00	6.7	60.0	79.0	61.3
Least mean difference$_{05}$	1.20	0.90	0.09	1.50	14.00	14.20	6.20

Comments. 1 — dry matters, %; 2 — sugars, %; 3 — total acidity, %; 4 — sugar to acidity index; 5 — vitamin C, mg/100 g; 6 — catechins, mg/100 g; 7 — anthocyanins, mg/100 g. For more detail see *Tenique*.

So, the foliar treatment with a complex mineral fertilizer and qrowth stimulants at different phases of strawberry vegetation increases the plant tolerance to weather stresses due to activating defence mechanisms. As a result, the yield increased by 32-46 %, and the parameters of quality in berries were 9-23 % higher with the nutritive value and medicinal properties also improved by 6-19 %.

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