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Address: build. 11, office 343, Dmitrovskoe sh., Moscow, 127434 Russia
Tel/fax: + 7 (499) 977-88-19, + 7 (499) 976-32-73
E-mail: agrobiol@mail.ru **Internet:** http://www.agrobiology.ru

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GENETIC DIVERSITY AND POPULATION STRUCTURE OF *Plum pox virus* IN RUSSIA

(review)

S.N. CHIRKOV[1], Yu.N. PRIKHOD'KO[2]

[1]*M.V. Lomonosov Moscow State University*, Department of Virology, Biological Faculty, 1/12, Leninskie gory, Moscow, 119234 Russia, e-mail s-chirkov1@yandex.ru;
[2]*All-Russian Plant Quarantine Center,* Federal Agency of Scientific Organizations, 32, ul. Pogranichnaya, pos. Bykovo, Ramenskii Region, Moscow Province, 140150 Russia, e-mail prihodko_yuri59@mail.ru

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A b s t r a c t

Plum pox virus (PPV) is the causal agent of Sharka that is considered the most detrimental viral disease of stone fruit crops. Regular monitoring of stone fruit plantings using enzyme-linked immunosorbent assay and reverse-transcription polymerase chain reaction for PPV detection and identification resulted in the findings of numerous focuses of the disease in European Russia. The virus was found in collections, variety test plots, nurseries, fructiferous and abandoned orchards, decorative plantings, private gardens, wild stone fruit trees growing in urban and rural areas. PPV was detected in naturally infected plum (*Prunus domestica*), peach (*P. persica*), nectarine (*P. persica* var. *nectarina*), myrobalan (*P. cerasifera*), blackthorn (*P. spinosa*), downy cherry (*P. tomentosa*), sour cherry (*P. cerasus*), sweet cherry (*P. avium*), apricot (*P. armeniaca*) and Canadian plum (*P. nigra*). PPV has been reported from Petersburg, Novgorod, Tver, Moscow, Tula, Voronezh, Tambov, Lipetsk, Belgorod, Rostov, Samara, Saratov, Volgograd, Astrakhan, Stavropol, Krasnodar, Karachay-Cherkessia, Dagestan, and Crimea regions. Six of the nine known PPV strains (D, M, Rec, W, C, CR) have been revealed in European Russia. Most isolates belong to the strains D (38 %), W (25 %), CR (23 %), M (7 %) and C (7 %). Two distinct PPV-Rec isolates have been found on myrobalan and plum in Crimea and Stavropol regions. Population of PPV in European Russia and, probably, all over the European part of the former USSR seems to be the most diverse in the world due to wide spread of PPV isolates belonging to the strains W, C, and CR that were never detected or only sporadically identified in other countries until now. Phylogenetic analysis of their genomes shows that these three strains constitute the supercluster divergent from other PPV strains. This evolutionary branch originated from a common ancestor and apparently developed mainly in Russia. The wide dissemination of PPV in Russia is a potential threat for newly bred stone fruit cultivars and further selection and biotechnological works.

Keywords: plant viruses, *Plum pox virus*, Sharka disease, genetic diversity, strains.

Plum pox virus, PPV (*Potyvirus*, *Potyviridae*) is the causal agent of Sharka disease of stone fruit crops. Because of reactive oxygen species produced in plants as a response to viral infection, the chloroplasts and photosynthetic apparatus are damaged resulting in visible symptoms appeared on leaves (Fig. 1), fruits, flowers and seeds [1]. PPV can infect, naturally and experimentally, many (or perhaps all) stone fruit crops from genus *Prunus* (*Rosaceae* family) and also from other taxa [2-5]. PPV is considered the most detrimental viral pathogen in stone fruit plants which causes significant yield losses in peach, apricot, plum, etc., as up to 100 % of fruits drop prematurely, being worse in quality and bad processed. In the susceptible varieties annual growth of the infected trees is suppresses. As a result, a lot of infected varieties are not in use despite high agronomic properties and quality indices [6, 7]. Annual losses in EUR reach hundreds of millions, and the millions of infected trees are eliminated [8]. PPV can

be transmitted mechanically, under vegetative propagation and by different aphids. Seed transmission is not found. For long distances PPV can be mainly transmitted via infected plants. The disease is recorded all around the world, except Australia, New Zeeland, South Africa and California [9-11]. In Russia PPV is a limitedly spread quarantine virus.

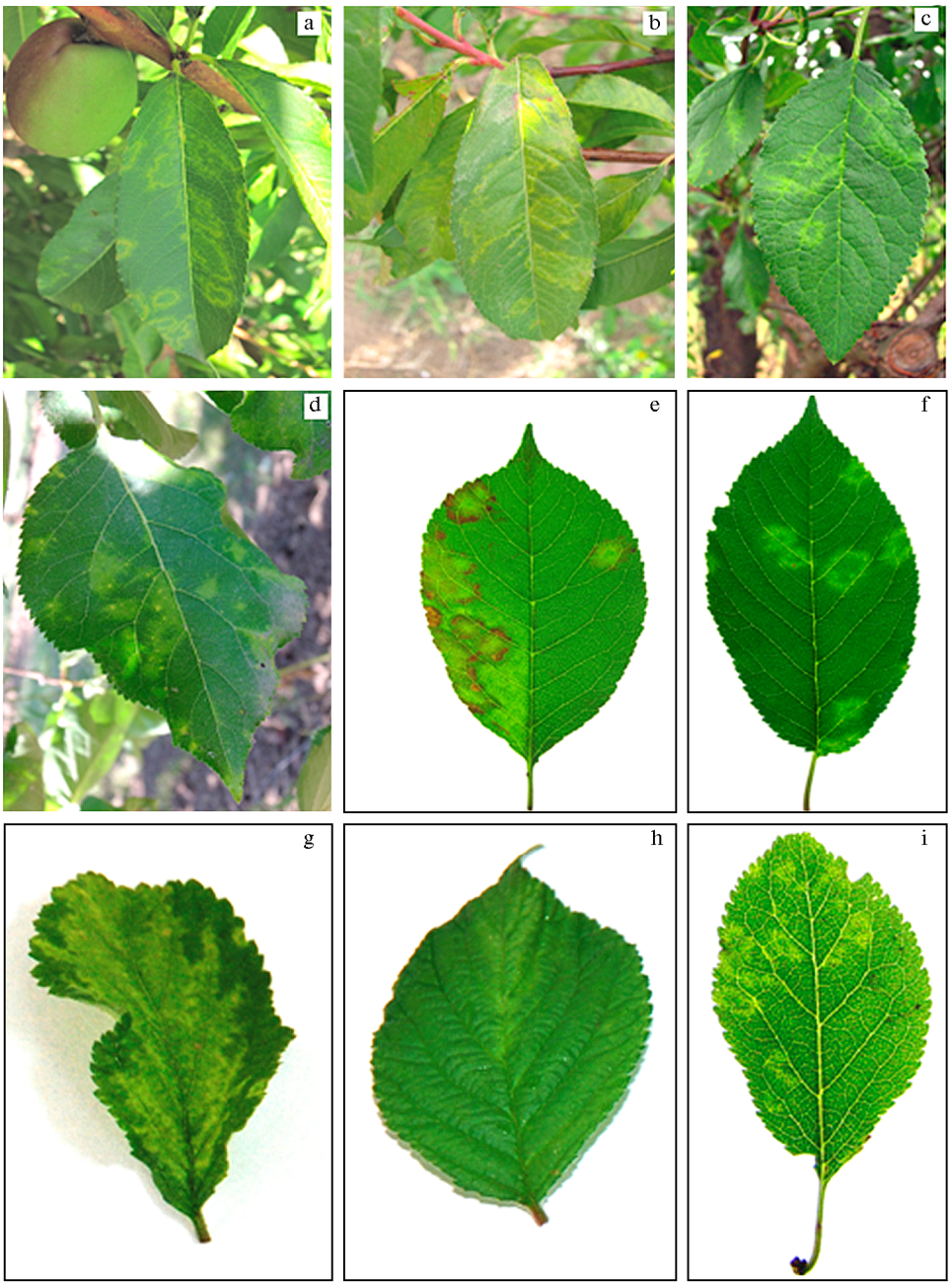

Fig. 1. Sharka symptoms on nectarine (a), **peach** (b), **plum** (c), **apricot** (d), **cherry** (e, f), **felt cherry** (g, h), **cherry-plum** (i). PPV strains: D (a-e, g), CR (e), C (f), W (h), Rec (i). Photo (a, b) courtesy of I.V. Mitrophanova.

Molecular biology of PPV. PPV virions are filamentous particles 750 nm long with 15 nm in diameter in which the +RNA of 9.8 thousand nucleotides is packed into 2,000 copies of virus capsid 36.5 D protein. PPV genome structure is typical for potyviruses. In the genome RNA there are two

open reading frames, 5′ and 3′non-coding regions (NCR) of 146 and 217 nucleotides, respectively, the viral protein (VPg) is covalently linked at 5′end and poly(A) sequence is attached to 3′end. In infected cells RNA is expressed into single precursor polyprotein than processed by virus-specific proteases into 10 functional proteins (P1, HcPro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and capsid protein CP). Another protein, the PIPO (Pretty Interesting *Potiviridae* ORF), is expressed due to the fusion of P3N and PIPO resulting from the shift in the P3 gene reading frame [9].

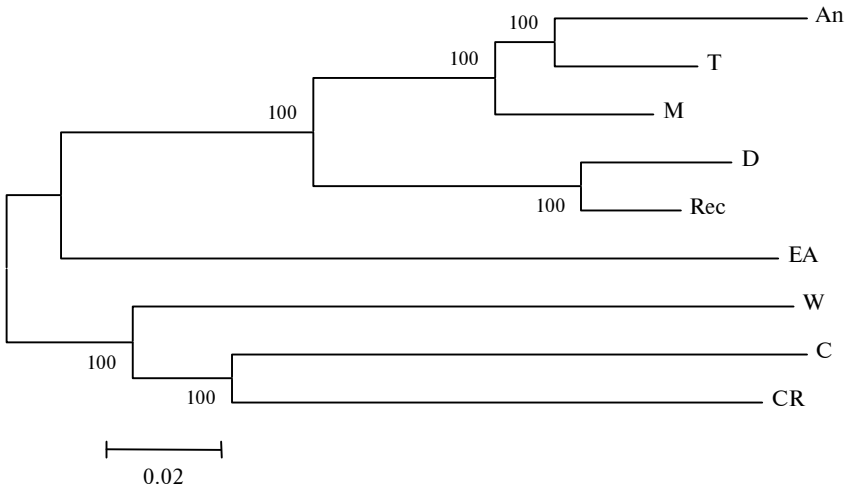

Fig. 2. Dendrogram of *Plum pox virus* (PPV). Grouped by the «neighbor-joining» method for full genome sequences of typical strains. The results of bootstrap analysis (%) for 1000 random samples are shown. Scale indicates the number of substitutions per nucleotide. An (AL-11pl, HF674399), T (AbTk, EU734794), M (SK68, M92280), D (Ou1, AB545926), Rec (BOR-3, AY028309), EA (AM157175), W (LV-145bt, HQ670748), C (BY181, HQ840518), CR (RU-17sc, KC020124) were analyzed (strain name and the record in GenBank are indicated). MEGA 5 was used for phylogenetic analysis [12].

To date, the analysis of full genome sequences of PPV allows distinguishing 9 strains. These are Dideron (D), Marcus (M), Recombinant (Rec), Cherry (C), Cherry Russian (CR), El Amar (EA), Winona (W), Turkish (T) and Ancestral (An) [9, 13], clustered into monophylic groups of closely related isolates. Recombination plays important role in the PPV evolution together with spot mutations. M and T probably appeared due to independent recombination between isolates of D and An strains [9]. Rec strain considered to be a recombinant of D and M isolates [14]. In the genome of the W317 isolate of W strain, there is an apparent evidence of recombination with D and M strains [15]. Most probable relationships are shown in the dendrogram based on full genome sequences of typical isolates of each strain (Fig. 2). The strains differ in antigenic and epidemiological properties, host plants range, geographic distribution and pathogenicity for different stone crop species. In Europe and Mediterranean basin where the majority of PPV isolates is revealed, D, M and Rec strains are spread widely while the rest one rarely and(or) endemically for specific territories. Thus, for the time being, the EA and T isolates were found only in Egypt and Turkey, respectively. The only known isolate of An strain, the AL-11pl, was found in Albania [16]. In other regions the D strain isolates are mainly detected. C, CR and W strains, with a few exceptions, are found in the ex-USSR territory.

PPV detection and identification. ELISA and different modification of RT-PCR are mostly used [17-19]. In ELISA the viral CP can be assayed. Most virus specific and strain specific epitopes are located at N-end of CP, which is exposed on the surface of the viral particle being the most variable

part of the molecule [9]. Numerous assay kits for PPV diagnostics based on sandwich-ELISA with polyclonal antibodies and conjugated alkaline phosphatase are well represented on the market. Indirect sandwich-ELISA [20] with 5B monoclonal antibodies to ^{94}DRDVDAG^{100}, a universal epitope found till now in the CPs of all studied isolates [21], seems to be the most reliable. In RT-PCR any PPV isolate can be found with universal primers specific for CP gene or 3′-NCR [22, 23]. The amplification results in 243 and 220 bp fragments. Immune-specific RT-PCR is deemed most specific detection technique [24]. Variability of PPV isolates can be studied differently [25]. In particular, the strain can be identified in indirect sandwich ELISA-test with monoclonal antibodies to D, M, C and EA [20, 26-28], in RT-PCR with primers specific to D, M, C, CR, EA, Rec, and W [29-34], and by sequencing CP gene or its hyper variable N-terminal fragment followed by phylogenetic analysis. Sometimes the PPV strain can be identified by sequencing and phylogenetic analysis of 243 bp product along with its restriction analysis using RsaI and AluI endonucleases [22]. Note, the strain specific monoclonal antibodies can reveal the epitopes located at the CP N-end, while the strain specific primers are designed to different regions of the PPV genome 3′-end, mostly for the CP gene. However, the An, T, Rec and M distinguishes are mainly located in the genome 5′-end. Besides, even single nucleotide or amino acid replacement may result in incorrectness of strain identification [13]. Therefore, several methods should be used, of which the phylogenetic analysis of full genome sequences is deemed the most reliable. Optimized protocols for ELISA, ER-PRC and other methods for PPV diagnostics and identification are approved by European and Mediterranean Plant Protection Organization (France) [35, 36].

PPV prevalence and genetic diversity in Russia. Charka symptoms in stone fruit crop plantations in the Russian territory were observed at least since 1970th. Nevertheless, till 2000th the end no molecular evidence of viral infection was reported with no disclosed isolate characteristics represented. Due do modern techniques of PPV diagnostics and identification the systemic resulted in the findings of numerous focuses of the disease in European Russia [37-39]. The virus was found in collections, variety test plots, nurseries, fructiferous and abandoned orchards, decorative plantings, private gardens, wild stone fruit trees growing in urban and rural areas. Collections and variety test plots where the cuttings for nurseries are produces seem to be most probable source of infection [37]. The isolates are mostly found in old gardens planted when the importance of total diagnostics and identification of plant viral infections were not understood enough and the means for its monitoring were not developed.

Till now, PPV has been reported from Petersburg, Novgorod, Tver', Moscow, Tula, Voronezh, Tambov, Lipetsk, Belgorod, Rostov, Samara, Saratov, Volgograd, Astrakhan, Stavropol, Krasnodar, Karachay-Cherkessia, Dagestan, and Crimea regions. Of note, no PPV was disclosed in about 130 samples from Kursk, Orel, Ryazan' and Penza regions.

PPV was detected in naturally infected plum (*Prunus domestica*), peach (*P. persica*), nectarine (*P. persica* var. nectarina), myrobalan (*P. cerasifera*), blackthorn (*P. spinosa*), downy cherry (*P. tomentosa*), sour cherry (P. *cerasus*), sweet cherry (*P. avium*), apricot (*P. armeniaca*) and Canadian plum (*P. nigra*). Of 370 PPV isolates, shown in 2007-2014 by experts All-Russian Plant Quarantine Center, 90 % were detected in plum and cherry trees. Nevertheless, these were the crops mostly surveyed. To date, no PPV was reported from *P. pumila*, *P. fruticosa*, *P. mahaleb*, *P. triloba*, and in the hybrids of *P. fruticosa* and *P. maackii*.

Six of the nine known PPV strains (D, M, Rec, W, C, CR) have been revealed in European Russia. Most isolates belong to the strains D (38 %), W (25 %), CR (23 %), M (7 %) and C (7 %). Since D strain prevails in the

world, obviously, Russia is not an exception. D strain has been mainly detected in plum, and few isolates have been disclosed in apricot, myrobalan and downy cherry. In Crimea a big number of D strain isolates were found in peach and nectarine plant collections. About 7 % of Russian isolates belong to the M strain. Importantly, the M-isolates have been revealed only in commercial plantation of plum (84 %) and peach (16 %) trees. Two distinct PPV-Rec isolates have been found on myrobalan and plum in Crimea and Stavropol' regions.

Wide prevalence of W, C and CR strains which are rare or even not found in other territories in the world seems to be most significant distinction of the PPV population in Russia. Thus, the molecular study of the Russian PPV isolates makes it possible to contribute stigmatically to our knowledge about PPV genetic diversity.

First isolates of W strains (W3174 and UKR44189) have been disclosed in Canada and USA but in the trees of the Ukrainian origin [40, 41]. For the long time, these isolates remained the only known representatives of new strain W. In our investigations the numerous genetically distinct isolates were revealed in Middle Russia, Chernozem zone, Volga region and in South Russia in all types of plantations and in a wide host plant range, including plum, apricot, myrobalan, downy cherry, blackthorn and Canadian plum [38, 42-44]. Few W-isolates have also been revealed in blackthorn and plum in Latvia [15]. For W-isolates poor manifested symptoms or symptomless infection is characteristic. Sequence analysis of genomes or their fragments approved the W strain to be most variable of all nine known PPV strains. The similarity at nucleotide or amino acid levels was 92.0-99.7 % and 96.4-99.9 %, respectively. Nevertheless, the biological properties of W-strain isolates can differ. Particularly, we have disclosed isolate RD4 in the naturally infected downy cherry [43]. However, the downy cherry can not be a systemic host plant for isolate UKR44189 [41]. Monoclonal antibodies to W strain [45] failed to distinguish the Russian W-isolates as the first asparagine acid (D) in the ^{2}DEEDD^{6} epitop is changed to asparagine (N) [43]. High genetic diversity along with wide prevalence in the European Russia territories and very wide host range allow to suggest the strain W to be one of the oldest in Russia.

C-strain isolates make about 7 % of all up-to-date PPV Russian isolates. These isolates were found in different types of cherry and sweet cherry plantings in Leningrad, Moscow, Belgorod, Samara, Saratov and Volgograd regions. They differ genetically that have been shown by molecular methods. The first C-strain isolate was disclosed in Moldova in cherry [46]. Few C-strain isolates have been revealed in Belarus [47]. Despite the relatively small number of the revealed isolates, the findings indicate broad spreading C strain in Russia and the neighboring countries.

First CR-isolates have been recently disclosed in cultivated cherry plants in Saratov and Samara regions, and in Moscow in wildly growing trees [29, 48, 49]. For CR-isolates the extremely low genome variability is characteristic, making 0.6-0.9 % for the whole genome sequences and 0.0-0.8 % for CP gene. Nevertheless, the isolates from Moscow and Volga regions are reliably distinct phylogentically under full genome sequence analysis. Their findings are located at the hundreds kilometer distance thus indicating wide area of CR strain in European Russia. A new strain disclosed improves scientifically the knowledge about genetic diversity in PPV. Moreover, until this finding, C strain considered the only strain able to cause a systemic infection in cherry (*P. cerasus*). Comparative study of two PPV isolates infecting cherry can possibly detect determinants involved in host range control [29]. An important peculiarity of CR-isolates consists in the ^{96}DRDVDAG^{102} universal epitope modification due to replacement of the asparagine acid (D) at 96 to glutamic acid (E). Such isolates can not be identified by ELISA with 5B monoclonal antibodies specific to the universal epitope and earlier

considered the universal testers for any PPV isolates. It obviously means the necessity to develop new universal monoclonal antibodies to provide reliable ELISA diagnostics of PPV.

Thus, to date there are six PPV strains disclosed in Russia. Three of them, the D, M and Rec, prevailed in Europe and Mediterranean regions. It is quite reasonable to assume their appearance in Russia due to introduction of European stone fruit crops infected by PPV. Sharka was first detected in plum in Bulgaria in 1917 [50], then Balkan PPV appeared in Europe, Mediterranean basin, Asia, North and South America [9]. For instance, wide dissemination of Rec strain in Europe was possibly caused by infected tolerant plum varieties from the former Yugoslavia [14]. M-strain isolates were found in peach in Krasnodar Krai and in plum in Stavrolol' region in the trees imported from the former Yugoslavia [38]. It is a probable reason of the M strain occurrence in the commercial garden only. Close similarity of the Russian and the European isolates are confirmed by phylogenetic analysis of their genomes.

The C, CR and W PPV strains are practically not found out of the ex-USSR, and to date the CR is found only in Russia. Two W-isolates from Canada and USA are of Ukrainian origin [40, 41]. Moreover, the isolate UKR44189, detected by the US quarantine guard in the in vitro plum culture hand carried from Ukraine is related closely to LV-145bt from Latvia [41]. LV-145bt and other Latvian W-isolates are obviously derived from Russia and Ukraine [15, 49]. C-isolates are described for sweet-cherry in Italy [51] and in cherry in Croatia, with the Croatian isolate being probably identic to that isolated in Moldova [52]. It also must be emphasized that sporadic findings of C and W PPV out of ex-USSR are in contrast with their frequency in Russia and neighboring countries.

Nevertheless, on the dendrograms based on partial or total genome sequences these strains form a distinct supercluster (see Fig. 2). Phylogenetic analysis indicates the common ancestor for W, C and CR strains, and this branch further evolved, mainly or exclusively, in the territory of contemporary Russia. Assumptions of East-Europe ancestry of W and C strains have been reported [13, 40, 53]. A presumable ancestor could appear in Russia or be introduced from Front and Middle Asia with infected plants. In this connection the two closely related D-isolates found in wild apricot and cultivated plum trees in Kazakhstan are of interest. These isolates posses an unique deletion of six nucleotides in the CP gene corresponding to CP N-end unknown in all PPV isolates for which this genome segment is sequenced. Wild apricot trees from Tien Shan are considered the most probable source of PPV isolates with this genetic marker [54].

It should be mentioned that for the long time in an single economic area of ex-USSR Russia the viral strains can move unlimitedly with infected planting material. Occurrence of W-isolates in Latvia and Ukraine [15, 40, 41], C-isolates in Moldova and Belarus [31, 46, 47], and D strain in Lithuania and Ukraine [55, 56] allows to assume the single PPV population formed in European Russia. Its characteristic feature is the highest genetic variability due to wide range of W, C and CR strains which are extremely rare or even not found in other regions in the world. The reasons of W, C and CR absence in other European countries are unknown. Probably, it may be due to limited practical use of stone fruit crop varieties derived from Russia. Thus, occurrence of two genetically distinct M-subpopulations in Europe of which one covers the area of France, Italy, Greece, and Cyprus, while another includes Bulgaria, Czech Republic, Serbia and Slovakia) one explains by no exchange with planting material for the long period of confrontation [57]. Also the areal of these strains is possibly limited by climatic factors at its western edge.

So, the recent results of *Plum pox virus* (PPV) monitoring its show prevalence all over the European Russia territory. PPV population in Russia and probably in European ex-UUSR is characterized by the highest genetic diversity in the world due to wide range of D, M, Rec, W, CR and C strains. Molecular studies of the Russian PPV isolates have already contributed significantly to the knowledge about PPV biodiversity and evolution, though the stone fruit trees in the territories to the east from Volga, in Ural and Altai regions, in Siberia and Far East still remained unstudied with special reference to PPV distribution. Our data show that such investigations of PPV isolates can result in numerous unexpected findings, since in Russia a probability of disclosing new isolates with unknown properties is extremely high. On other side, so broad spreading PPV in Russia is a potential threat for newly bred stone fruit cultivars and further selection and biotechnological works.

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FORMATION OF PLANTS NONSPECIFIC INDUCED IMMUNITY AT THE BIOGENOUS STRESS

(review)

N.N. KARPUN, E.B. YANUSHEVSKAYA, Ye.V. MIKHAILOVA

All-Russian Research Institute of Floriculture and Subtropical Crops, Federal Agency of Scientific Organizations, 2/28, ul. Yana Fabriciusa, Sochi, 354002 Russia, e-mail nkolem@mail.ru
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Abstract

Because of pesticide pollution and violation of protective reactions in biosystems, the ways to increase a nonspecific natural resistance in plants is relevant. For the recent decades the mechanisms of pathogens-to-plant cell interaction were revealed. To identify chemical signals arising in the spots of plant infection by pathogenic microorganisms, the term «elicitor» was suggested (M. Yoshikawa et al., 1993; M. Thakur et al., 2013). Cell innate immunity is based on the recognition of phytopathogenic surface molecules, which is a primary signal for actuating the complicated network, including induction and phytoimmunity regulation (I. Tarchevskii, 2000). During signaling the essential role is played by proteins and small molecule messengers (salicylic acid and jasmonic acid, hydrogen peroxide, nitric oxide). Salicylic acid is involved in amplification and multiplication of the signals coming from the receptors into the plant cells, which ensures the timely activated protection. The earliest plant organism response to the pathogen introduction is a local generation of reactive oxygen species (oxidative burst), triggering a chain of subsequent defense mechanisms (S. Tyuterev, 2002). A significant increase in the level of reactive O_2 and H_2O_2 has an inhibitory effect on the pathogenic microorganisms. The reactive oxygen species (ROS) are also suggested to play significant role in the membrane lypooxidation, cell wall modification and signal transduction (C. Richael et al., 1999; T. Pietras et al., 1997). A key role in ROS regulation is played by an antioxidant defense system, which function is to slow down and prevent intracellular oxidation of organic substances. In this, the antioxidant enzymes (superoxide dismutase, catalase, peroxidase) and low molecular weight antioxidants (ascorbic acid, glutathione, tocopherol, carotenoids, anthocyanins) are mainly involved (S.S. Gill et al., 2010). A defensive effect of peroxidases is due to oxidation of phenolic compounds to quinones (B. Barna et al.; 1995, E.N. Okey et al., 1997). The correlation was found between peroxidase activity in plant tissues and plant resistance to pathogens (T.B. Kumeiko et al., 2009; N. Radhakrishnan et al., 2009). An increase in catalase activity is a defense reaction in cells during the next stages of biotic stress development (F.M. Shakirova, 2001). Starting from reception of signaling molecules of phytopathogens on the cell membrane all metabolic processes are controlled by resistance genes that regulate complex defense reactions (V. Repka et al., 2004). As a consequence, plants produce large variety of substances, carrying protection functions. The main ones are phytoalexins and PR-proteins (Yu. D'jakov, 2012). Due to stress proteins, the enzymes get activated, the membrane stabilization occurs, the activity of mitochondria and chloroplasts increases, and, therefore, the energy level rises (T. Chirkova, 2002). The data summarized herein are the basis for developing new concept for protection of agricultural crops by means of biologicals with eliciting effect that boost plant immune state.

Keywords: elicitors, phytoalexins, genome, resistance genes, salicylic acid, jasmonate acid, peroxidase, catalase.

Long use of ecotoxic pesticides in intensive agriculture resulted in an increased destabilization of agrocenoses and a decreased plant resistance to pathogens [1]. These obviously need to improve cardinally the understanding of plant pathogenesis, and, primary, diagnostics of immune state which determines the course of pathological processes and adequate defense measures [2, 3].

Article provides an overview of the data concerning the main regularities of an induced nonspecific immunity and the mechanisms which make it possible to estimate plant resistance to phytopathogens [4-6]. These results make the base for development of new concept in plant defense with a view to promoting agro-

cenose immunity and environmental improvement due to lowering pesticide load by means of immune-stimulating preparations.

The pathathogenic microorganisms being in touch with plants are shown to excrete the compounds providing infestation of plant tissue. Chemical signals produced in the locus where infection occurs are defined as elicitors [2, 7]. Elicitors are the initial signals and triggers of phytoimmunity induction and regulation [8]. Nonspecific cell immunity in plants is based on recognition of surface molecules of phytopathogens, the nonspecific elicitors [9, 10]. Polysaccharides, proteins, polypeptides, glycoproteins, lipid-containong copounds can serve the biogenic nonspecific elicitors [11, 12]. The polysaccharide elicitors from fungal cell wall are mostly studied. Glucans and chitosans posses an expressed elicitor effect [13]. Interaction between elicitors and the receptors of cell plasmalemma is the firs step in a signaling chain together with cell response to phytopathogen [14]. A possible number of molecular receptors of the same types can reach few thousands per cell thus providing reliable transmission of information from the elicitors of different chemical structure [4]. Presumably, the Ca^{2+} input and the K^- и Cl^- output, the membrane depolarization, NADPH-oxidase activation, and cytosol acidation are involved in signal transmission into the cell [15].

Despite the individual mechanism for recognition of each elicitor, the complex of phosphorilation reactions is commonly involved, resulting in transfer of phosphoric acid residue to inner part of a receptor thus activating enzyme which is associated to [16, 17]. Receptors for all elicitors have the same structure and consist of an outer fragment located out of the cell, an intra-membrane fragment and a fragment located in the cytoplasm. The outer N-end of the receptor is elicitor-specific while the inner C-end possesses the specificity to the receptor-associated enzyme determining choice of a signaling system to be involved in interaction.

Transmembrane signaling from the outer receptors into the cell is one of the main mechanisms of metabolic regulation which area basic for intercellular signaling system [18-20]. In this, proteins and relatively small molecular messengers such as salicylic and jasmonic acids, hydrogen peroxide, nitric oxide, etc., prevail, being functional intermediates between the receptors and cell response manifested in metabolic modifications which result in increased immunity in plants [21]. Necrotrophic pathogens can induce jasmonate signaling pathway [14, 22]. Jasmonates are assumed to enhance the eclicitors' effects as they are an integral part of signaling system [23, 24]. Plant infection caused by pathogenic microflora is accompanied by ethylene production, the hormone important for increasing plant resistance [31].

Biotrophic microorganisms have been shown to induce the salicylate signaling pathway [7, 25]. Salicylic acid well meet the features of systemic signaling molecules, particularly can easy move over the phloem vessels due to physical characteristics perfectly adjusted to distant transport via the sieve tubes [4, 26-29]. Under the influence of pahogens its level rises tens-folds, and it can induce defense mechanisms in plants [30-32]. Salicylic acid is involved in enhancement and multiplication of the signals from receptors into cell thus guaranteeing relevant defense [14, 33]. Besides, a positive influence of salicylic acid on intracellular molecular processes should be mentioned. It participates in transport of newly synthesized proteins into nucleus, chloroplasts, mitochondria and vacuoles. Salicylic acid activates synthesis, protection and restoration of structures of nucleic acids and proteins important for plant viability [4].

Generation of active oxygen species (AOS) is one of earliest plant cell response to the elicitors [34-37]. Of them, the superoxide anion and hydrogen peroxide are od special importance [38, 39]. The main potential sources of AOS are

NADPH-oxidase, peroxidase, amino oxidase, flavin-containing oxidases, polyamino oxidases [40, 41]. AOS are assumed to possess a direct antimicrobial effect and be significant for other defense mechanisms, such as membrane lypooxidation, cell wall modification, and signal transduction thus inducing cell resistance or death from oversensitivity [42, 43].

Activation of oxidative burst is a core component of highly amplified and integrated signaling system [41]. These reactions make a base for formation not only local resistance but also induced systemic immunity due to which plant resistance to microorganisms, fungi and viruses increases [44]. Under optimal conditions the AOS are produced in small concentrations mainly in chloroplasts, mitochondria and peroxisomes [45]. Intensive AOS production in cell is a universal nonspecific response to pathogens as any other stressors such as high temperature, drought, frosts, eco-toxicants [46]. Obviously, H_2O_2 and another AOS can be «double agents», either inducing directly an oxidative stress which results in cell destroying and death, or act as signaling molecules which induce molecular, biochemical and physiological reactions contributing to plant adaptation and resistance [47-50]. Salicylic acid is most powerful inducer of AOS [46, 51].

A key role of AOS level regulation in cell belongs to antioxidant defense system of which the main function is to slow down and to prevent oxidation of intracellular organic matters, to protect biological structures, and to provide detoxication od secondary metabolites [52-54]. During initial defense reaction to stress the AOS are neutralized and the free radical chain is interrupted [55]. In this the superoxide dismutase, catalase and peroxidase, etc., are important together with low-molecular antioxidants such as ascorbic acid, glutathione, tocopherols, carotenoids and anthocyanins [56]. For plant viability under different stresses a balanced AOS generation and degradation is crucial [57, 58], therefore catalase and peroxidase involved in AOS degradation and utilization are important, too [59].

In publications there are special references to multiple roles of peroxidases in plant resistance to phytopathogens [60]. An increased level of AOS in plant tissues initiates peroxidase activation and expression of the genes involved in control of induced systemic resistance. Genes determining peroxidase level in tissue are disclosed in many plant species. Peroxidase activation mainly serves to avoid the adverse AOS effects on cell structures. Peroxidases oxidate phenolic compounds to highly reactive quinones [61, 62]. Peroxidase was shown to enhance antibacterial activity of phenols in the presence of hydrogen peroxide. Particularly, it was reported that plant resistance to phytopathogens increases due to more solid plant cell wall as the lignin synthesis is activated by peroxidase. It was also reported that infection was a «catalyst» of some peroxidases. Their activation results in AOS production and is a defense mechanism together with NADPH-oxidase activity. So peroxidases, being a part of signaling system in plant cells, provide defense response which is adequate to the infection.

A direct correlation is revealed between the activity of plant tissue peroxidases and the resistance to pathogens [63-66]. Special attention is paid to peroxidases as an element of superoxide dismutase system for an elicitor signal transduction which, as a result, determines the character of cell response to infection [67, 68]. Peroxidase catalytic systems are considered the most important of biotic defense factors in plant protection against pathogenic microorganisms [69].

Catalases which control H_2O_2 level in plant tissues are also involved in a defense response [70-72]. Under biotic stresses the catalase activity of pathogen indicates its aggressiveness as it represses AOS in host plant tissues thus decreasing biocide effect of H_2O_2 [60, 73]. At the initial phase of pathogenesis it is neces-

sary to put dawn the catalase activity in plant cells. As a result, the H_2O_2 concentration in the tissues remains enough to kill pathogens. Due to salicylic acid binding catalases the H_2O_2 is accumulated and involved in the immune response [74-76]. Jasmonic acid demonstrates the same activity of catalase inhibiting.

Otherwise, catalase activation is a mechanism of decreasing plant defense potential [77]. As the catalase activity increases, the H_2O_2 level is putting down so that resistance is not developed and, in contrary, the plants become more susceptible to pathogens [78-80]. Since AOS are toxic both to pathogen and the host plant, the intracellular AOS level is controlled stringently by antioxidant system the catalase is a part of [81]. Besides, catalases restrict the AOS lifetime thus preventing their adverse effects in cells. Therefore, an increase in catalase activity is considered a positive defense response toward cell safety when biotic stress is in progress [82, 83]. Though the oxidative stress is an integral part of a developed infection, the enough antioxidant level enables plants to withstand it [12].

All these events are controlled by resistance genes which regulate the defensive mechanisms. Signaling systems and genome are related in two ways. On one side, all enzymes and other proteins are encoded by the genome, on the other side, gene expression or suppression is under control of the signaling systems. These include signal reception, multiplication and transduction to gene promoters, programmed gene expression, control of adequate response in cells, and induced plant immunity to phytopathogen [84, 85]. A number of interrelated metabolic processes are developed due to which the tolerance is expressed and the plant possesses the power to withstand phytopatogenes [86].

Plants can produce a lot of defensive substances, particularly, phytoalexins [12]. To date, there are about 350 these plant antibiotics which are synthesized in response to elicitors [87, 88]. These are mostly lypophilic compounds located around the infected zone. Phytoalexin production is closely related to oversensitivity [89], and, in this, the phytoalexins are accumulated intensively in the necrotized cells where they kill phytopathogens due to expressed antibiotic activity.

PR-proteins related to pathogenesis are synthesized in plant tissue as a response to fungal, bacterial and viral infection [90-92]. For instance, the β-1,3-glucanases can destroy cell walls in fungi, and also the suppressors are blocked due to them. Chitinases are involved significantly in nonspecific induced resistance to phytopathogens. The expression of chitinases and β-glucanases are usually correlates with plant tolerance to biotic stressors.

Reported mechanisms of plant immunity are tightly linked to intracellular metabolic processes of restoring energy which was used for immunization. Due to stress proteins' action the enzyme systems becomes more powerful, the stabilization of cell membranes occurs, and the functional activity of mitochondria and chloroplasts rises, resulting in more energy production [93]. Changes in intracellular metabolism make a biochemical basis for a nonspecific plant tolerance to different stressors [94].

Recently, phytoimmune correction due to the treatment with elicitors used to form reliable immunogenic agrocenoses with high adaptability and reproduction rate is considered promising for cultivated crops [1, 4, 12, 13, 51]. Particularly, in All-Russian Research Institute for Agricultural Microbiology (St. Petersburg) the concept of chitosan-based preparation has been developed. In this concept the chitosan is a nonspecific resistance inducer used together with a few more ingredients. A novelty consists in using signaling molecules to widen the range of defensive reactions, particularly, to intensify the AOS production, and to promote octadecanoid pathway of antibiotic synthesis in plants the same as salicylate pathway. It results in similar efficiency against necrotrophic and biotrophic organisms which attack plants. Different preparations under the Chi-

tozars trade name have been developed for the stimulation of plant tolerance to fungi, bacteria and viruses [14]. In a view of activating natural mechanisms of plant tolerance to biotic stresses, Immunocitofit and Bioduks, the commercial preparations based on arachidonic acid and its derivates, have been developed. These compounds stimulate the phytoalexins production in plant tissues providing increase in tolerance to phytopathogens. Immunity activated by arachidonic acid or the arachidonic acid-based biolgicals is bimodal in character depending on used concentration of the elicitor, so that at low level it causes a long systemic resistance and reliable defensive effect, while only a short local induction occurs when the concentration is higher. Developed immunostimulants are being used widely in crop farming [13].

Studying more detail about the effects of salicylic acid as a key factor of plant immunity resulted in practical recommendations according to its use for the defense stimulation [4]. High stimulating activity is characteristic to Albit, the preparation of poly-β-butyric acid. It induces a systemic resistance to wide range of diseases. After the treatment with Albit, significant biochemical and physiological changes are observed due to induced immune response. Particularly, the peroxidase activity and salicylic acid level rise reliably. Cytological study reveals a significant increase in the number of mitochondria in protoplasts. After sensitization the polymorphic leucoplasts are detected around the nucleus, and the production of carotenoides, terpenoids and phenols increases in the tissues. Besides, Albit suppresses diseases due to promoting high immune state in plants for a long time [51].

A special feature of the inducers is their ability to sensitize the plant to further infestations. Activation of tolerance mechanisms along with specific physiological and biochemical impact on cell nucleus determine the rate and the type of cell response. This event is «recorded» in epigenetic programming thus providing prompt response under repeated attacks of the pathogen [12]. Moreover, at least 2-fold decrease in pesticide level must be regarded too as a significant positive effect of elicitors which contribute to improvement of environmental condition and functions of agro-ecosystems.

Thus, a concept of nonspecific induced immunity in plants is quite well developed. According to this concept, the mechanisms involved in plant immunity formation are i) the interaction between signaling molecules produced by phytopathogen and the receptors in plant cell membrane; ii) signal transduction through intercellulat signaling system; iii) expression of resistance genes which activate the defense mechanisms, such as oversensitivity, synthesis of key enzymes, signaling molecules, proteins, phytoalexins. Also the levels of salicylic and jasmonic acids, phytoalexins, peroxidase and catalase activity in tissues must be assayed to estimate plant response to the biogenic stress. Due to eliciting biologicals and optimized plant protection measures it is possible to boost immune state of plants thus making agrocenoses environmentally friendly.

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RESERVES OF AGRO-TECHNOLOGIES AND BREEDING FOR CEREAL YIELD INCREASING IN THE RUSSIAN FEDERATION

V.P. YAKUSHEV, I.M. MIKHAILENKO, V.A. DRAGAVTSEV

Agrophysical Research Institute, Federal Agency of Scientific Organizations, 14, Grazhdanskii prosp., St. Petersburg, 195220 Russia, e-mail yakushev@agrophys.ru, ilya.mihailenko@yandex.ru, dravial@mail.ru

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Abstract

The authors consider new approaches to cereal yield increasing by relevant agro-technologies and breeding methods. Earlier it was shown that the yield of agrophytocenosis is determined by seven genetic and physiological systems, the GPS (A.B. Dyakov and V.A. Dragavtsev, 1994), the breeders are de facto faced to increase the crop yield. There was the first genotyping of each GPS of productivity which showed the ways to optimal strategy of yield management via GPS. In this paper for the first time it is shown that effect of some systems can be increased both by agro-technological and breeding methods. In the future the agro-technologies should be improved by the development of precise agriculture. In this, it is necessary to provide i) monitoring of phytocenoses, ii) indication of limiting factors for each stage of ontogenesis which impact the traits starting their development, iii) development of new agro-methods precisely influencing the phytocenosis by biologicals which can level the negative effects of specific limiting factors. In other words, «phase-specific agro-technologies» must be developed to protect sensitive phases of plant ontogenesis. To obtain maximal effect from precise breeding technologies, it is necessary to create mathematic models allowing to estimate quantitatively haw each GPS contributes to yield increasing at each stage of ontogenesis. Moreover, these models should be further used both in classical field breeding and, most effectively, in special phytotron where the dynamics of environment limiting factors typical for the territory of interest can be simulated. On the base of first created models the important quantitative algorithms for breeders were suggested which provide rapid identification of individual genotypes by their phenotypes and optimal selection of the parents to predict and obtain the transgressions (for self-pollinated plants). In this paper it is shown that, though certain GPSs can be improved both by agro-technologies and breeding, there are some traits which can be changed only by breeding. Analysis of unique breeding results of V.S. Pustovoyt, P.P. Lukyanenko and Norman E. Borlaug showed the GPSs improved by these great breeders and those ones unimproved. Breeding and agro-technology programs for each GPS must result in further yield increase. The paper summarizes the perspectiveness of hormones used during different phases of ontogenesis to deepen roots into soil, to increase attraction and to improve stress tolerance. Agro-technologies and breeding technologies must work together, because the high genetic potential of newly bred varieties can not be realized with improper agro-technology, and, in contrary, the best agro-technology will be limited by low potency of the variety. Modern agro-physical equipment (in particular, special phytotron accelerating breeding process), IT-technologies, mathematical modeling used to control crop yields are the main factors for optimal teamwork of agro-technologists and breeders with the view of effective agriculture in Russia.

Keywords: agro-technologies, breeding, reserves of cereal yield increase in Russia.

Yet 100 years ago, the farmers of the world used the following levers to increase the gross output of crop production: plowing new land up; optimized location of genera, species and varieties of agricultural plants in macro, meso- and micro-ecological niches; improvement of agricultural technologies; breeding new varieties and hybrids using genetic breeding technology; creation of variety, species and genera mixes to maximize the use of environmental resources by complex agrophytocenoses. Currently, the first and second approaches have almost exhausted their possibilities. There are almost no new lands left, optimizing

the placement of cultures on the territories has been finished in general (it was a long empirical process of «trial and error»), and just the system of variety zoning is continuously running. According to recent data, the ratio of contribution in the yield increase due to improved agricultural technologies and breeding technology development in the different zones of the Russian Federation is from 50 %:50 % to 30 %:70 %. Creation of variety, species and genera mixes is highly developed, for example, in Brazil, where up to 80 % of the area are seeded with genotype mixtures; in Russia, this approach is just beginning to develop, but it is experiencing difficulties due to the lack of new machinery modifications and special systems for monitoring and maintenance of complex agrophytocenoses.

A global crisis of agricultural production arose in the 21st century because technological intensification of crop production could not solve the problem of further yield increase but was associated with the growth of energy costs and ecological imbalance. Solving the problem of hunger and malnutrition of the growing population of the world and reduction of the negative impact on the biosphere are the two main problems of the contemporary agricultural sector. Therefore, identification of the reserves of inexpensive, environmentally safe and accurate agricultural technologies, as well as opportunities to radically improve crop breeding, is very important.

Priority Russian phenotyping (subdivision of the final yield into its seven contributions as a result of functioning seven genetic and physiological systems) and presented approaches to improve the efficiency of each system by agrotechnological and breeding methods for the first time demonstrate the quantitatively assessed significant potential for increasing crop yields in the Russian Federation.

New approaches to the estimation of reserves of agricultural technologies and breeding technology in solving problems of increasing yield. The effectiveness of the above five «arms» to increase yields has not been assessed in a rigorous manner so far. This can be assessed based on the currently available knowledge about the prospects of agricultural, genetic and breeding techniques which contribute in the yield improving.

A.B. D'yakov and V.A. Dragavtsev have shown [1] that the yield of agrophytocenosis is determined by the following genetic and physiological systems (GPS): Attraction (ATTR, System 1), or redistribution of photosynthesis products and elements of mineral nutrients from the stem and leaves to the centers of attraction, i.e. the ear (in cereals), the flower basket (in sunflower), etc.; microdistribution of attracted plastic substances (MIC, System 2) between the grains and the chaff in the wheat ear, between the seed core and peeling in sunflower, etc.; adaptability (AD, System 3) as total adaptation to the specific conditions of field and trials year, or specific adaptation in case of provocative background, such as drought, cold, heat, salinity, changes in soil pH, etc.; horizontal immunity (IMM, System 4) as a horizontal (polygenic) resistance to diseases and pests; «payment» by plant dry biomass for the limiting factor unit in soil nutrition (EFF, System 5), i.e. nitrogen, phosphorus, potassium, etc.; tolerance to thickening (TOL, System 6) as the ability to withstand a high density of planting and maintain a large number of ears per 1 m^2 of the area; genetic variability for the duration of ontogeny phases (ONT, System 6).

This experimentally tested approach described back in 1994 [1], in fact, has become the priority Russian phenotyping (phenotyping is a recently arisen line of research abroad that attempts to divide the final phenotype signs that formed during growth and get to the primary elements of productivity that are related more closely with the gene products than the resulting complex proper-

ties of productivity and yield) [2-6].

Domestic phenotyping appeared to be significantly more effective compared to foreign approaches [7, 8]. However, it contained no information on how to increase the efficiency of GPS based on agro- and/or breeding techniques.

Analysis and experiments have shown that the efficacy of the contribution in the yield of the systems 1, 2 and 3 can be increased by both agricultural and genetic breeding techniques, and of the systems 4, 5, 6 and 7 can be mainly increased by genetic breeding only.

What are the provisions to increase yield while improving the agricultural and breeding techniques? Let us consider the summary table of the cereal areas of the world, represented by S. Borojevic [9] in a special lecture at the International Congress of Genetics (Moscow, August 21-31, 1978).

Average and record productivity values in the agricultural production of the major cereal regions of the world [9]

Crop, production	Yield		Record to average value ratio
	average	record	
Corn, cwt/ha	44.4	189.4	4.3
Corn, cwt/ha	18.8	145.2	7.7
Wheat, cwt/ha	16.1	73.9	4.6
Sorghum, cwt/ha	18.6	197.4	7.1
Oat, cwt/ha	17.2	106.3	6.2
Barley, cwt/ha	20.4	114.1	5.6
Potato, cwt/ha	282.2	940.8	3.2
Sugar beet, cwt/ha	469.1	1,333.0	2,8
Milk (annual yield), kg/head	4,635	22,500	4.9
Chicken egg (1 laying hen), pieces/year	230	365	1.6

As is evident, the wheat and sorghum yield, in principle, can be increased by more than 7 times by improving agricultural technologies and genetic improvement of breeding assortment.

Prospects for the development of precision agrotechnologies and their possible contribution to improving yields. In 2007-2008, the precision farming technique saved about 20 % of fertilizer and ensured a 15 % yield increase on spring wheat (Menkovskaya Experiment Station of Agrophysical Institute — API) compared to conventional technology. Under the conditions of the North-West of Russia, the yield reached 60 kg/ha [10]. This was one of the first steps in mastering precise techniques of differentiated mineral fertilizer application. Fractional application of nitrogen fertilizers (with dose and treatment timing optimization) should be the next step. It is necessary to optimize the regimes of the use of fertilizers and ameliorants that are effective for several years in several crops in the rotation. To solve the above problems, reliable information on the status of crop and soil is required which will be provided by the accelerated development of mobile ground-based measuring instruments and more extensive use of optical and radar equipment for remote sensing [11]. The use of precision technologies such as delicate controls of the state of crops, biological preparations and hormones, required greater differentiation in the treatment with liquid and loose agrochemicals. Design and construction of such machines have been designed and patented in API [12]. In general, all of the above areas of high-precision technology development can become the basis for increasing yields by 20-25 % and more and fully developing the productive potential of varieties created using contemporary breeding and genetic methods.

Speaking of precision technology, we should not lose sight of the underlying innovative agricultural practices. Thus, K.G. Alimov, discovering the most sensitive phases of development during the growing season of crops and influencing plants by technological means, has achieved the roots going deep and

consuming moisture from a depth of 1 meter. He used the well-known works of plant physiologists to stimulate root growth [13] which showed that with the water deficit in the soil, roots begin to move to the deep moist horizons. Deepening is stimulated by abscisic acid (ABA) the synthesis of which starts in plants during drought, inhibiting the growth of the aerial part but contributing to the development of the roots. If the crop is additionally treated with the ABA hormone in the appropriate phase (prior to the formation of the «number of grains per ear» trait) the root deeping is more intense. Nutrition optimization (manipulation by fractional application of nitrogen, effect of growth regulator and fungicide in the phases of ontogeny) results in the formation of 90-98 grains per ear instead of the usual 15-20 grains in the spring-summer drought in Siberia. At this, productive tillering remains not great (no more than two ears), which makes it possible to harvest crops before the snow. The unfertile stem ears are known to ripen 1 week later, and if the agronomist is waiting for their ripening (and they provide up to $^2/_3$ of the harvest), the field may go under the snow. This original technique is limited to the precise monitoring of developmental phases and adherence to the timing of the operations by the stages of organogenesis in accordance with them, i.e. it does not require any additional cost. Unfortunately, the assessment of the crop status is performed by eye, it is necessary to improve and gradually turn it into a fine instrumental estimation. In 1990, in the work-study unit NGAU Tulinskoe (Novosibirsk Region), K.G. Aliyev's technique provided 62-76 cwt/ha in the spring wheat and 62-63 cwt/ha in the spring varieties Kantegirskaya 89 and Lutescens 70 that emerged from the cooperative Siberian DIAC (diallel crossing) program. About 70-74 cwt/ha of the spring wheat have been produced for 4 years at the Ekaterininskaya station of the Institute of Plant Genetic Resources (VIR) (Tambov Region). In the field seasons of the 2000s, the spring wheat variety Esther provided yields of up to 75 cwt/ha in Ul'yanovsk Region. Under extreme drought, this technique provided yields of 30-35 cwt/ha in 70 % of experiments, while in the adjacent fields, where the conventional technique was applied, 10-12 cwt/ha only were produced.

These facts illustrate the improvement of drought resistance (due to artificial deeping of active roots into the moist soil horizon) resulting from technological methods. Of course, the depth and shape of the root system can be improved by genetic and breeding methods. Thus, in the drought-resistant Kazakhstanskaya 10 spring wheat variety of V.V. Novokhatin, R.A. Urazaliev, the originators (V.R. Villiams Kazakh Institute of Agriculture, Kaskelen, Almaty Region, Kazakhstan), the root system penetrated to a depth of 2.42 m in bogharic lands. Currently, intensive work is underway on the breeding management of the depth and shape of root systems starting with the phase of seedlings [14].

As mentioned above, fractional fertilization is of great importance. The same small fertilizer ($N_{30}P_{45}K_{45}$) dose was applied under spring wheat in two ways in the experiment performed by V.V. Kuznetsov and G.A. Dmitrieva [13]. These were the application completely or partially in the soil at plowing ($N_{15}P_{30}K_{30}$) and partly in the rows at sowing ($N_{15}P_{15}K_{15}$). In the second variant, as early as in the tillering stage, the total and active absorbing root surface increased by 1.5 times, and the grain yield increased by 1.5 times finally [13, p. 424].

There are real opportunities to improve the efficiency of attraction not by breeding methods only, though, as noted by V.V. Kuznetsov and G.A. Dmitriev, their effectiveness is high enough to made it possible to increase the percentage of grain to the total dry matter weight from 24 to 47% in corn and from 43 to 57 % in rice due to reached redistribution of assimilates (attraction gain) [13, p. 334]. According to them, under the technological impact on attraction intensity

with hormonal treatment, the influx of sugar to the flowers increased 5 times, the influx of nitrogenous substances increased more than 2 times in 5 days after spraying tomatoes with 2,4-dichlorophenoxyacetic acid (2,4-D) [13, p. 331]. The authors note that assimilates move in the direction of higher hormone content since it is the hormones that create the attracting zones, regulate ATP synthesis and the H^+ pomp [13, p. 331]. The research on ear treatment with kinetin prior to the grain filling phase is very promising to enhance the attraction. At attraction, a competition for plastic material arises between the stem and the leaves. Healthy leaves prevent the outflow of assimilates to the ear. V.F. Altergot (Novosibirsk) proposed to treat the fields from aircrafts in the phase of the beginning of grain filling with senicats, the cheap minerals accelerating leaf aging. At senication, weakened leaves gave earnings the stock of plastic substances easier, and the yield increased by 4-5 cwt/ha. This aviatechnological method was widely used in the fields in Siberia in 1960-1970s, until the varieties with genetically enhanced ear attraction (including the ones resulting from the DIAC program) have been created.

Prospects for the development of breeding agrotechnologies and their possible contribution to improving yields. As noted by S. Borojevic, harvests once considered a record, became average in 20 years. It is known that as a result of breeding, the yield of soft winter wheat varieties has increased in Kuban by 44.9 cwt/ha, or 218.8 % since 1930 [15]. According to L.A. Bespalova, the yield of the winter wheat varieties created in the P.P. Lukyanenko Krasnodar Research Institute of Agriculture (Krasnodar NIISKh) sometimes reached 130 cwt/ha in 2013 (against the basic technology background). According to the information from the Head of the Tyumen Selection Center V.V. Novokhatin (personal communication, 2014), the yield of the recently created spring wheat variety Icarus averaged 45 cwt/ha and up to 75 cwt/ha with fertilized fallow (with basic technology) within the last 6 years in the area of several thousand hectares; the yield of the Tyumen zernokormovoy variety of triticale produced by V.V. Novokhatin is currently up to 120 cwt/ha in Tyumen Region.

There was a unique Przewalski variety test plot in the USSR Gossortoset system (at the eastern end of the Issyk-Kul lake in Kyrgyzstan). There, the yield was always 80-90 cwt/ha in all spring wheat varieties from Western Siberia with watering indicating quite normal systems of photosynthesis, respiration, energy transfer, and all ontogeny processes. In Siberia, the same varieties yielded 11-18 cwt/ha which clearly indicates their poor resistance to typical Siberian spring and summer drought which reduces the number of grains per ear and reduces the weight of 1000 grains against under the autumn cold. Today, various types of drought are the main lim-factors that limit the crop yield in the Russian Federation [16].

Genetic and breeding efforts to improve the drought tolerance of cereals have not resulted in serious findings for many years because the drought tolerance is a very complex multi-property feature formed by more than 20 traits; each of the traits is defined by tens and even hundreds of genes. It is almost impossible to find the «big» Mendelian gene which dramatically increases drought tolerance. To genetically enhance drought tolerance, other approaches based on the ecological genetics of quantitative traits are required. Most modern conventional and molecular geneticists tend to operate within a genetically centered paradigm, that is, continue to search for specific genes of drought tolerance, which is hardly promising. At the same time, there are more adequate systemic approaches to improve drought tolerance [17]. Poor genetic drought tolerance of modern cereal varieties results in annual losses to the economy of the Russian

Federation in the amount of 5.7 billion rubles.

Analysis of unique achievements in breeding. Let us consider the mechanisms for obtaining unique breeding results from the standpoint of the seven above-mentioned genetic and physiological systems.

Thus, V.S. Pustovoit increased the yield of sunflower oil from 20 to 55 %, which was not only an outstanding domestic but also a global achievement in breeding. The mechanisms of this revolutionary success have been carefully studied by A.B. D'yakov and V.A. Dragavtsev [18]. At the first stages of his research, V.S. Pustovoit estimated the percentage of oil per the achene core weight unit in various varieties using a special device purchased in the United States. He failed to find a significant breeding genetic diversity for these traits and even questioned the possibility of increasing the yield of oil through breeding. Then, it has not been known yet that (as in cultivars) oil content per the achene core cell is the same in wild forms (if the amount of oil per cell is increased, the cell just dies). However, he did not notice an interesting fact of a much greater yield of oil with 1 kg of seeds in thin-peeling varieties compared to thick-peeling ones («crunchable»). V.S. Pustovoшt understood that with the selection for the seed peeling thickness reduction (this meant lignification of a small number of outer layers only), the inner peeling layers that remain alive, increase the number of cells in the achenes core and consequently increase the yield of oil per flower baskets (or per plantation area unit). The peeling thickness can be felt by tongue, so the need for expensive American equipment disappeared. Thus, a unique breeding result was obtained by V.S. Pustovoшt mainly due to the MIC system. His associates used the IMM system as well, and managed to significantly enhance the immunity of varieties-populations. At the same time, the ATTR, AD, EFF, TOL, and ONT systems were not improving genetically (since no one worked on them) indicating substantial prospects for further breeding improving of sunflower.

The American Nobel Peace Prize winner Norman Ernest Borlaug, who worked in Mexico, made the first «green revolution» in the world (mainly with wheat) creating stunted (short-stem) varieties that did not lodge with high doses of nitrogen and abundant moisture (brought by irrigation and sub-tropical and tropical rains). He used only the «big» Mendelian genes of the short stem (and sometimes of dwarfism) but he never worked with the ATTR, MIC, AD, IMM, EFF, TOL, and ONT systems. Consequently, there are significant prospects for the second «green revolution», not only for wheat, but also for other genera and species of agricultural plants.

A famous scientist and breeder P.P. Luk'yanenko started his research at the Krasnodar Research Institute of Agriculture carefully studying the «bottlenecks» of the production process in the Kuban wheat winter varieties. According to L.A. Bespalova [19], P.P. Luk'yanenko conducted a thorough analysis of the factors that limit the yield of wheat in the Kuban. He was the first to realize that the reasons for low yields of winter wheat were brown rust, lodging, lack of early ripeness, and low ear productivity (add poor winter hardiness). The lack of early ripeness due to grain filling accounted for the unfavorable July heat was changed by genetical shifting the phase of filling to June. Selection for winter hardiness was carried out in special concrete containers (troughs) placed above the surface of the soil to ensure stronger root freezing in the mild winters compared to the soil. Mendelian genes of rust resistance were introduced in the varieties. In this way the Bezostaya 1 variety, a masterpiece of world wheat breeding, was created. Its yields immediately exceeded all the Kuban winter varieties by 10-15 cwt/ha. Obviously, P.P. Luk'yanenko perfected the AD, IMM and ONT systems, but the ATTR, MIC, EFF, and TOL

systems were not improved in the creating the Bezostaya 1 variety.

The following radical increase in the yield of winter wheat in Kuban took place in 1980-1990-ies when Yu.M. Puchkov and L.A. Bespalova began to increase tolerance to thickening (TOL) by breeding methods. While the Bezostaya 1 variety is capable of supporting about 200 ears per 1 m^2, this value is 600 and 800 ears in the Spartanka and Skifyanka varieties, and 1,000 ears in the Kroshka variety created by L.A. Bespalova, with the yield gain reached 10 cwt/ha compared to the Spartanka variety. Thus, genetic improvement of only one of the seven systems (MIC in sunflower, TOL in winter wheat) may result in a drastic increase in yields.

The improvement of breeding and agricultural technologies. The models that make it possible to quantitatively describe strictly the contributions of all seven systems to increase yields were created in API [20-22]. The algorithms for the identification of genotypes and phenotypes and optimal selection of the parental pairs important for successful breeding were proposed based on these models. The next step is creation of special computer programs for automatic incremental implementation of the algorithms to optimally select the pairs for crossing, predict transgressions (and heterosis effects) and the algorithms for accurate identification of genotypes (by the productivity and yield components).

By 2013, in the API the development was completed of the priority theory of ecological and genetic organization of quantitative traits (TEGOQT) based on the discovery (1984) of a new epigenetic phenomenon. It was a change of the spectrum and the number of genes that determine any component trait of productivity under the changing environmental factor (lim-factor) that retards the growth of plants. 24 selection important consequences and nine breeding know-how were derived from this theory and experimentally studied [23-25].

Back in 1984, the first time in the world science, based on the great experimental material of the DIAC program, rigorous calculations of the possible breeding increase of the spring wheat yield in the territory from the Urals (Krasnoufimsk) to Transbaikalia (Ivolginsk) (along the parallel), and from Tyumen to Ust-Kamenogorsk (along the meridian) were made [26]. According to mathematical calculations, the limits of the genetic potential of yield increase (GPYI) variation versus the best variety in the sample of 15 varieties and 225 hybrids were 21-60 % (average of 41 %) between eight geographical points. Quantitatively, GPYI averages 8.5 cwt/ha with the average yield of the whole area of research of 21 cwt/ha. This potential can be rapidly realized in a breeding phytotron only with simulating the typical dynamics of various lim-factors for any region selecting parents for priority API algorithms and using ultra-precise methods of genotype identification by phenotype.

However, the above examples (i.e., search and evaluation of the years typical in the dynamics of the main lim-factors in a given geographical point for breeding and precise genotype identification by the maximum «genotype—environment» interaction effect by the component and resulting productivity traits against the background of the typical lim-factor dynamics) are only two possible approaches to ecological and genetic improving of productivity. The third area involves elimination of the effect of environmental lim-factors due to the stress AD systems involved in the critical phases of ontogenesis. This lever is capable of raising the yield by 30-40 %. Finally, the fourth possibility is to overcome the effect of limits in the daily dynamics of physiological processes of growth and development. The extension of their active state for 2 hours per day alone will provide an increase in the vegetative biomass equal to that of a late (9 days later) variety within 100 days of vegetation, and that is about 20-30 %. It is known

that while one corn hybrid ripens just 10 days later than the other, the productivity of dry biomass may increase 2 times over this period compared to that accumulated by this hybrid plants by the time of the earlier one's ripening. The total potential ecological and genetic improvement of crop yields for spring wheat by four innovative breeding techniques in the extreme zone of Western Siberia is 50-70 %, and in the European part of Russia it is 70-90 %. Again, the implementation of these objectives should be based on new breeding techniques, with the most important element of the breeding phytotron.

Currently, the work is under intensive development to improve the agricultural and breeding technologies in the following areas: agrophysics, ecological genetics of quantitative productivity traits, plant physiology and biochemistry, phenotyping, ontogeny genetics, plant genetic resources, etc. [27-35].

Thus, we have found seven genetic and physiological systems (GPS) earlier, namely attractions, micro distribution of attracted plastic substances, adaptability, horizontal immunity, «payment» by plant dry biomass for the limiting factor unit in soil nutrition, tolerance to thickening, and genetic variability for the duration of ontogeny phases. Breeders and agricultural engineers have never really worked to improve each system separately, although this approach can provide the maximum increase of productivity. Some of these systems have been shown to be possibly improved through both agro- and breeding techniques, while the other part can be improved through the breeding methods only. Analysis revealed those GPS that have been perfected by the great breeders who have obtained outstanding results, and made it possible to understand what GPS have not been genetically improved, and therefore, retain the potential to increase yields in the future. The impact of hormones on the different phases of ontogenesis may be considered a promising area to manage the deepening of root systems, increase attraction and increase plant tolerance to stressors. It is important to note that the considered breeding and agro-technical reserves of the cereal yield increase should naturally complement each other, but not to be opposed to in any case: To implement a high crop genetic potential achieved by breeding methods, enhanced precision agricultural techniques are required, and vice versa, the most advanced technological methods may not give the desired result due to limited genetic potential of varieties. To combine agrotechnological and breeding reserves in solving the problems of agricultural sector, the creation of appropriate agrophysical instruments and equipment, construction of breeding phytotrons, development of information technologies, means of mathematical modeling and control of the processes of productivity and yield formation are required.

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BIOTECHNOLOGIES AND MOLECULAR METHODS IN VEGETABLE CROP BREEDING (to 95th Anniversary of VNIISSOK)

N.A. SHMYKOVA, T.P. SUPRUNOVA, V.F. PIVOVAROV

All-Russian Research Institute of Vegetable Breeding and Seed Production, Federal Agency of Scientific Organizations, 14, ul. Selektsionnaya, pos. VNIISSOK, Odintsovo Region, Moscow Province, 143080 Russia, e-mail shmykovanat@mail.ru

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Abstract

The resource consumption and costs for an increased agricultural production can be lowered only due to effective breeding for which biotechnologies and molecular genetics provide powerful tools. Among them, tissue cultures, clonal micropropagation, androgenesis, gynogenesis, and genetic transformation are widely used to obtain diversified forms and homozygous constant lines, and to speed up breeding process (J.M. Dunwell, 2010). Androgenesis and gynogenesis involve individual gametoclonal variability, rare recessive alleles, and unique genetic recombinations into breeding (T. Winkelmann et al., 2006). An interspecific hybridization is a way to develop initial breeding material. Due to it, novel traits (for example, resistance to biotic and abiotic stresses) can be transferred from wild species to cultivated crops, and the range of genetic variability expands (R. Hajjar et al., 2007). An interspecific hybridization incompatibility can be overcome by biotechnological methods, too. Molecular markers are helpful for detecting DNA changes and desired genes' introgression from one genotype to another, for germplasm fingerprinting, gene mapping, etc. Marker-assisted selection is powerful facility to maintain germplasm collections, to plan crosses, to predict useful gene combination, and to protect varieties and hybrids authenticity. In the review we summarized the results on biotechnology, molecular genetics and their practical use in All-Russian Research Institute of Vegetable Breeding and Seed Production (Moscow Province). In particular, onion (Izumrudnii, Sigma, Zolotye Kupola, Tseparius), salad (Izumrudnii, Tvorets, Aleks, Korall, Malakhit), and physalis (Lakomka, Desertnii) varieties were recently obtained by distant hybridization. Carrot and wild aubergine are involved into interspecial crossings, and pepper plants as sources of resistance to viral infection are selected. Microclonal protocol has been developed for unlimited in vitro propagation of male sterile cabbage. Clonal micropropagation and in vitro cultivation were used to provide embryo viability under interspecial hybridization. A novel protocol developed for in vitro cell cultivation made it possible to create double haploids in carrot and pepper. Due to optimized conditions, double haploids in rape, Chinese cabbage, broccoli and white cabbage were selected. Double haploids of carrots, onion, cucumber, pumpkin, sugar beet, etc., were derived from in vitro cultivated seed-bud without pollination. In this, the ISSR, IRAP, AFLP и SSR markers are involved to assess genome variability and genotyping in vegetable crops.

Keywords: interspecific hybridization, clonal micropropagation, androgenesis, gynogenesis, molecular markers.

The Gribovskaya Vegetable Breeding Research Station founded in 1920 was transformed into the All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops (VNIISSOK) in 1971. Hear, within 95 years, consistent formation of vegetable breeding on a scientific basis was in process in close cooperation with the development of life sciences in the world. At the beginning of the last century, N.I. Vavilov noted in his writings that the selection as a science is a synthesis of the data of many disciplines and linked to genetics, systematics, embryology, cytology, ecology, biochemistry, physiology, and technology [1]. At the Gribovskaya station, in 10 years after its foundation, new theoretical laboratories were established: the Laboratory of Physiology and Bio-

chemistry in 1931, and the studies of plant resistance to disease started at the same time, Laboratory of Cytology in 1933, Laboratory of Plant Protection in 1943, Laboratory of Genetics and Cytology, on the basis of the Laboratory of Cytology, in 1972.

The most important method of enriching the gene pool of plants is distant hybridization, through which valuable traits are transmitted from wild species to cultured ones. This direction is given much attention in world breeding practice. Over the past 20 years, about 100 beneficial traits have been transferred from 60 wild species to various crop varieties [2]. As a result of the research on distant hybridization started at the Gribovskaya vegetable experimental breeding station in 1930, the first forms of onion interspecific hybrids of *Allium cepa, A. fistulosum, A. vavilovii, A. oschaninii, A. schoenoprasum,* and *A. nutans* were created [3-6]. The varieties of Emerald, Sigma, Golden Domes, Tseparius, highly resistant to peronosporosis and stably high yield were obtained on their basis [7-9]. Interspecific hybridization of the *Lactuca* genus made it possible to isolate the original forms with new morphological characteristics and resistance to *Alternaria*, on the basis of which the lettuce varieties of Emerald, Creator, Alex, Coral, and Malachite were created [10]. The Gourmand and Dessert varieties characterized by resistance to abiotic stressors, by high content of sugar, vitamin C, pectin, and the lack of bitterness have been obtained in VNIISSOK from the interspecific hybridization of tomatillo *Physalis longifolium* (non-alkaloidal small-fruited form) and *Ph. angulata* (low-alkaloidal large-fruited form) [11-12]. In the studies on involving wild aubergine species in breeding for the transfer of resistance to abiotic stressors, interspecific hybrids of *Solanum melongena* L., *S. integrifolium* L. and *S. aethiopicum* L. have been obtained in vitro using the method of embryo culture; the offspring has been assessed by morphological and economically valuable traits, the promising samples have been selected [13, 14].

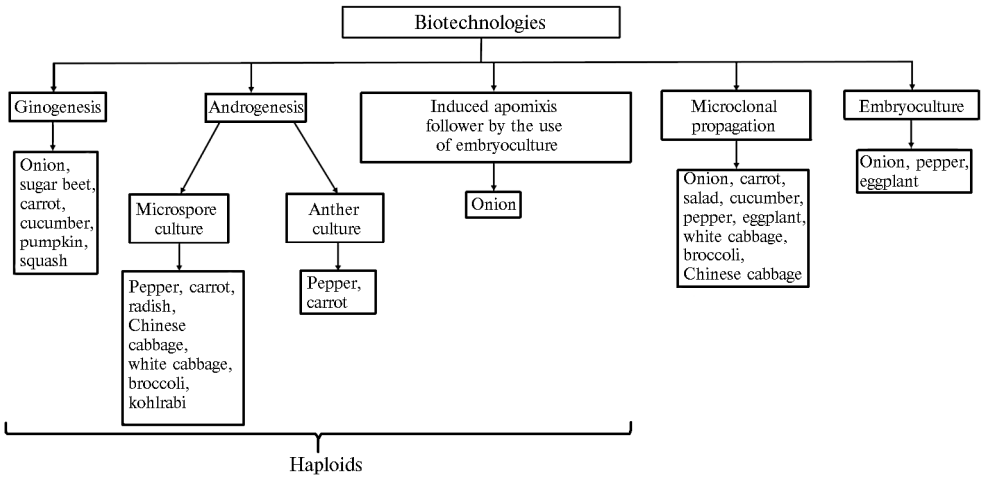
A method of producing pepper source material resistant to viral diseases has been developed using the interspecific hybridization. The *Capsicum annuum* L., *C. frutescens* L. and *C. chinense* L. species have been involved in hybridization. In this, both the classical techniques of breeding and evaluation of the resulting material, and biotechnological techniques (e.g., in vitro culture of isolated embryos to overcome the incompatibility) and modern molecular genetic approaches (i.e., molecular control for R-resistance genes in wild species, and interspecific hybrids using the RGA-marking) were applied. As the result, the pepper, lines resistant to the tomato spotted wilt virus (TSWV) were obtained: L-(Health × *C. frutescens*); L-(Health × *C. chinense*); L-[Chimes × (*C. annuum* × *C. frutescens*)]; L-(*C. annuum* × *C. chinense*); Л-[*C. annuum* × *C. frutescens*) × Health] [15] (the research was supported by the GK № 1282/13 grant from the Ministry of Agriculture ofthe Russian Federation). Studies on the interspecific hybridization of carrots are being performed in VNIISSOK within the past 20 years. Among the hybrid offspring of the combinations of crosses *Daucus carota* × *D. hispidifolius*, *D. carota* × *D. gi-ngidium*, *D. carota* × *D. carota* ssp. *libanotifolia*, etc. the forms with a new combination of high resistance to *Alternaria*, intense orange root-crops and other features of cultural carrots have been selected [10].

The development and evaluation of interspecific hybrids is not impossible without cytogenetic studies, which are conducted in VNIISSOK with the involvement of high-performance methods of hybrid form diagnostic (genomic in situ hybridization, GISH; fluorescence in situ hybridization — FISH) [16, 17].

One of the most important purposes of today's selection is the fast achievement of constancy of breeding material, which is especially important when creating heterosis hybrids that require homozygous lines with high combin-

ing ability. Usually, these lines are obtained with prolonged inbreeding (5-10 generations), but modern biotechnological approaches can almost twice reduce this process. Among the methods of cell technology, the most demanded ones are clonal micropropagation, androgenesis, gynogenesis, and genetic transformation that are widely used in agricultural programs to obtain diversified forms, create constant lines with the specified characteristics, and to speed up the breeding process [18].

The methods of androgenesis and gynogenesis can realize the individual gametoclonal variability in individual plants, detect rare recessive alleles, and create unique forms. Clonal micropropagation subject to high rates of reproduction and preservation of genetic stability can be included in the selection process and occupies an important place in the world crop practice [19]. In the breeding of vegetables it is especially important to maintain the male-sterile or self-incompatibility plants.



Biotechnological methods used in the creation of agricultural varieties in the All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops (Moscow Region)

The in vitro tissue and cell culture techniques (Fig.) have been actively developing in VNIISSOK since the end of the 1980s, the start for the research was given by obtaining the virus-free seed garlic in the meristem culture [20]. The use of improved seed garlic increases the yield (70 % maximum growth) and is widely used throughout the world [21]. Virus-free seed stachys and yakon were also obtained through meristem culture with heat treatment and clonal micropropagation using antibiotics [22, 23].

In the Institute the range of vegetable crops under biotechnological research increased over time. The methods of cell technology were improved. Thus, cauliflower clonal micropropagation using perifloral meristem has been optimized [24]. In world practice, the first study on clonal micropropagation of cabbage crops started at the beginning of the 1970s, however, the achievement of a high rate reproduction in vitro failed. Effective protocol for clonal micropropagation of Chinese cabbage, broccoli and rape were developed in the last decade only [25-27]. Microclonal protocol has been developed for unlimited in vitro propagation of male sterile cabbage in the Laboratory of Biotechnology of VNIISSOK [28] with support by GK № 566/13 grant from the Ministry of Agriculture of the Russian Federation. Clonal micropropagation may become the basis for many cell technologies. Thus, in aubergine and pepper, our clonal micropropagation technology [29, 30] was used for embryo cultivation to provide embryo viability under interspecies hybridization [31].

Producing doubled haploids (DH-technology) makes it possible to

quickly create homozygous lines and speed up the breeding process. By 2010, about 300 varieties have been produced in the world using the DH-method [32]. However, this approach was less effective in vegetable crops, so now they have become the objects for the experiments on the induction of haploid embryogenesis [18, 33, 34]. First doubled haploids in carrot and cabbage in anther culture were obtained in the Laboratory of Biotechnology of VNIISSOK in the early 1990s [35, 36]. The cytological study of embryogenesis in the anther culture of carrot has identified the regularities of embryoid formation of microspores, and the change in ploidy has been shown to start in the early stages of primary embryoid development [37]. Later, cultivation of carrot anthers was optimized, doubled haploid variety samples of different origin were produced (NIIOH 336, Vitamin, Moscow Winter A-515, Losinoostrovskaya 13, Leander, Shantane 2461, Nape, Rondo, F_1 hybrids Karatan and Calisto, etc. The obtained regenerated plants possess gametoclonal variation which has been confirmed by morphological and molecular genetic analysis [38].

Currently, the methods for producing doubled haploids in microspore culture for a variety of vegetables are being improved in the Laboratory of Biotechnology. A domestic technique for creating doubled haploid lines of pepper via anther (microspores) culture was developed [39], and doubled haploids of different varieties and interspecies hybrids were obtained based on it [40]. This technique is not inferior to the best foreign methods developed for hot pepper [41, 42]. This research was supported by a grant from the Ministry of Education and Science of the Russian Federation GK № 16.M04.11.0004 and continued with funding from the Ministry of Education of the Russian Federation, a grant GK № 14.M04.12.0013. The first hybrids of sweet pepper (Natalie, Hussars) have been produced with doubled haploid lines.

The methods for obtaining doubled haploids in the microspore culture of vegetable cabbage crops are actively developing abroad [43-48]. The basic protocol for the rape microspore culture was optimized, and doubled haploid lines of Chinese cabbage [49], broccoli line BR 1-1 and white cabbage involved in the creation of heterotic hybrids were produced in the Laboratory of Biotechnology of VNIISSOK.

Despite the numerous attempts to obtain doubled haploids in the carrot anther culture [50-54], the progress in the development of microspore culture technique for these important vegetable crops was achieved just in the recent years [55, 56]. A technique for producing DH-plants in the carrot microspore culture is under development in our Institute, with regenerated plants have been grown.

To produce haploid plants, female gametophytes are also used in breeding. In vitro cultivation of non-pollinated ovaries and ovules makes it possible to produces haploids when anther or microspore cultures do not provide good results. Sometimes this is the only way to isolate the DH-lines (for example, in plants with cytoplasme male sterility) [57]. The developed methods mostly are cultivating non-pollinated ovules in onion [58-60], cucumber [61, 62], red beet [63], pumpkin [64], and zucchini [65]. These studies have been conducted in VNIISSOK over the past 20 years. There are technologies for producing doubled haploids in the culture of non-pollinated ovules for carrot, onion, cucumber, zucchini, squash, and red beet [37, 66-69]. The effectiveness of the doubled haploid cucumber plant technique developed in the Laboratory is a few times superior to the foreign analogues [68], and the study is supported by grant of Russian Foundation for Basic Research 08-04-13513-ofi_ts.

In the Laboratory of Biotechnology, genetic transformation of plants is also performed. In collaboration with the scientists from the branch of the Insti-

tute of Bioorganic Chemistry of the Russian Academy of Sciences (Pushchino), transgenic carrots with the genes of *GUS*, defensin *Rs* and thaumatin II have been obtained, and the thaumatin II gene expression has been identified in the leaves and root-crops, the families of transgenic plants that are resistant to the *Fusarium avenaceum* pathogen have been selected [70].

Over the past decade, physical and functional organization of genomes of many crops has been studies due to the significant advances in molecular genetics. The molecular marking technologies make it possible to monitor the transfer of commercially valuable genes from one organism to another, and allow to accelerate and optimize the breeding process [71]. The systems of molecular marking of vegetable crops began to be developed in VNIISSOK in the 1990s together with the worldwide development of DNA technology. While at the initial stage the RAPD-markers (random amplified polymorphic DNA) only were used in tomato, aubergine, and onions [72-75] for the identification of intra- and interspecies polymorphisms, currently ISSR- (inter-simple sequence repeat), IRAP- (inter-retrotransposon amplified polymorphisms), AFLP- (amplified fragment length polymorphism) and SSR-labeling (simple sequence repeat) are used in VNIISSOK to study the variability of genomes, to genotype varieties (lines), and determine the purity of hybrid offspring in tomato, cabbage, onion, parsley, aubergine, peppers and other vegetables [76-81]. To maintain and improve the collection of varieties, copyright, authenticity verification of high-grade material and seed certification, molecular-genetic passports for pepper varieties have been composed on the basis of multilocus marking [82]. The possibility of using the data of molecular genetic analysis (AFLP and SSR) in the selection of pairs for hybrids with high heterosis effect has been shown, and promising heterotic hybrid combination of sweet pepper with a complex of major economically important traits have been selected [83].

In VNIISSOK, the development and application of DNA markers for vegetable crops are mainly of applied nature and are aimed at identifying genetic donors of economically important traits, one of which is the cytoplasmic male sterility (CMS) widely used to create hybrids on the sterile basis. Using molecular SCAR-markers (sequence characterized amplified region), *coxII* and *atp6* mitochondrial genes responsible for the CMS have been identified in samples of sweet pepper bred in VNIISSOK and in the *Capsicum frutescens* and *C. shinense* interspecies hybrids obtained using the embryo culture [84].

Screening of onion samples from the VNIISSOK collection using PCR markers for the mitochondrial genes of *orfA501* and *cob* made it possible to identify the samples with sterile and fertile cytoplasm, as well as to determine the types of sterile cytoplasm (S- or T-plasma-type) that do not differ phenotypically [85]. The selected samples of onion with a sterile S-plasma type are recommended for obtaining hybrids on the basis of CMS, as the system of male sterility in onions is easier to inheritance and stable in various environmental conditions [86]. Using multiplex PCR, the type of sterility in cabbage, broccoli, Beijing, radish and daikon have been identified, and the primer system allowing selecting the plants with the cytoplasm type of *Ogura, Ogu-NWSUAF, nap, pol, cam,* and *rad* has been matched. A new subtype of sterile cytoplasm of *Ogura* has been found in cabbage based on the *orf138* nucleotide sequencing [87].

Indirect selection based on molecular marking makes it possible to detect the desired alleles and haplotypes in the early stages of plant development, which significantly reduces and simplifies the selection process [88]. The *pun1* gene markers responsible for the hotness of pepper became an example of the practical application of this approach (*C. annuum* L.) [89]. The screening of the segregating population produced as a result of sweet and hot peppers crossing made with the help of this marker made it possible to select the genotypes with the desired com-

bination of alleles of *Pun1/Pun1* at the stage of seedling [90].

Thus, the results of theoretical research on biotechnology and molecular genetics along with traditional methods are used to accelerate the breeding objectives, create qualitatively new varieties and heterosis hybrids of vegetable crops combining high productivity and quality with complex resistance to the most dangerous diseases, pests, and abiotic stressors.

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EVALUATION OF THE MEASURE OF POLYMORPHISM INFORMATION OF GENETIC DIVERSITY

Yu.V. CHESNOKOV, A.M. ARTEMYEVA

Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources, Federal Agency of Scientific Organizations, 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail yu.chesnokov@vir.nw.ru

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Abstract

Gene identification and mapping are one of the main goals of plant and animal genetics. Upon verifying genetic linkage it is usually found which marker loci (markers) possess alleles co-segregated with the alleles of the desired locus. Marker utility for these purposes depends on the number of alleles, which the marker possesses, and their relative rates. There are two indexes, or measures, usually used for the polymorphism degree evaluation. They are the heterozygosity (H) for which the evaluation method and variability formula are well known (M. Nei et al., 1974, 1979), and polymorphism information content (PIC) (D. Botstein et al., 1980). Based on published data, we described the statistical approaches which are used for analysis of polymorphism information. Herein, the value of polymorphism information content, heterozygosity and some associated values detected upon evaluation of genetic diversity on interspecific and intraspecific population levels are considered. PIC shows haw the marker can indicate the population polymorphism depending on the number and frequency of the alleles (D. Botstein et al., 1980). So the PIC reflects a discriminating ability of the marker and, in fact, depends on the number of known alleles and their frequency distribution, thus being equal to genetic diversity. PIC maximal value for dominant markers is 0.5. Note, that for the markers with equal distribution in the population the PIC values are higher. They are much higher for markers with multiple alleles, and, however, also depend on the frequency distribution of the alleles. Using 135 SSR (simple sequence repeats) and 123 S-SAP (sequence specific amplified polymorphism) primers, we found 135 SSR и 123 S-SAP polymorphic markers among 96 *Brassica rapa* L. samples from the VIR (N.I. Vavilov Institute of Plant Genetic Resources) core collection. The PIC values for both markers, SSR and S-SAP markers were 0.316, 0.257 and 0.379 (50 % higher on average), respectively. Expected heterozigosity (H_E) is usually used to describe the genetic diversity because it is less sensitive to the sample size compared to observed heterozigosity (H_O). **T**he crossings in the population are occasional, if H_O and H_E are similar (i.e., no reliable differences found). They are related as $H_O < H_E$ in inbred population, and as $H_O > H_E$ in case of occasional crossing prevailing compared with inbreeding. Effective multiplex ratio (EMR) is calculated as total number of polymorphic loci per primer multiplied by the rate of polymorphic loci from their total number (W. Powell с соавт., 1996; J. Nagaraju с соавт., 2001). Marker index (MI) is a statistical parameter used to estimate total utility of the maker system; the higher MI, the better method is used) (W. Powell et al., 1996; J. Nagaraju et al., 2001). Resolving power (Rp) is a parameter characterizing ability of the primer/marker combination to detect differences between large numbers of genotypes (J.E. Gilbert et al., 1999; A. Prevost et al., 1999). The information about some software which can be used for calculation of polymorphism information content value and heterozygosity is also summarized. The formula for effective multiplex ratio, marker index calculation, and resolving power calculation are shown.

Keywords: heterozygosity, polymorphism information content value, effective multiplex ratio, marker index, resolving power, software.

Identification and mapping of genes responsible for the expression of the traits that are of the interest to the researcher are one of the main goals of plant and animal genetics. There is a large number of marker loci visualized using various marker systems (briefly referred to as markers) the positions of which and their order within a chromosome are well known. Upon verifying genetic

linkage it is usually found which marker loci (markers) possess alleles co-segregated with the alleles of the desired locus. Marker utility for these purposes depends on the number of alleles, which the marker possesses, and their relative rates. Qualitatively, a marker is characterized as polymorphic if it contains at least two alleles, and the most common allele has a frequency in a population of at least 99 %. The polymorphism degree evaluation is usually measured by two indexes (measures). One of them is known as heterozygosity (H) for which the evaluation method and variability formula are well known [1, 2]. The other measurement unit in the polymorphism information content (PIC) [3].

Molecular markers have become an effective tool and a means by which both intra- and inter-species genetic diversity is evaluated and characterized. Marker systems are distinguished by the extent (i.e., magnitude) of their informativeness, which in turn depends on the degree of polymorphism. The concept of polymorphism is used to determine the genetic variability in the population, which in recent decades has become the subject of intense study by various disciplines (genetics, ecology, botany, zoology and some others). Examples of this are numerous and obvious [4-10]. However, when planning the use of molecular markers for any research or for the practical use in breeding programs, the questions arise inevitably, and the researchers often have to look for the answers. How difficult is it to find polymorphic loci that are suitable for the planned work? How many markers will be required to be used? How polymorphic should any selected marker be? All these questions can be answered by estimating the measure of the marker informativeness. The two main parameters determined for this purpose are heterozygosity (H) and the polymorphism information content (PIC). In addition to these, there are some associated indicators with the help of which the effectiveness of the chosen system of «primer-marker» and/or of the chosen methodological approach can be determined.

In this paper, we summarized the published data on the statistical approaches which are used for the analysis of polymorphism information, heterozygosity and some associated values determined in the assessment of genetic diversity in both interspecies and intraspeciees population levels.

H e t e r o z y g o s i t y (H). Locus heterozygosity which is defined as the probability of an individual's heterozygosity in the population in this locus [11] can be calculated by the formula:

$$H = 1 - \sum_{i=1}^{l} P_i^2, \quad (1)$$

where P_i is the frequency of the i-th allele among the total number of alleles l. In other words, heterozygosity may be considered as an average portion of loci with two different alleles at one locus in a single individual. Usually, this applies to the whole population or some part of it and is divided into the observed and expected heterozygosity. The expected heterozygosity (H_E), or genetic diversity according to M. Nei [1], is the expected probability of an individual's heterozygosity for the relevant locus in multilocus systems (for all analyzed loci). In other words, it is the determined fraction of all individuals that were heterozygous for any randomly selected locus. It is often calculated based on determining the square root of the frequency of the null allele (recessive) as follows similar to equation (1): $H_E = 1 - \sum_i^n p_i^2$, where p_i is the frequency of the i-th allele, n_i is the total number of alleles at all loci. The observed heterozygosity (H_O) is the part of heterozygous genes in the population. It is calculated for each locus as the total number of heterozygotes divided by the sample size. The values of H_E and H_O range from 0 (no heterozygosity) to substantially 1 (a large number of alleles

with equal frequency). The expected heterozygosity is usually defined when describing genetic diversity, because it is less sensitive to the sample size than the observed heterozygosity. When H_O and H_E are similar (not significantly different), the crossing in the population is almost accidental. When $H_O < H_E$, it is an inbred population. When $H_O > H_E$, the random mating system dominates inbreeding in the population.

Polymorphism information content (PIC). The measure or value of the polymorphism information content (PIC) is determined by the ability of a marker to establish polymorphism in the population depending on the number of alleles detected and on their distribution frequency [3]. Thus, PIC identifies the discriminatory ability of the marker, actually depends on the number of known (established) alleles and their distribution frequency, and thus it is equivalent to the gene diversity. In its simplest form, the value of PIC can be calculated like heterozygosity, see equation (1):

$$\mathrm{PIC}_j = 1 - \sum_{i=1}^{n} P_i^2, \tag{2}$$

where i is i-th allele of the j-th marker, n is the number of the j-th marker's alleles, P is allele frequency. Sample calculations for the value of PIC and multiallelic markers are shown in Table 1. At the same time, for dominant markers, equation (2) can be represented as follows [12]:

$$\mathrm{PIC} = 1 - ({}^{k}\textstyle\sum^{i=1} P_i^2) - {}^{k-1}\textstyle\sum^{i=1\ \mathrm{k}}\sum^{j=1} 2\, P_i^2\, P_j^2, \tag{3}$$

where k is the number of alleles, P_i and P_j are frequency of the i-th and j-th alleles in the population, respectively. For dominant markers, the PIC value is calculated as described [13]:

$$\mathrm{PIC} = 1 - [\mathrm{f2} + (1 - \mathrm{f})2], \tag{4}$$

where f is the marker frequency in the data set. For the dominant markers, the maximum PIC value is 0.5. Note, that for the markers with equal distribution in the population the PIC values are higher. The values are much higher for the markers with multiple alleles, however, the PIC value also depends on the distribution frequency of the alleles (see Table 1).

1. Sample calculations for PIC for biallelic and multiallelic markers

Allele frequency	Formula for calculating from the equation (2)	PIC value
Biallelic marker		
$P_1 = 0.5$; $P_2 = 0.5$	$1 - (0.5^2 + 0.5^2)$	0.50
$P_1 = 0.4$; $P_2 = 0.6$	$1 - (0.4^2 + 0.6^2)$	0.48
$P_1 = 0.3$; $P_2 = 0.7$	$1 - (0.3^2 + 0.7^2)$	0.42
$P_1 = 0.2$; $P_2 = 0.8$	$1 - (0.2^2 + 0.8^2)$	0.32
$P_1 = 0.1$; $P_2 = 0.9$	$1 - (0.1^2 + 0.9^2)$	0.18
Multi allelic marker		
$P_1 = 0.33$; $P_2 = 0.33$; $P_3 = 0.33$	1 – (0.332 + 0.332 + 0.332)	0.67
$P_1 = 0.4$; $P_2 = 0.3$; $P_3 = 0.3$	1 – (0.42 + 0.32 + 0.32)	0.66
$P_1 = 0.4$; $P_2 = 0.4$; $P_3 = 0.2$	1 – (0.42 + 0.42 + 0.22)	0.64
$P_1 = 0.5$; $P_2 = 0.3$; $P_3 = 0.2$	1 – (0.52 + 0.32 + 0.22)	0.62
$P_1 = 0.5$; $P_2 = 0.4$; $P_3 = 0.1$	1 – (0.52 + 0.42 + 0.12)	0.58
$P_1 = 0.6$; $P_2 = 0.2$; $P_3 = 0.2$	1 – (0.62 + 0.22 + 0.22)	0.56
$P_1 = 0.6$; $P_2 = 0.3$; $P_3 = 0.1$	1 – (0.62 + 0.32 + 0.12)	0.54
$P_1 = 0.7$; $P_2 = 0.2$; $P_3 = 0.1$	1 – (0.72 + 0.22 + 0.12)	0.46
$P_1 = 0.8$; $P_2 = 0.1$; $P_3 = 0.1$	1 – (0.82 + 0.12 + 0.12)	0.35

Note. PIC — polymorphism information content.

In our research, in evaluating the core collection of *Brassica rapa* L. (96 samples) stored in the VIR, using 21 pairs of SSR (simple sequence repeats), and 12 pairs of S-SAP (sequence specific amplified polymorphism) of primers, we found 135 SSR and 123 S-SAP polymorphic markers that were used

to construct the phylogenetic tree [14]. PIC values were calculated for any marker (Table 2).

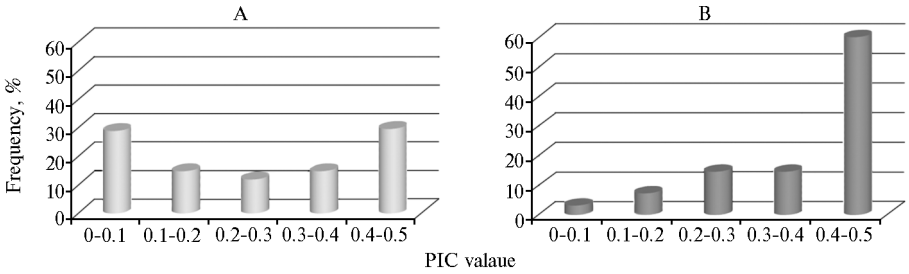
2. Identified SSR and S-SAP alleles molecular markers and their PIC among the VIR *Brassica rapa* L. core collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources)

Number of alleles	Proportion of alleles, %	PIC value
	SSR markers	
39	28.9	0-0.1
20	14.8	0.1-0.2
16	11.9	0.2-0.3
20	14.8	0.3-0.4
40	29.6	0.4-0.5
Total		
135	100	
	S-SAP markers	
4	3.2	0-0.1
9	7.3	0.1-0.2
18	14.6	0.2-0.3
18	14.6	0.3-0.4
74	60.0	0.4-0.5
Total		
123	100	

Note. SSR — simple sequence repeats, S-SAP — sequence specific amplified polymorphism. PIC — polymorphism information content.

Their frequency distribution for each type of markers is shown in the graphs (Fig.). The PIC value for both markers was 0.316, while it was 0.257 for microsatellite markers and 0.379 for S-SAP markers, i.e. 50 % higher on average, respectively. Thus, in our studies on the evaluation of genetic polymorphisms in the *B. rapa* collection using two markers, the most informativeness was noted in S-SAP markers.

At the same time, when determining the genetic diversity in rice (*Oryza sativa* L.), the average PIC value was 2 times higher in the case of using SSR markers (0.66) than with the use of RFLP (restriction fragment length polymorphism) markers (0.36) [15].



Distribution of PIC (polymorphism information content) **values for SSR** (simple sequence repeats, A) **and S-SAP** (sequence specific amplified polymorphism, B) **molecular markers in the evaluation of the *Brassica rapa* L. core collection stored in VIR** (N.I. Vavilov All-Russian Institute of Plant Genetic Resources).

In the study of molecular genetic diversity in sweet corn (*Zea mays* L.) using RAPD (random amplified polymorphic DNA) and SSR markers, the results indicating a higher similarity between the studied populations than within them, were obtained [16]. The authors note that the RAPD markers had lower average PIC values (0.17) than the SSR markers (0.57). The absolute PIC values for RAPD and SSR in the above case could not be compared due to the maximum PIC value of 0.5 and 1.0 for RAPD and SSR loci, respectively. RAPD and ISSR markers were also used to assess the genetic variability and relationships among Tunisian local varieties of barley (*Hordeum vulgare* L.) [17]. Despite the high level of polymorphism revealed for both RAPD and SSR markers and the mean PIC values of 0.477 and 0.533 for the RAPD and SSR markers, respectively, the authors conclude that the SSR markers are better suited to assess the genetic diversity of barley than RAPD markers, as SSR markers have greater polymorphism (90.7 %) compared with the RAPD markers (74.0 %).

And finally, another example of PIC value estimation to which we would like to draw your attention. The researchers from Argentina and the USA have

analyzed the genetic diversity of the Argentinean varieties of wheat (*Triticum aestivum* L.), created in the period from 1932 to 1995 [18]. Using the SSR and AFLP (amplified fragment length polymorphism) markers, they found that there were no significant differences in the genetic diversity between the group of varieties created until 1960 and the groups produced in each of the next three decades. The mean diversity estimated with the SSR markers was virtually identical for all the four time periods. The genetic diversity identified through the AFLP markers confirmed the absence of genetic diversity reduction over time. However, significant differences ($P = 0.01$) were found between the varieties of soft wheat created in the 1970s (PIC = 0.28) and 1980s (PIC = 0.34). The overall results obtained with PIC indicate that the Argentinean varieties of soft wheat were maintained almost at the same level of genetic diversity for over 60 years, and their differences were largely due to the implemented breeding programs, but not to the degree of genetic diversity of the derived varieties. Thus, the measure of polymorphism information content is an important component in the planning of breeding programs and one of the key information and statistical indicators in their implementation.

Software for calculation of H and PIC. To correctly plan a genetic research and evaluation of the results, calculation of heterozygosity (H) and polymorphism information content (PIC) values is often required to describe the marker informativeness, but until recently there have been no simple and public calculators for such computations. To simplify the work on the marker research, in 2012, a group of Hungarian scientists proposed their interactive online PICcalc program (http://w3.georgikon.hu/pic/english/default.aspx) [19]. The program makes it possible to calculate the values of H and PIC over allele frequencies with the manual introduction of the values or using a special file containing a binary data matrix. The additional options make it possible to calculate the values for the certain number of loci using a simple text file which ensures estimation of H and PIC for the primer or primer sets used for the analysis of different genetic marker systems associated with binary data. For multilocus markers such as AFLP, ISSR (inter simple sequence repeats) or RAPD, it is theoretically assumed that the fragments of equal length are amplified at the corresponding chromosome loci and that they represent a single dominant locus with two possible alleles (amplicon presence/absence). The maximum value for H and PIC for dominant markers in this case is equal to 0.5, as only two alleles per locus are permitted for this type of markers and both values are influenced by the number and frequency of alleles [13, 20, 21]. Given this characteristic of dominant markers, the program specifically provides for the possibility of calculating H and PIC for them [19].

Earlier, a group of American scientists also offered a computer program (http://darwin.cwru.edu/pic) makes it possible to calculate the H and PIC parameters [22]. The main difference with the above-mentioned Internet resource is the uniformly distributed minimum deviation of the unbiased PIC estimation in accordance with the exact dispersion value. To estimate this, the authors derived the formula for the calculation of any polynomial in the set of variables distributed multinomially.

Associated values. Effective multiplex ratio (EMR) is calculated as total number of polymorphic loci (per primer) multiplied by the proportion of polymorphic loci per their total number [23, 24]:

$$EMR = n_p(n_p/n), \quad (5)$$

where n_p is the number of polymorphic loci, and n is the total loci number. The higher the value of EMR, the more efficient the «primer—marker system» is.

Marker index (MI) is a statistical parameter used to estimate the total utility of the maker system. Marker index is the product of the polymorphism information content value (or expected heterozygosity, H_E) and effective multiplex ratio [23, 24]:

$$MI = PIC \times EMR, \quad (6)$$

The higher MI, the better the method is. Resolving power (Rp) is a parameter used to characterize the ability of the primer/marker combination to detect the differences between a large numbers of genotypes [25, 26]:

$$Rp = \sum I_b, \quad (7)$$

where $I_b = 1 - (2 \times 0.5 - p$ is an amplicon informativeness, p is a proportion of individuals with identified amplicon I.

Thus, there are several approaches that are designed to measure the polymorphism information content and the associated values. DNA markers are now recognized as a rather convenient and high-quality tool for assessing genetic diversity at the molecular level. However, before using a particular marker system, it is necessary to evaluate the technical equipment of a laboratory, the need for the use of the selected marker system and its compliance with the current tasks, staff professionality, as well as the upcoming maintenance cost and the available means of support services. The required software should be selected based on the calculation of its suitability for solving problems faced by the researcher, including the problems of population genetics when it comes to assessing the information polymorphism. The morphological parameters are important for the interpretation of the results. The estimation of statistically significant association and correlation between morphological and molecular genetic parameters is the key factor in the final decision. And of course, we cannot ignore the biological characteristics of the species studied in the evaluation of genetic parameters, as one and the same parameter can be different in different species not only in phylogeny but also in ontogeny. The latter is particularly important for the effect of the «genotype—environment» interaction.

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ANALYSIS OF EPIGENOMIC AND EPIPLASTOME VARIABILITY IN THE HAPLOID AND DIHAPLOID SUGAR BEET (*Beta vulgaris* L.) PLANTS

S.I. MALETSKII, S.S. YUDANOVA, E.I. MALETSKAYA

Institute of Cytology and Genetics of SD of Russian Academy of Science, Federal Agency of Scientific Organizations, 10, pr. Lavrentieva, Novosibirsk, 630090 Russia, e-mail stas@bionet.nsc.ru

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Abstract

Reproduction of cells, individuals, populations is a principal concept of biology. This process is characterized by two properties: heredity and variation. The concept of inheritance indicates the identity of the parents and the offspring's; the concept of variability indicates the incompleteness of this identity. There is a direct proportional relationship between the genome level ploidy, the cytoplasm volume and the cell size (nuclear-plasma ratio). Variation of chromosome or chromatid numbers in the cell nuclei determines the epigenomic variability and variation of intracellular organelle numbers in the cell (for example chloroplasts) determines epiplastome variability in plants. Relationship of the chloroplast number in stomata guard cells and nucleus ploidy level in sugar beet is well known that permit to compare an epiplastome variability in plants and different ploidy of genomes. Chloroplast number in the cells varies, partly due to the asymmetric organelle distribution during cytokinesis. However the epiplastomic variability is related not only with a random organelle distribution during cytokinesis, but also with a genome number variation per cell nucleus (epigenomic variability). An endohaploidy, i.e. an appearance of haploid cells in cell population, is one of the variant of epigenetic variability display. Sugar beet may form the haploid seeds spontaneously both by biparental and uniparental reproduction. In the work the diploid (control, generation A_0), dihaploid and haploid (generation A_1) seed progeny were used. Dihaploids and haploids were obtained by the parthenogenetic reproduction mode (pollen less condition). In the paper we considered a variability of chloroplast number in stomata guard cells and integral tissue characteristics which are compared with harmonious proportions (Fibonacci number, golden ratio). It was studied following parameters: a) a chloroplast number in stomata the guard cells; b) a plastotype number in the epidermal tissue. And it was determined the average value of chloroplast (M) and plastotypes (Pt) number in stomata guard cells of haploid, diploid and dihaploid plants. On the base of obtained data the ratios of epigenetic stability (D-ratio) in haploid, diploid and dihaploid (control) sugar beet plants were estimated. D is logarithmic ratio of the chloroplast number to plastotypes and indicates the physiological and epiplastome stability of cell populations. It was shown the differences between the experiment simples: D-ratio in dihaploids is always above than one in the haploids. It was established for the first time that integral tissue characteristic (D-ratio) corresponds to harmonious proportions (bio-logical invariants), i.e. Fibonacci numbers (golden ratio). In diploids and dihaploids this ratio corresponds to the first terms of the harmonious series (from first to fifth), in haploids D-ratio corresponds to eighth and higher terms of the harmonious sequence.

Keywords: apozygoty, haploids, dihaploids, variability, harmonious proportions, fractals, endopolyploidy, epigenetics.

Reproduction of cells, individuals, populations is a principal concept of biology. This process is characterized by two properties: heredity and variation. The concept of inheritance indicates the identity of the parents and the offspring's [1], and the concept of variation indicates the incompleteness of this identity. There are two distinct types of variations, the inherited genetic variations derived from genome mutations and epimitations, and the non-inherited paratypical modifications due to inner or outer factors influencing biogenesis. At cell level the heredity means the competence of cell components to provide

structural and functional similarity (symmetry) in the next offspring due to reproduction, i.e. self-reduplication of DNA, chromatids, chromosomes, and chloroplasts and mitochondria organelles. As the self-duplication is completed, the nucleus and chromosomes divide by means of the division spindle (cariokinesis, mitosis) and cytoplasmic membrane septum formation followed by separation of daughter cells (cytokinesis). The daughter nuclei produced in cariokinesis are usually the copies of the parent cell while exact distribution of organelles between daughter cells is impossible.

In mitosis intercellular variation does not occur, and therefore in the Mendelian inheritance paradigm the daughter cells are considered parent clones [2]. Random nucleotide substitutions in the DNA molecules (gene mutation) and reciprocal or nonreciprocal exchanges (recombination) of homologous chromosomes during meiosis are usually deemed the main sources of intercellular variations in the tissues. These basic assumptions form the basis for a genocentric paradigm (GCP) of inheritance and variability and associate trait variation and the chromosome structure, but do not apply to all groups of features [3].

There are discrete (qualitative) and continuous (quantitative) traits. The discrete traits are alternative and their variation is usually caused by mutations in genes of the nucleus or protoplasm, while the variation of the continuous traits (i.e., number of flowers, seeds and fruits on plants, etc.) is not directly linked to the gene activity being influenced by outer signaling derived from growth conditions, density of planting in phytocenosis, etc.). A clear description of the inheritance of continual traits within GCP is very difficult, therefore, their polygenic determination is declared.

Epigenetic paradigm (EGP) does not bind cellular and individual variations solely to changes in the nucleotide sequences of DNA, chromosome structure and recombination of chromosomes in meiosis [3, 4]. Variability in cell populations may occur due to the volatility in the chromosome number (mixoploidy) or DNA amount in the nucleus. Mixoploidy in plants was reported in 1910, and in 1935 this phenomenon was found in members of the family *Chenopodiaceae* to which the beet plants belong.

Another mechanism of intracellular variation in somatic cells is the variability in the number of chromatids in chromosomes when along with monochromatid chromosomes duple and quadruple chromosomes are also found. The variations of the DNA amount, the number of chromosomes or chromatids are very common mechanisms of epigenomic and epigenetic variation in plants [5-7]. There is a direct proportional relationship between the genome level ploidy, the cytoplasm volume and the cell size (nuclear-plasma ratio). A volatility of DNA level in somatic cells is confirmed cytometrically [8-14].

Epidermal cell variability in organelle number is partially related to endoploidy due to spontaneous changes in chromosome ot chromatid number in the diploid cell nucleus proportionally to the number of endomitosis [6, 15]. Endopolyploidy does not affect the nucleotide sequences in DNA and chromosome structure and occurs during DNA replication in the cell nucleus not followed by its division. These processes lead to volatility in the DNA levels in the cell populations which is the main source of cell and tissue variation in plants [6-13]. Note, the first researchers, the same as modern scientists, confirmed high prevalence of this phenomenon in the family *Chenopodiaceae* [10-14].

Endohaploidy (the appearance of cells with single set of chromosomes) is a mechanism of epigenomic variation in somatic cells. Haploidy is experimentally effective for genome homozygotization. Sugar beet may form the haploid seeds spontaneously both by biparental and uniparental reproduction with

the haploid seedling frequency of 10^{-4}-10^{-6} and 0.5×10^{1}, respectively [16, 17].

According to EGP a special group of traits, the fractal or geometric ones, inherent in cell or plant as a whole can be distinguished. These include, in particular, the structure of the vascular system (xylem and phloem), the root system [18], and embryonic and tissue characteristics. Mathematically, the discrete, continuous and fractal characteristics can be clearly distinguished by a geometric dimension (D). In the discrete and continuous traits D is expressed in whole numbers (one-, two-, or three-dimensional features), while in the fractal traits the fractional dimension is characteristic [18].

Variation in chromatid and chromosome number affects directly their distribution in meiosis, and, therefore, offspring splitting on traits [3, 17]. Moreover, an increased DNA content in nucleus can influence the cell size and number of organelles (chloroplasts) in the cytoplasm [19]. Chloroplasts possess their own genome. This plastid number per cell in plant tissues varies from several ones to hundreds of chloroplasts. Prior to cell division, the self-replication of chloroplasts occurs, and daughter cells are assumed to posses the same organelle number as the parent cell. In case of exact splitting in a series of cell offspring, chloroplast number in each plant cell must be the same as in initial zygotic cell, however, but it has never been. Plastid distribution in cytokinesis is asymmetric. Epiplastom variation is due not only to random distribution of organelles in cytokinesis but also to DNA amount and genome number per cell nucleus (epigenomic variation), since the nucleus size, cytoplasm volume, the number of organelles are in a direct proportional relationship.

The relationship between chloroplast number, ploidy and cell size is well known in sugar beet plants. In the studies focused on the production of polyploids an indirect convenient procedure for plant grouping according to ploidy has been disclosed. Thus, in stomata guardian cell of triploids and tetraploids there were reliably more chloroplast (from 17 to 22 and from 22 to 28, respectively) compared to diploids (from 12 to 16). This finding permits to compare epiplastome variation in haploids, dihaploids, tripoids, tetraploids, etc.

In this article we compared integral tissue parameters (i.e. the ratio of its variation to an average number of organelles) and showed for the first time that these are in a concordance to harmonic proportions (biological invariants) denoted as ρ-numbers or the Fibonacci numbers, which, in turn, correspond to the golden ρ-proportion.

So our study was focused on experimental estimation of chloroplast number variability in stomata guard cells in haploid, dihaploid and diploid sugar beet plants.

Technique. The seeds of sugar beet (*Beta vulgaris* L.) dioloid male sterile (MS) hybrids Roksana, Lenora, Iris (F_1, or A_0), and their apozigotic dihaploid and haploid seed progeny (A_1) were used. The study was carried out from 2009 to 2012 in field condition (Novosibirsk). To produce apozygotic seed progeny (memo: apozygoty means embtyo parthenogenesis from unfertilized cells of the embryo sac) the roots were planted on an isolated plot, and in each plant at flowering the phenotype of anthers and pollen grains was recorded. The plants of MSII phenotype (semi-fertile) were eliminated [19] and the MS0 (complete pollen sterility) and the MSI (pollen semi-sterility) individuals were remained for apozigotic seed reproduction.

The seed samples from the progeny (A_1) of each MS-hybride set after 2 day rinsing in tap water were incubated in thermostat at 25 °C, and 2 day after the germination the seedlings were separated morphobiologically as haploids and dihaploids (in vivo production of haploids) [16, 20]. Then seedlings were grown in individual pots at a climatic chamber Biotron 4 (Russai) under controlled

humidity, temperature and lightening for first 100 days of life. Further, haploids and dihaploids were undergone vernalization at t = 4 °C and planted in a field.

Chloroplasts were counted in epidermal cells of 1 year leaves. Selected leaves were average in size. Epiderma was removed from the underside leaf part. Chloroplasts were stained with $AgNO_3$ and counted in each of 50 epidermal cells. The number of plastotypes (Pt) was estimated in the tissue, and an average chloroplast number in cells (M) was calculated. Epidermal tissue was characterized using M and Pt parameters as M:Pt. An average number of cell organelles and number of plastotypes in the cell population a related as $M = Pt^D$. Thus, D (fractional dimension) being the integral parameter of epidermal tissue [18] was calculated as D = ln M/ln Pt.

Data were processed using common methods of variation statistics [21]. For each plant, we found σ^2, the arithmetic mean of organelle number per cell (in 50 cells for each leaf) and $M \pm m$, also weighted average M_w in a sample was calculate and Cv was determined as

$$Cv = \frac{100\sigma}{M} \times 100\ \%.$$

Besides, in leaf samples of each specimen an average number of plastotypes with the error of mean ($Pt \pm m$) were estimated. The coefficient of linear correlation (r) was calculated according to the formula:

$$r = \frac{\sum a_X a_Y}{n\sigma_X \sigma_Y},$$

with a_X and a_Y as variance deviations from arithmetic mean, n as sample size, and σ as standard deviation.

Using goodness of fit test (G) multiple-tables were analyzed statistically comparing discrete distributions with null hypothesis. G was calculated as

$$G = 2\left(\sum_i f_i \ln \frac{f_i}{f_i'}\right) = 2\sum_i f_i \left(\ln f_i - \ln f_i'\right),$$

with f_i и f_i' as empirical and theoretical frequency of discrete distributions of specific trait [21].

Fig. 1. Diploid (left) **and haploid** (right) **seedlings of sugar beet (*Beta vulgaris* L.).**

Results. A multividual variation is inherent in sugar beet plant, that is the flowers of the main shoot and side-shoots can differ in the anther phenotype [22]. There are three distinct phenotypes according to anthers and pollen: i) complete pollen sterility due to defective anthers and pollen (MS0); ii) pollen semi-sterility when uninucleate pollen grains are unable to form pollen tube (MSI); iii) semi-fertility when pollen grains are partly viable (MSII). The seeds were obtained from MS0 and MSI plants.

Apozygotic seed progenies are usually diploids (dihaploids) as derived from endotetraploid cells of archesporium. In addition to dihaploids, some apozygotic seeds were haploids [16, 17]. Haploid seedlings are 4-fold shorter and smaller in diameter compared to dihaploid sibs (Fig. 1).

Chloroplast number in an individual epidermal cell we have designated as plastotype (Pt). There were several plastotypes different in the chloroplast (Fig. 2). In contrast to chloroplast number the number of plastotype is an integral trait inherent in a discrete leaf (a discrete plant). The M:Pt ratio changes stochastically during plant ohtogenesis in accordance to

the harmonic proportion principle [23, 24].

Fig. 2. Chloroplasts in the stomata guardian cells in leaves (epidermal tissue of sugar beet *Beta vulgaris* L.).

Earlier we have shown this proportion not to be the same in hybrid and inbred plants [16].

An average (weighted average) chloroplast numbers in MS-hybrids Roksana and Lenora (A_0) were about the same (12.79±0.35 and 13.12±0.42, respectively). In Iris MS-hybrid the cells were larger and comprised about 15 chloroplasts (14.92±0.53) which was significantly higher compared to other hybrids (the *t*-criterion of 2.87 и 2.69, respectively; P > 0.95). An average number of plastotypes in the epidermal tissue of all three MS-hybrids (A_0) was statistically indistinguishable (7.2±0.35, 6.5±0.41, 7.3±0.54) (Table 1).

In dihaploid offspring (A_1) the chloroplast and plastotype number in all variants did not change reliably compared to parent MS-hybrids (A_0) (see Table 1). Therefore, apozygotic seed reproduction did not affect this parameters in stomata guard cells of dihaploids.

1. Chloroplast (Chl) **and plastotype** (Pt) **number in the leaf epidermal tissue in diplod** (A_0)**, dihaploid** (A_1)**, and haploid** (A_1) **plants of three sugar beet (*Beta vulgaris* L.) male-sterile hybrids**

Ploidy	Progeny	Plant number	Chl number		*Cv*, %*	Pt number		$D_{average}$ (min-max)
			$M_w \pm m$	min-max		Pt±*m*	min-max	
Hybrid Roksana								
Diploids	A_0	10	12.79±0.35	11.1-14.9	12.35	7.2±0.35	6-10	1.30 (1.18-1.44)
Dihaploids	A_1	10	13.16±0.41	11.3-15.6	14.69	8.2±0.47	7-10	1.24 (1.07-1.47)
Haploids	A_1	55	12.32±0.30	10.3-16.0	17.43	8.8±0.17	6-12	1.16 (1.04-1.37)
Hybrid Lenora								
Diploids	A_0	11	13.12±0.42	10.8-15.4	13.05	6.5±0.41	5-9	1.40 (1.19-1.70)
Dihaploids	A_1	15	14.22±0.41	13.1-15.5	9.50	7.2±0.45	5-10	1.50 (1.19-1.69)
Haploids	A_1	38	11.85±0.35	9.4-19.2	17.96	8.8±0.31	6-12	1.16 (1.03-1.31)
Hybrid Iris								
Diploids	A_0	10	14.92±0.53	13.9-17.5	10.89	7.3±0.54	5-9	1.39 (1.15-1.64)
Dihaploids	A_1	12	14.54±0.40	12.0-17.1	8.33	6.2±0.33	5-7	1.51 (1.39-1.70)
Haploids	A_1	40	12.27±0.31	10.4-18.0	15.69	8.7±0.22	6-12	1.17 (1.04-1.46)

Note. $M_w \pm m$ is weighted average mean and the error of mean for chloroplasts, Pt±*m* is average mean and the error of mean for plastotypes, *Cv* is coefficient of variation, D is epigenome stability index.
* Weighted average mean.

In Lenora and Iris haploids the chloroplast number mean was reliably lower compared to parent plants (A_0) and dihaploid sibs (A_1). The Roksana male-sterile hybrid was the only one with no reliable difference in chloroplast number in diploids, dihaploids and haploids (12.79, 13.16 and 12.32, respectively). Haploids also were characterized by higher variation on plastotype number being from 6 to 12 where as in diploids and dihaploids its varied from 5 to 10. The means of this parameter in haploids in all three hybrids in fact coincided (8.7-8.8) being significantly higher then in diploids (7.2-7.3) or dihaploid Lenora and Iris plants (6.2-7.2). In Roksana plants no significant difference was found between dihaploids and haploids (8.2 and 8.8, respectively).

D (fractal dimension) is associated with cell epigenomic and epiplastome instability in leaf tissue, so that D is the parameter of cell population variability on chloroplast number per cell and plastotype number per tissue. D differed significantly in diploids and haploids (see Table 1). The ranges of D value in diploids (1.30-1.40) and dihaploids (1.24-1.51) overlap. Therefore, a single parthenogenetic reproduction had no expressed effect on the parameter, too. Nevertheless, in haploids D was much lower (1.16-1.17) when compared to diploids and dihaploids. At that, no interference was observed, and D values were close to those early reported in inbred sugar beet lines [15].

2. Distribution of produced sugar beet (*Beta vulgaris* L.) haploids on genome stability (D) in cell populations in three male-sterile hybrids (A_1)

Group	Hybrid			Total
	Lenora A_1	Iris A_1	Roksana A_1	
1.01-1.10	13	9	20	42
1.11-1.20	14	21	21	56
1.21-1.30	9	8	8	25
1.31-1.40	1	1	6	8
1.41-1.50	1	1	–	2
Total	38	40	55	133

D distribution in three haploid populations (133 plants) is shown in the Table 2. D mostly varied within 1.11-1.20, and in minor fraction of plants it was higher or the same as in diploids. Comparing D distribution in three samples of haploids, we calculated *G* and found it to be 13.6 (df = 12; 0.50 < P < 0.30), thus indicating random distribution for D (Table 3). Thus, the combination is possible which allows comparing D distribution in the haploids, diploids and dihaploids.

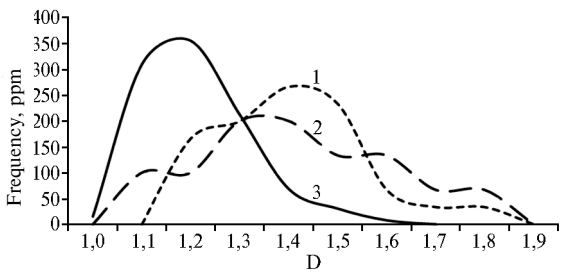

Fig. 3. Distribution of epigenomic stability (D) in diploid (1), dihaploid (2) and haploid (3) sugar beet (*Beta vulgaris* L.) plants.

Substitution of the number of diploids, dihaploids and haploids by their frequencies in ppm made it possible to plot graphs of D distribution in a total sample over all hybrids. In diploids and dihaploids the variation rang was about the same, while in haploids it differed significantly from that in both diploids showing more cytological uniformity (Fig. 3). D means in three haploid samples were identical (1.16-1.17) and differed from corresponding parameter in diploids, though haploids and diploids derived from the same seed progenies (see Table 3).

3. Statistical parameters for cell populations in leaf epidermal tissue in haploid and diploid male-sterile hybrids of sugar beet (*Beta vulgaris* L.) (A_1)

Hybrid	*N*	*M*±*m*	*Cv*, %	Pt±*m*	D	$r_{D/Cv}$
Haploids						
Iris	40	12.27±0.31	15.69	8.7±0.22	1.17	–0.77±0.07
Lenora	38	11.85±0.35	17.96	8.8±0.31	1.16	–0.87±0.04
Roksana	55	12.32±0.30	17.43	8.8±0.17	1.16	–0.86±0.03
Dihaploids						
Iris	12	14.54±0.40	8.33	6.2±0.33	1.51	–0.50
Lenora	15	14.22±0.41	9.22	7.2±0.45	1.50	–0.82
Roksana	10	13.16±0.41	14.69	8.2±0.47	1.24	–0.93

Note. *N* is plant number, *M*±*m* is mean and the error of mean for chloroplast number, *Cv* is coefficient of variation, Pt±*m* is mean and the error of mean for plastotype number, D is epigenomic stability, $r_{D/Cv}$ is coefficient of liner correlation.

In Table 3 where statistical parameters for haploid and dihaploid plants of three male-sterile hybrids are summarized, *Cv* as index of cell variability on chloroplast number correlated negatively with D as coefficient of epigenomic stability in cell population with *r* varying from –0.77 to –0.93 and, thus, approaching –1 in some cases.

Polyploidy is widely spread in plants causing different intra- and interspecial variation [9]. Polyploid series in species of many botanical genera and families are a common example. Different manifestations of individual variation which is widely used in plant breeding are also related with polyploidy [18, 19].

Mixoploidy also occurs when in the minor fraction of the cell population the chromosome number is more or less than in the dominant one [5-7, 10].

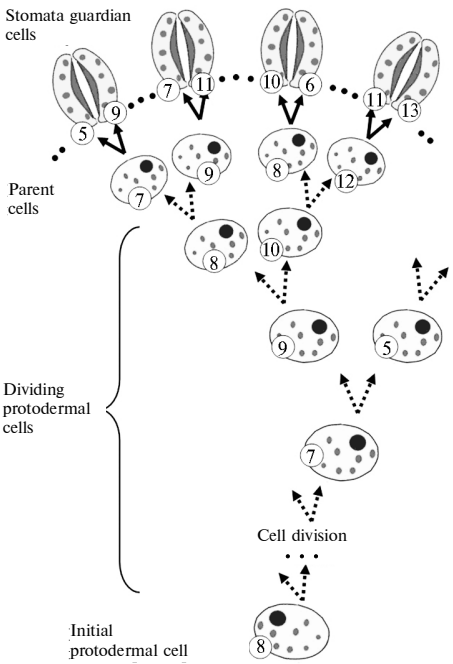

Fig. 4. Scheme of genealogical tree of stomata guardian cell (cyto-fractal) **in leaf epidermal tissue** (chloroplast number per cell is indicated).

Variation of chloroplast and plasotype number in epidermal tissue is an expression of epigenomic and epiplastom cell instability in ontogenesis. During cell division a protodermal cell becomes parent one and produces two daughter cells which further form guardian cells fixing features of parent protodermal cell such as linear size, ploidy and the number of organelles in cytoplasm [19].

Modeling different biological processes is possible due to fractals [25, 26]. Mathematically, fractal is a set in which Hausdorff-Bazilevich dimension is strictly higher than the topological dimension [18]. In Euclidian geometry, a dimension means the number of spatial coordinates determining location of the point (i.e., one-, two- and three-dimensional objects). Fractional number for D parameter is an attributive feature of any fractals.

Epidermal cells can be considered a tree-like fractal [24]. Plant tissue is a set of cells with the same or different number of organelles. It is like a genealogical tree started from a primordial cell with definite number of plastids. Each cell grows and then divided into two daughter cells. The set of cells in the tissue form geometric structure (Fig. 4), or «plastid fractal» [15]. As genealogical tree is growing (see Fig. 4), the same iteration occurs. A cell divides into two ones with equal or unequal plastid number, and several cell divisions result in appearance of the cell set (tissue) with different chloroplast number. Variation in the cell population is determined by the ploidy and the mode of seed reproduction (i.e., due to fertilization or parthenogenetically) [10].

As it can be seen from Table 1, variation in in chloroplast number in stomata guard cell is associated with epigenimic instability in the cell nuclei in haploids which, in its turn, correlates with homozygosity in self-pollinated progenies. For inbred beet plants, we earlier reported a significant increase in average numbers of organelles in the stomata guard cells [8] and plastotypes in the leaf epidermal tissue [16]. A M:Pt ratio allows us to link the trait of two distinct levels, the tissue level (an average number of chloroplasts per cell) and an individual level (an average number of plastotypes per tissue), so that this ratio is a new geometrical trait peculiar to epidermal tissue of leaf (tissue) as a whole.

Changes in cytological variables of epidermal tissue in inbred sugar beet plants results in decreased fractal D value if inbred and hybrid plants are compared. In other words, higher epigenomic variation (instability) is peculiar to inbred lines compared to hybrids. The epigenome stability was the lowest in the inbred plants and the highest in male-sterile hybrids.

Monogenomic character of cell nucleus in haploids leads to increased cell mixoploidy (endopolyploidy) affecting on the number of chloroplasts [8, 15]. An increased number of plastotypes in the epidermal tissue of haploids compared

to dihaploids is the evidence of more unstable genome in haploids. D values in haploid Lenora and Roksana hybrids were the same (1.16), and in haploid Iris plants it was 1.17. In haploids D values were significantly lower compared to dihaploid sibs (A_1) and diploid meal-sterile hybrids (A_0). In all three haploid populations D values, in fact, were the invariant being significantly different from D invariants in diploids and dihaploids.

Chloroplast number variation in epidermal cells is related to variability of nuclear size determined by endopoliploidy. High mixoploidy in inbred lines is obviously due to different irregularities in cell divisions leading to an increased level of mixoploidy in somatic cell population and also to variability in the number of organelles (epiplastome variation). Under an infringement in mitosis, both endopoliploid cells (due to chromosome replication without karyokinesis) and endohaploid cells (because of karyokinesis without chromosome replication) can appear in cell populations. In the meristem tissue, triploid and tetraploid cells, and the cells of other ploidy are found along with diploid ones [5-8]. So at cell population level the epigenomic variation occurs due to ploidy determining also epiplastome cell variation.

In our experiments *Cv* values in haploid plants were higher than in dihaploids and diploids. *Cv* is not related directly to activity of distinct genes, gene blocks or external conditions being determined by all intracellular genetic, physiological and biochemical mechanisms. Therefore, the observed variation should be considered epigenetical one, i.e. occurred during plant ontogenesis. Depression is known to be peculiar to homozygous and haploid plants. It is particularly expressed in instability and growth abnormalities compared to hybrids. Such instability is observed under epiplastome variation in sugar beet plants.

D values found for different beet samples, varied from 1.0 to 1.7 and corresponded to the golden ρ-proportion [23], the geometric parameter which describes division of the whole subject to two unequal parts, for instance, the division of a line segment into extreme and mean ratio. A.P. Strakhov suggested a generalized principle of golden ratio according to which the division of a subject as a whole comprises a set of structure invariants, particularly the dichotomic mode of division ($\rho = 0$) and the golden ratio ($\rho = 1$). These D values were observed in our investigation (see Table 3), nevertheless, another values were also reported which corresponded to harmonic proportions occurred at $\rho \geq 2, 3, 4$, etc. For initial ρ value the harmonious series (structure invariants) are as follows: 1.618, 1.465, 1.380, 1.324, 1.285, 1.255, 1.232, etc. [26]. D value in hybrids corresponded to the first terms of the harmonious series ($\rho \geq 1, 2, 3, 4$), while in haploids the D-ratio corresponded to more far terms of the same sequence ($\rho \geq 8, 9, 10$ ...).

Harmonious proportion and golden ratio are, in fact, invariants which reflect the principle of self-organization in living material. The system, as a whole, can be subjected to changes, however, some its properties remain unchanged.

Thus, found D ratios in haploid and diploid sugar beet plants make it possible to estimate epigenomic stability and epigenome variation in cell populations. Epigenome and epiplastome variation evaluated as geometrical D parameter characterize physiological state of cell populations in plants during ontogenesis. Heterosis, or hybrid power, is peculiar to one group of plants, the male-sterile hybrids, which possess epigenomic stability with high D value, while in another group, the haploid plants, a depression with low D value is characteristic.

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THE ASSESSMENT OF THE VARIETY AND ROOTSTOCK GENOTYPES INTERACTION IN APPLE (*Malus domestica* Borkh.) GRAFTED TREES USING BIOMETRIC METHODS

I.A. DRAGAVTSEVA[1], V.A. DRAGAVTSEV[1], I.L. EFIMOVA[1], S.N. SHCHEGLOV[2], V.V. DOMOZHIROVA[1], A.S. MORENETS[1]

[1]*North-Caucasian Zonal Research Institute of Orcharding and Viniculture,* Federal Agency of Scientific Organizations, 39, ul. 40 let Pobedy, Krasnodar, 350901 Russia, e-mail i_d@list.ru, dravial@mail.ru, vetch-dv@yandex.ru, efimiril@mail.ru;
[2]*Kubanian State University,* 149, ul. Stavropol'skaya, Krasnodar, 350040 Russia, e-mail gold_finch@mail.ru
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Abstract

In grafted fruit plants with vegetative propagation the scion (variety) and rootstock influence each other in newly-formed variety-rootstock combination. Under intensive technologies of fruit production based on maximal realization of grafted fruit plants' bio potential it is very important to obtain accurate knowledge about quantitative traits which characterize productivity and biometrical parameters of fruit plants. To forecast the productivity of apple (*Malus domestica* Borkh.) trees, we studied the possibility of math modeling for the prediction of variety and rootstock influence on formation of quantitative traits in grafted plants using formulas offered by biometric genetics to estimate the same indexes in parent forms and their F_1 hybrids. The data of apple trees productivity obtained in Prikubanskaya zone of Krasnodar region in 1983 to 2003 years (a total of 22 years) were analyzed. We studied the variety-rootstock combinations (VRC) of four apple varieties (Idared, Golden Delicious, Jonathan, Korah) as scions and seven rootstocks (I-48-1, I-47-55, I-48-46, M2 , M3, M4, M7) with regard to yields, the width of the crown from North to South and from West to East, the tree height and trunk diameter. An impact of the year conditions, the genotypes of the variety and the rootstock and their interaction on the yield of the variety-rootstock combinations was proven using math statistics. It was shown that the conditions of the year have the greatest effect (37 % of the total variance). Strength of the variety influence on the VRC yield was determined to be expressed with a several years interval. The rate of the rootstock impact and the cumulative effect of the scion and the rootstock were found to be roughly equial. For the first time for biological objects, which are characterized by non-linear relationship of traits, it is revealed that the forecasting models of VRC productivity based on multiple linear regression analysis with a linearized model is more effective and promising approach which takes into account the inadequacy of linear models previously considered. The histogram of residuals showed their normal distribution that is in conformity with correct use of the applied regression analysis. It provides a basis for adequate non-linear (quadratic) model of the yield production in each variety-rootstock combination as related to morphological and anatomical characteristics of grafted trees. Thus, based on theoretical analysis and the 22 year survey, we suggested the mathematical models for the variety and rootstock genotypes influence on quantitative traits in a grafted plant, primarily its productivity. It has been developed for the first time. This model enables more accurate control of stable and effective yield production in perennial crops.

Keywords: math modeling, methods of the math statistics, biometric genetics, fruit crops, apple tree, variety, rootstock, variety-rootstock combinations, regularities of the influence of variety and rootstock, the quantitative traits of the grafted plant, the yield forecast for variety-rootstock combinations, productivity, management.

In fruit plants that propagate vegetatively, the scion and rootstock are known to have a mutual effect on a newly formed scion-rootstock combination [1-5]. When using intensive technologies based on the maximum realization of

the biological potential of understock, obtaining accurate knowledge of quantitative traits that characterize the yield and biometrics of the obtained combinations is actual. In horticulture, extensive experimental data have been accumulated on the effect of rootstocks on the viability and adaptability to growing conditions, longevity, productivity, production quality and other properties of grafted plants [6-13]. At the same time, gardeners need to know about quantitative changes of agronomic traits in various combinations [14, 15].

In world practice, the ability of stocks to influence the size of plants and improve the productivity of understock trees is detected by means of long-term costly empirical testing [16-20]. The novelty of our approach is to predict the interaction between the genotypes of the scion and rootstock using biometric methods. Analysis of published sources [21-37] demonstrates the lack of the information on such studies, and the proposed approach is used in gardening for the first time.

Our purpose was to develop techniques for assessing the interaction between the genotypes of the scions and apple rootstocks based on the analysis of biometric parameters to identify the most economically promising combinations.

Technique. The research has been carried out in the Kuban area of the Krasnodar Territory at Experimental Production Farm Tsentral'noe of the North Caucasus Regional Research Institute of Horticulture and Viticulture (SKZNIISiV) within 22 years (1982-2003). Scion-rootstock combinations of apple (*Malus domestic*a Borkh.) were studied based on the four varieties (scions) (Idared, Golden Delicious, Jonathan, Korah) and seven rootstocks (I-48-1, I-47-55, I-48-46, M2, M3, M4, M7). The yield of understock trees was estimated annually using the weight method. Biometric studies were performed in 1983 and 1985. In combination of Korah and Idared varieties with the I-48-1, I-47-55, I-48-46, M2, M3, M4, M7 stocks, the width of the crown in the direction from north to south and from west to east (cm), height (cm), the diameter of the trunk (cm) were determined.

Statistical calculations were performed using the StatSoft Statistica v. 10.0 and Statgraphics XVI program [38]. The method of three-way analysis of variance was used to assess the contribution of the study year, the scion varieties, the rootstock and their interaction in various combinations to the variability of the yields. The two-way analysis of variance was used to verify the significance of differences between rootstocks, scions and the presence of the «rootstock-scion» interaction in the years of research. Average long-term data were analyzed using the Box & Whisker plot graphical representations of the data. The proportion of the effect of the study year, scion and rootstock varieties and their interactions on the morphological and anatomical characteristics of ubderstock trees were also determined by analysis of variance. To assess the strength of association of traits studied, Spearman and Pearson correlation analysis was used; to construct the models for yield forecasting in scion-rootstock combinations, multiple linear regression analysis was used.

Results. Analysis of variance is one of the most used methods in biology [39, 40]. Two-factor analysis of variance (Table 1) showed that the conditions of the year, the scion variety, the rootstock and their combination affected the yield significantly. The conditions of the year had the greatest effect (31.7 %), the variety and the stock accounted for about 1.0 %. The cumulative effect of the year and variety (17.9 %) was the second most important, whereas the combined effect of the year and the stock was almost 7.5 times lower (2.4 %). We succeeded to detect a slight but statistically significant effect of the combination of variety and rootstock (0.5 %). Interestingly, the cumulative effect of all three factors was the third most important (4.9 %). This once again confirms the complexity of the

mathematical modeling of laws describing the role of the scion and rootstock in the formation of quantitative traits.

1. Assessment of the effect of the conditions of the year, the scion and rootstock on the yield of obtained apple combinations by analysis of variance (EPF Tsentral'noe of SKZNIISiV, Krasnodar, 1982-2003)

Variability	df	mS	*F*	σ^2	The proportion of the effect in the total variance, %
Inter-year	21	235629.45	394.2*	465.41	31.7
Inter-variety	3	38581.43	64.6*	13.68	0.9
Inter-rootstock	6	22935.62	38.4*	14.07	1.0
Year × variety	63	33730.03	56.4*	262.43	17.9
Year × rootstock	126	3194.65	5.3*	36.00	2.4
Variety × rootstock	18	3671.20	6.1*	7.75	0.5
Year × variety × rootstock	378	1906.06	3.2*	72.54	4.9
Residual	10494	597.69		597.69	40.7

N o t e. df is a degree of freedom, mS is the mean square, *F* is the Fisher's variance ratio, and σ^2 is a variance.
* $p < 0.01$.

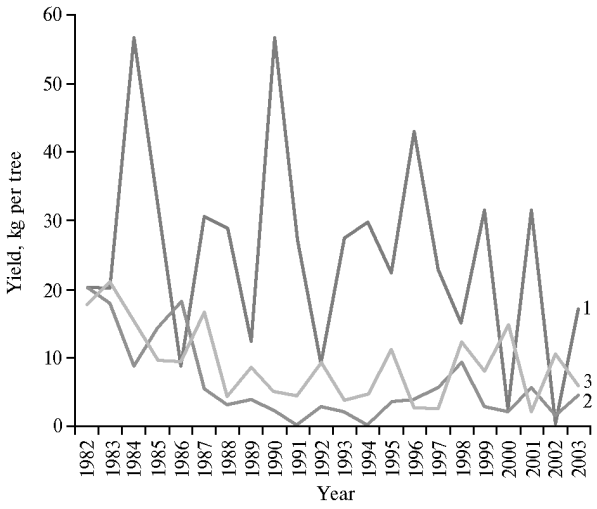

Fig. 1. The proportion of variety (scion) (1)**, rootstock** (2) **and combination of these factors** (3) **effect on the yield of apple scion-rootstock combinations on years of research** (EPF Tsentral'noe of SKZNIISiV, Krasnodar, 1982-2003).

Two-way analysis of variance showed a significant effect of the scion, rootstock and their interaction on the yield. This regularity was traced in all the years of research and in an average sample. The proportion of the scion effect on the yield ranged from 2.6 (the year of 2000) to 56.6 % (the year of 1984). The year of 2002 was an exception. The proportion of the rootstock effect was from 1.6 (the year of 2002) to 20.2 % (the year of 1982). Special conditions of 2002 that caused a sharp decline in variability in different scion-rootstock combinations should be noted. The effect of stock was not found in 1991 and 1994.

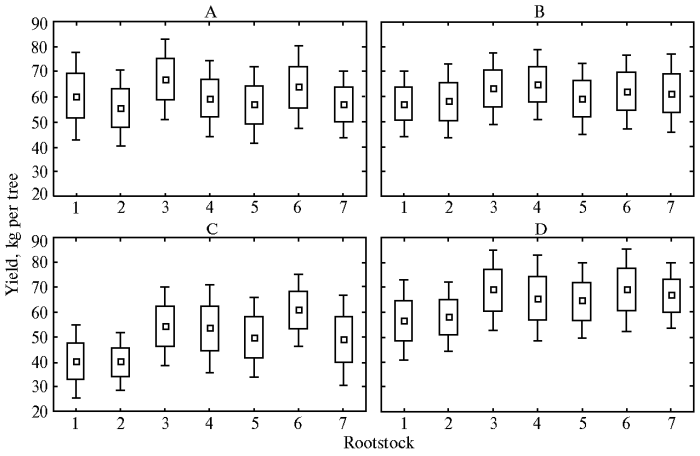

Fig. 2. Distribution of the statistical characteristics of yield in scion-rootstock combinations in the Idared (A)**, Golden Delicious** (B)**, Jonathan** (C)**, and Korah** (D) **apple tree varieties:** 1 — I-48-1, 2 — I-47-55, 3 — I-48-46, 4 — M2, 5 — M3, 6 — M4, 7 — M7 rootstocks (Krasnodar, EPF Tsentral'noe of SKZNIISiV, 1982-2003).

Annual effect of the rootstock varieties on the pro-

ductivity of scion-rootstock combinations was pronounced every few years (Fig. 1). Perhaps this is due to the periodicity of fruiting that is characteristic of apple trees, climatic conditions and their combination.

The shares of the effect and interaction of variety and rootstock on the yield were approximately equivalent. Average long-term data are shown by Box & Whisker plot graphical representations (Fig. 2). In the graphs of this type, points represent the arithmetic mean, the borders of the rectangle represent the error of the mean, the lines outside the borders of the rectangle represent confidence intervals which makes it possible to graphically describe the statistical characteristics of the samples. It is evident that the best yield was provided by I-48-46, I-48-1 and M4 rootstocks in Idared, by I-48-46, M2 and M4 rootstocks in Golden Delicious variety, by M4, I-48-46 and M2 rootstocks in Jonathan variety, and by I-48-46, M2 and M4 rootstocks in Korah variety (see Fig. 2).

The results of analysis of variance based on biometric studies demonstrated a very strong effect of the year conditions on all the traits studied. The proportion of variance ranged from 9.9 (height) to 63.4 % (trunk diameter). The effect of variety genotype was also found for all of the analyzed features. The variability due to this factor ranged from 0.1 (trunk diameter) to 17.8 % (yield). The effect of the stock appeared to be 3 times lower than in the variety, but statistically significant ranging from 0.8 (crown width from north to south) to 5.9 % (trunk diameter). The combined effect of the scion and the year conditions was from 2.0 (height) to 32.0 % (yield), and the combined effect of the rootstock genotype with the year conditions varied from 1.8 (crown width from north to south and yield) to 5.7 % (height). The crown width from west to east was the exception (the effect on this feature has not been determined).

In our opinion, detection of statistically significant interactions involving the variety and rootstock genotypes which was shown for the first time for all traits without exception was of the most importance. The cumulative effect of the variety and rootstock genotypes was from 2.1 (crown width from north to south and yield) to 7.2 % (height), and the cumulative effects of the variety and rootstock genotypes and of the year conditions varied from 0.9 (trunk diameter) to 14.0 % (height). Therefore, the effects of the variety and rootstock genotypes and of environmental conditions were approximately similar.

The effect of the year condition on the traits studied is of interest, and this assessment is important for creating effective selection programs. The interaction of the genotype with the environment in a population (set of varieties) results in the changes in the total phenotypic, genotypic and additive variance that can be estimated if the tests are performed at different places in the same year or within several years in the same locality. Where the test genotypes do not change the grades of efficiency in all environments, then the interaction of «genotype-environment» is zero (absent). In the experiment performed in the same location or within one year, the variance of genotype—environment interactions will be mixed with a random variation. The impossibility of their separation results in the displacement of the estimates of genetic parameters. Even if the error variance is estimated under ideal experimental conditions, genotypic variance and the variance of genotype—environment interaction remain combined which results in the wrong conclusions. In this regard, the need to perform all the genetic and selection experiments for a number of years or in a few locations is obvious.

Calculation of relative values of the various components of genotype—environment interactions in practical selection should provide a more rational distribution of resources in experiments. In particular, it will make it possible to pre-

dict the importance of setting tests in a larger number of locations or in a single location for a longer period of time. For the genotype reliable ranking the tests performed in the same environment but with a larger number of replications (as in our case) are as informative as the costly experiments in various environments. In addition, the study of the genotype and environment interaction helps to select varieties or hybrids with more adaptive capabilities.

Until now, the main methods for the study of the genotype-environment interaction effects are the ones based on the analysis of variance and the regression analysis that provide a sufficiently reliable estimate of the variability. So, it is possible to trace the nature of the variation of additive and non-additive gene effect depending on the growing conditions in the analysis of combining ability in diallel crosses. More attention should be paid to the contribution of the non-additive component in the effects of the genotype—environment interaction as an additively functioning gene does not interact neither with the genes in the cell, nor with the varying environmental factors.

The first step in constructing a mathematical model was to find correlations between traits. To do this, we used a pooled biometric sample of trees of various scion-rootstock combinations. This sample (about 800 trees) suggests that the traits will be subject to the normal distribution (this condition is mandatory for the use of parametric methods). The normality of trait distribution was estimated by χ^2 test ($\chi^2 = 561.77$, $p < 0.01$ for tree crown width from north to south; $\chi^2 = 1924.13$, $p < 0.01$ for tree crown width from west to east; $\chi^2 = 50.28$, $p < 0.01$ for height; $\chi^2 = 109.83$, $p < 0.01$ for trunk diameter; $\chi^2 = 1594.72$; $p < 0.01$ for yield).

2. Trait correlation coefficients in scion-rootstock apple tree combinations (EPF Tsentral'noe of SKZNIISiV, Krasnodar, 1982-2003)

Trait / Trait	Crown width		Height	Trunk diameter	Yield
	north to south	west to east			
Crown width					
north to south		0.80*	0.49*	–0.46*	0.47*
west to east	0.76*		0.42*	–0.27*	0.47*
Height	0.39*	0.39*		–0.07*	0.10*
Trunk diameter	–0.51*	–0.28*	–0.03		–0.13*
Yield	0.39*	0.35*	0.03	–0.08*	

N o t e. Pearson correlation coefficients are above the diagonal, Spearman correlation coefficients are below the diagonal.

* Association of traits is reliable at the 5 % significance level.

The analysis was performed using Pearson's correlation test parametric and non-parametric Spearman rank correlation test (Table 2). Pearson correlation coefficient was more effective as it is intended for such a distribution. Spearman's correlation coefficient was calculated for the two pre-ranked variables. An inadequate result obtained with it can be attributed to frequent same values. When ranking these, there is a problem of tied ranks. In this case, a special rule applies according to which the objects with the same values are attributed to the same average rank. In the presence of similar (tied) ranks, the Spearman rank correlation formula cannot be used.

Mathematical modeling to analyze the relationship of the scion and rootstock requires the detection of significant variables of the system and setting links between them, so that the model yielded the same result of behavior as the object under study. This model is able to predict the behavior of the system in different environments, especially those poorly understood, such as the interaction of the scion and the rootstock in a new two-component fruit plant. When it comes to phenomena and processes with a complex structure and diversity of inherent relationships, this analysis is very complicated.

For further studies, the following linear models were used that suggest the observed values to be interconnected by dependence:

$$y_i = b_0 + b_1 x_i + c_i, \quad (1)$$

Where b_0, b_1 are unknown parameters (coefficients of the equation), c_i is independent normally distributed random variables with a zero expected value and a σ^2 variance. This procedure was needed to construct a b_1, b_0 model and confidence intervals for b_1, b_0 according to the observations of x_i, y_i in the best way, and to test the hypothesis on the significance of the equation and the regression coefficients, as well as to assess the adequacy of the obtained dependence.

A multiple regression model with several predictors was used:

$$y_i = b_1 x_{1i} + b_2 x_{2i} + ... + b_p x_{pi} + b_0 + c_i, \quad (2)$$

where b_0, b_1, b_2, ... b_p are unknown model parameters.

A linear multiple regression in the Statgraphics XVI software package resulted in the following model:

$$\text{Yield} = -30.0149 + 0.183946 \times \text{crown width from north to south} + 0.137848 \times \text{crown width from west to east} - 0.145484 \times \text{height} + 0.564993 \times \text{trunk diameter}. \quad (3)$$

The analysis of results show a weak association of the response and the predictors ($R^2 = 0.28$), the constructed linear regression adequately described the association of the response and the predictors, and the absolute term was statistically significant.

There are several reasons to assume the linearity of trait association in regression analysis. Often, such an assumption is the simplest, so it becomes a natural to start the analysis with it. Many mathematical methods adapted to the solution of linear problems, forcing the use of linear circuits, even in cases where there are serious grounds to expect that the real association is significantly different from the linear one. Moreover, as a rule, all dependencies in the surrounding nature are nonlinear. Nevertheless, there are dependencies, the linearity of which in the considered applications is practically significant with any reasonable degree of accuracy. In the construction of mathematical models, the assumption of linearity is much more likely to have a distinct nature of the assumption, though it is not always stated as such. Therefore, when modeling the association of the processes and phenomena studied, nonlinear regression models are appropriate to be considered along with linear regression models. Typically, the need for a nonlinear regression appears if the researcher obtains data about the inadequacy of the linear model, and some nonlinear terms are added to the equation to clarify it [38].

In general, the regression model can be expressed as follows:

$$Y = F(X_1, X_2, ..., X_n). \quad (4)$$

To model the association of the yield and morpho-physiological characteristics of plant stocks the nonlinear coupling of these features that we have discovered should be taken into account.

Physiologists have found that the association of the productivity of an object and the degree of its physiological arousal is expressed by regression excitation equation:

$$Y = b_0 + b_1 X + b_2 X^2, \quad (5)$$

where b_0 is an absolute term, b_1 and b_2 are regression coefficients, Y and X are the values that characterize productivity and excitation, respectively. The nonlinearity of the model is expressed by the X^2 term. This model is called nonlinear in the variables. It allows linearization which can be done by the replacement: $X = X_1$, $X^2 = X_2$. The equation takes the following form:

$$Y = b_0 + b_1 X_1 + b_2 X_2. \quad (6)$$

Using the Fixed Nonlinear Regression module, multiple linear regres-

sion analysis of the linearized model was performed. Due to the software limitations on the number of variables, the trait of the crown width from north to south had to be excluded from the analysis, and the second trait of this category (crown width from west to east) was left in the analysis. Of the various options for linearizing transformations, the best one was:

$$X = X^2. \quad (7)$$

To assess the adequacy of the resulting model, a histogram of residual was constructed (Fig. 3). Residual is the difference of observed and fitted (or predicted by the model) values. One of the conditions for the correct use of regression analysis is the matching of residuals obtained and the normal distribution. Figure 3 shows that this term was satisfied, that is we constructed an adequate nonlinear (quadratic) model of the dependence between the yield in variety-rootstock combinations and morphological and anatomical features of grafted trees. The resulting model was as follows:

$$\text{Yield} = 171.3953 - 0.4599 \times \text{crown width from west to east} - 0.7876 \times \text{height} + 5.5432 \times \text{trunk diameter} + 0{,}0008 \times \text{crown width from west to east}^2 + 0.0009 \times \text{height}^2 - 0.0628 \times \text{trunk diameter}^2. \quad (8)$$

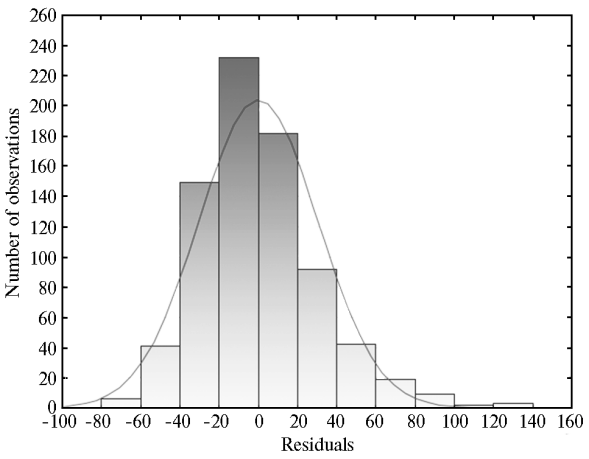

Fig. 3. Histogram of residuals in the evaluation of the correctness of the regression analysis used to model the association of the yield and morphological and physiological features of grafted apple trees according to long-term observation (Krasnodar, EPF Tsentral'noe of SKZNIISiV, 1982-2003).

We note that the adjusted multiple regression equation with a linearized model has a coefficient of determination $R^2 = 0.53$ which refers to the average power of association of the response and the predictors. In other words, the proposed model describes 53 % of the original variation, allowing further improving the efficiency of the yield prediction in the grafted fruit trees.

Thus, we first used biometric methods to identify the interaction between the stock and scion genotypes in apple varieties. It was found that the studied traits are significantly affected by the conditions of the year (37 % of the total variance). The power of the grafted variety effects on the yield of rootstock-scion combinations has pronounced intervals of several years. The proportion of the rootstock effect and the cumulative effect of variety and rootstock on the analyzed traits were approximately equivalent. For the first time, for the biological objects which are characterized by nonlinear trait associations, it was found that multiple linear regression analysis with the linearized model was more effective in the construction of prognostic models of the yield of scion-rootstock combinations. It is a promising methodological approach which takes into account the inadequacy of the previously considered linear models. The correctness of regression analysis application was confirmed by the histogram of residuals. Their distribution was proven to correspond to normal. It provides a basis for the construction of an adequate nonlinear (quadratic) model for the dependence of the productivity of scion-rootstock combination on morphological and anatomical features of grafted trees. The approach to the mathematical modeling of the regularities describing the effect of the scion and the

rootstock genotype on the formation of quantitative traits in grafted plants (primarily its yield), which was for the first time theoretically proven and developed by us using the data from 22 years of observations, will allow to more accurately and reasonably control the stability and efficiency of the production process in perennial crops.

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MAIZE (*Zea mays* L.) INBRED LINES AND HYBRIDS OF SERBIAN SELECTION WITH HIGH EFFICIENCY OF PHOTOSYNTHESIS, RICH IN PIGMENT CONTENT AND INCREASED NUTRITIVE VALUE

Ch. RADENOVICH[1, 2], N. DELICH[1], M. SECHANSKY[1], Zh. JOVANOVICH[1], G. STANKOVICH[1], A. POPOVICH[1]

[1]*Maize Research Institute, Zemun Polje*, ul. Slobodana Bajicha 1, 11185 Belgrade-Zemun, Serbia, e-mail radenovic@sbb.rs;
[2]*Faculty of Physical Chemistry, University of Belgrade,* Studentski trg 16, 11000 Belgrade, Serbia

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Abstract

This paper presents results of several different studies that confirm the hypothesis that maize inbred lines rich in proteins and with exceptional nutritive values can be bred. This is also supported by a medicinal standpoint of programmed need for maize-based food and feed. With such an experimental approach, the maize inbred lines ZPPL 146 and ZPPL 159 and hybrids derived from them (ZP 633, ZP 735 and ZP 737) rich in proteins have been systematically tested. Based on concrete results the following we can be conclude as follows. Selected maize inbred lines ZPPL 146 and ZPPL 159, rich in carotenoids, yellow pigments, also have significant amounts of other relevant bioactive compounds. Observed inbred lines have erect top leaves and are classified into a group of maize inbreds with significant properties of the photosynthesis model, are resistant to high temperatures and tolerant to drought. Spectral bands pointing to conformational characteristics of molecules of carotenoids but also of other compounds (phosphates, glutens and amides III) were established by the resonance Raman spectroscopy method applied to the leaf of the maize inbred line ZPLL 146. Physiological, biochemical and biophysical traits of elite maize inbred lines and their hybrids were observed in this study with a special emphasis on their efficiency of photosynthesis, productivity and suitability for broad use of nutrient values of grain and other essential biogenic matters, first of all pigments that express antioxidative properties. Relevant traits, properties and parameters of observed maize inbred lines that can be used in the process of selection are presented. These prestigious maize inbred lines were used to develop high-yielding and high-quality maize hybrids (ZP 633, ZP 735 and ZP 737) that are recognisable for their quality in human nutrition (children and the elderly), that are confirmed by medical observations related to their use in food and feed. Relevant agronomic and morphological traits of maize hybrids are presented. Moreover, results on grain structure and yields obtained in the regions of south-eastern Europe are also displayed.

Keywords: maize (*Zea mays* L.), inbred lines, hybrid, thylakoid membrane, photosynthetic model, delayed chlorophyll fluorescence, pigment properties, nutritive value.

The period since 1954 to date is historically significant due to numerous accomplishments in maize breeding and selection of maize hybrids and the production of hybrid seeds of high quality. As a result of such activity there are more than 1,400 developed grain and silage maize hybrids as well as hybrids for the industrial processing [1-4]. During this period, up-to-date technical and technological prerequisites for the modern process of breeding, the production of hybrid seeds and sufficient amounts of commercial seeds, have been provided [5-9]. Certainly, diverse interdependent studies of several scientific disciplines (biophysics, biochemistry, biotechnology, photosynthesis and Raman spectroscopy) were intermingled into the complex of developmental trends with the aim to modernise and efficiently implement contemporary programmes in maize breed-

ing and seed production [10-16] In addition to the exceptional results obtained in breeding of standard maize hybrids for grain, silage and industrial processing, there was the need to develop elite inbred lines and quality maize hybrids with improved chemical composition of essential biogenic compounds [17-22].

To meet many demands and justifiable needs for quality nutrition of people (mainly children and the elderly), domestic animals, as well as for industrial processing (semi- and final products) it was necessary to select maize inbred lines with significantly richer pigment-complex properties and the exceptional nutritional value. With such inbred lines it was possible to develop high-quality maize hybrids, which would meet established medicinal criteria regarding healthy nutrition of people, domestic animals, as well as broadly developed industrial processing.

The purposes of the present study were to compare physiological, biochemical, biophysical features of the maize elite inbred lines and their hybrids, to assess the effectiveness of plant photosynthesis, productivity and suitability for wide practical use, nutritive value of grain and accumulation of essential biogenic substances, in particular, pigments, possessing high antioxidant activity.

Technique. The studies were performed with two elite maize inbred lines, ZPPL 146 and ZPPL 159, and the hybrids ZP 633, ZP 735 and ZP 737 developed from them. The observed maize inbreds and hybrids belong to the collection of the Maize Research Institute, Zemun Polje, Belgrade, Serbia.

Methods applied to determine the grain chemical composition of maize inbred lines and hybrids are generally accepted and standardised and already described in detail in previous papers [17, 22-26]. The resonance Raman spectroscopy of maize inbred line leaves were done in accordance with the procedure and the method described in our previously published manuscripts [27-30]. The angle and the leaf area of maize inbred lines estimation was related to studying the erect position of top leaves in maize inbred lines. A specially designed protractor was used to measure the angle between the position of the above-ear leaf and the position of the plant stalk on maize inbred lines. The leaf area was measured by the LI-3000 portable leaf area meter (LI-COR Biosciences, USA). Measures of the angle between the above-ear leaf and the stalk and the leaf areas were carried out on 126 plants for each inbred line during the three-year period. These procedures had been described in previously published papers [15, 26].

The series of the experiments was related to photosynthetic-fluorescence measurements, including thermal processes of delayed chlorophyll fluorescence (DF), critical temperatures of phase transitions and activation energies. The test maize inbreds were grown in the experimental field of the Maize Research Institute, Zemun Polje. These inbreds were brought to the laboratory between 7 a.m. and 8 a.m. Plants sampled in the field were transversally cut in the ground internode. In the laboratory, plants were placed in water up to the second internode. Prior to the fluorescence experiment, all plants were kept under the black ball glass for two hours. A segment of intact above ear leaves was taken from such plants and placed into a chamber of the phosphoroscope. The intact leaf segments were kept in the chamber (in the dark) for at least 15 minutes, and then thermal processes of DF were measured. These tests were performed on 118 plants of each maize inbred line. An improved, non-invasive photosynthetic-fluorescence method was applied for these measurements. This method was developed at the Maize Research Institute.

The equipment for measurement of chlorophyll DF has been already described in the previously published papers [12-14, 26, 31-33]. However the device for recording of thermal induction processes of chlorophyll DF, critical

temperatures of phase transitions and activation energies is much different and has been described in detail in the previously published paper [33]. The distinctive components of this devise are thermistors, embedded in the phosporoscope, i.e. the dark chamber, and a temperature controller PRT 3000 (Sefram, France). The temperature controller maintains a constant, preset temperature of the sample (leaf segment) or it changes the temperature within the range of 25 °C to 60 °C at a predetermined time interval, while the thermistor in contact with the surface of a leaf segment provides the accurate temperature measurement.

Numerous and long-term studies of yields ($t \cdot ha^{-1}$) of the three high-yielding and high-quality grain and silage maize hybrids (ZP 633, ZP 735 and ZP 737) were performed in many different locations in Serbia and other countries of south-eastern Europe. Standard methods for contemporary maize production, tinning and processing were applied in these studies [17, 20, 34].

Data obtained were processed statistically using relevant software.

Results. Empirical efforts to acquire knowledge about the need for maize diet in human nutrition were initiated a long time ago, perhaps 200 years ago. Much later, in the 1950s, scientific literature related to this topic emerged, primarily in medicinal institutions. However, the authors of this study became interested in this topic in the early 1990s [35].

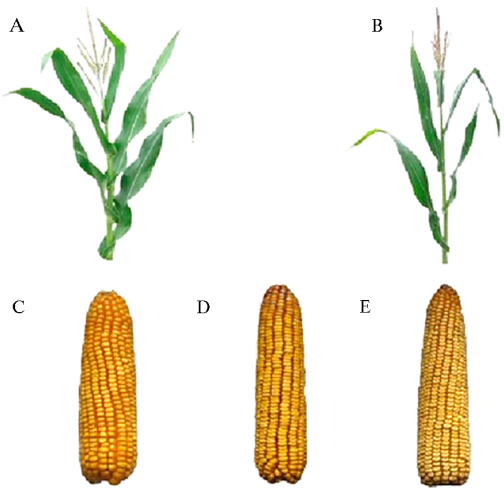

Fig. 1. Actual appearance of elite maize (*Zea mays* L.) inbred lines with erect top leaves: ZPPL 146 (A) **and ZPPL 159** (B)**, and ears of high-quality hybrids ZP 633** (C)**, ZP 735** (D) **and ZP 737** (E) **of Serbian selection.**

As these are inbred lines with significant breeding traits and specific chemical properties and maize hybrids (Fig. 1) with their use programmed for nutrition of people, domestic animals and for industrial processing, their traits will be separately presented in this manuscript. Note, comprehensive studies of the elite inbred lines and hybrids with erect top leaves developed from these inbreds encompassed several series of experiments in which both conventional and new own modern methods were applied.

1. Chemical composition of grain in maize (*Zea mays* L.) elite inbred lines and hybrids of Serbian selection (an average for 3 years, trial field of Maize Research Institute, Zemun Polje, Beograd, Serbia)

Parameter	Published data (24)		Average grain chemical composition				
	range	average	lines		hybrids		
			ZPPL 146	ZPPL 159	ZP 633	ZP 735	ZP 737
Moisture, %	7-23	16.0	10.24	10.12	9.90	9.84	10.15
Starch, %	61-78	71.7	67.80	66.26	68.23	64.39	67.86
Protein, %	6-12	9.5	10.22	12.57	11.11	12.27	11.57
Fat (oil), %	1.0-5.7	4.3	7.53	5.38	6.11	5.82	7.16
Ash, %	1.1-3.9	1.4	1.48	1.45	1.51	1.54	1.47
Ash, %		3.0	2.26	2.33	2.37	2.43	2.00
Pentose (ribose and desoxyribose), %	5.8-6.6	6.2	–	–	–	–	–
Fibres, %	8.3-11.9	9.5	–	–	–	–	–
Cellulose + lignin, %	3.3-4.3	3.3	–	–	–	–	–
Total sugars (as glucose), %	1.0-3.0	2.6	–	–	–	–	–

Continued Table 1

Yellow pigment, μgβCE/g d.m.	–	–	19.00	18.10	27.30	21.90	21.60
Total carotenoids, mg/kg	12-36	26	33.2	31.8	32.4	28.3	27.8

N o t e. Yellow pigment assessment (d.m. mrans dry matter) was done by the AACC method: AACC (Amer.Assoc. of Cereal Chem. USA), 1995. Pigment. Methods 14-50 [25]. Dashes mean low influence of the rest parameters on nutritive value in the inbred lines and hybrids.

Results on overall studies of grain chemical composition of observed maize inbred lines and hybrids are presented in Table 1. Obtained results relate to important chemical constituents and are supplemented with results of chemical compositions of vitamins, dietary fibres and other biogenic and medicinal compounds [24, 25].

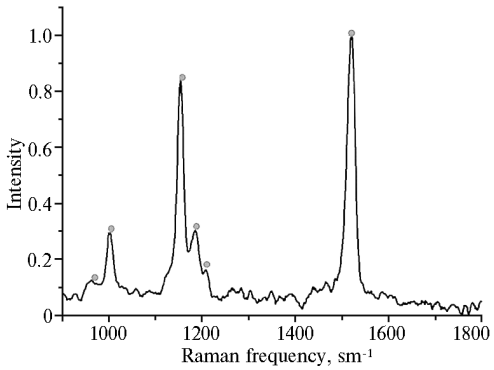

Fig. 2. Resonance Raman spectrum of the leaf of the maize (*Zea mays* L.) elite inbred line ZPPL 146 of Serbian selection.

This paper presents a typical example of a leaf resonance Raman spectrum of the maize inbred line ZPPL 146, i.e. a carotenoid molecule placed on a non-polar phase of a thylakoid membrane (Fig. 2).

The following six characteristic resonance Raman spectral bands were established within the 900-1800 cm^{-1} interval of Raman frequencies: 962, 1026, 1160, 1187, 1206 and 1520 cm^{-1}. Four spectral bands with smaller intensity (I_{926}, I_{1026}, I_{1187}, I_{1206}) were caused by conformational changes of phosphates, glycogens, amides III. The remaining two spectral bands with a significantly higher intensity (I_{1160}, I_{1520}) are regularly analysed in relation to the conformational changes in the carotenoid molecule. It is common to analyse the differences in the intensities of spectral bands (I_{1520} and I_{1160}), and even more often to analyse the differences in their ratio (I_{1520}/I_{1160}). Figure 2 presents the resonance Raman spectrum of the leaf of the inbred line ZPPL 146 with dominant spectral bands (I_{1520} and I_{1160}) that condition the carotenoid molecules placed in the non-polar phase of the thylakoid membrane of the leaf of the inbred line ZPPL 146. In this paper, the effort was made to emphasise the application of resonance Raman spectroscopy in studying important vital functions of leaves of maize inbred lines, especially under agroecological conditions atypical for the maize growing region. Carotenoid molecules (β caroten, $C_{40}H_{56}$, with the activity of vitamin A, but also two xanthophylls: cryptoxanthin $C_{40}H_{56}O$ and zeaxanthin $C_{40}H_{56}O_2$), since localised in non-polar phase of the thylakoid membrane of maize inbred leaves, showed to be a very suitable natural probe, capable to contribute to registering not only higher and more significant, but also smaller and finer conformational changes. These changes in the molecular structure of carotenoids may be expressed in the form of bending, stretching, compressing and physical disruption of chemical bonds, which is caused by intensive actions of environmental factors, first of all of unfavourable critical temperatures. In the end, each conformational change in the carotenoid molecule unconditionally changes the function not only of the carotenoid molecule but also of the thylakoid membrane in leaves of maize inbred lines. Conformational changes in chemical bonds −C=C− are reflected in the spectral band at 1520 cm^{-1}. In addition, conformational changes in chemical bonds =C−C= are reflected in the spectral band at 1160 cm^{-1} (Fig. 2) [36].

Results on the measures of angles between the above-ear leaf and the stalk, as well as, the average leaf areas are presented in Table 2. Based on obtained results on the measures of angles it can be stated that the observed maize elite inbred lines belong to the group of recently developed inbred lines with erect top leaves and a trait of a photosynthetic model.

2. Angle between the above-ear leaf and the stalk and the leaf area in maize (*Zea mays* L.) inbred lines of Serbian selection with efficient photosynthesis (an average for 3 years, trial field of Maize Research Institute, Zemun Polje, Beograd, Serbia)

Line	FAO maturity group	Heterotic origin of inbred line	Angle of the above-ear leaf, °		The above-ear leaf area, sm^2	
			$\bar{X}$	σ	$\bar{X}$	σ
ZPPL 146	650-700	BSSS, USA, Zemun Polje	20.8	1.21	3762.7	238.00
ZPPL 159	550-600	Local population from Argentine (S13) crossed to the inbred PE25-10-1, Zemun Polje	21.3	1.23	2378.1	241.00

N o t e. Observed maize elite inbred lines represent good heterotic pairs, have good combining abilities for grain yield and silage, their propagation is well and they are highly yielding inbreds. These inbreds are rich in pigments and have extraordinary nutritive qualities (for detail see Table 6).

The experimental measures of changes in the stationary level of delayed chlorophyll fluorescence (I_{DF}) in dependence on the temperature, ranging from 25 to 60 °C, were performed. The dynamics of temperature dependence for observed maize inbred lines is presented in Figure 3 (A, B).

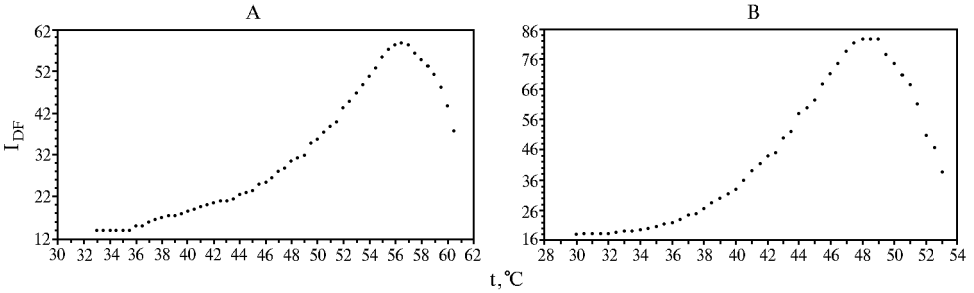

Fig. 3. Dynamics of the intensity of the delayed chlorophyll fluorescence (I_{DF}) of thermal processes in dependence on the effects of temperatures in chloroplasts and the thylakoid membrane of the intact above-ear leaf of the maize (*Zea mays* L.) elite inbred lines of Serbiam election with erect top leaves: ZPPL 146 (A) and ZPPL 159 (B).

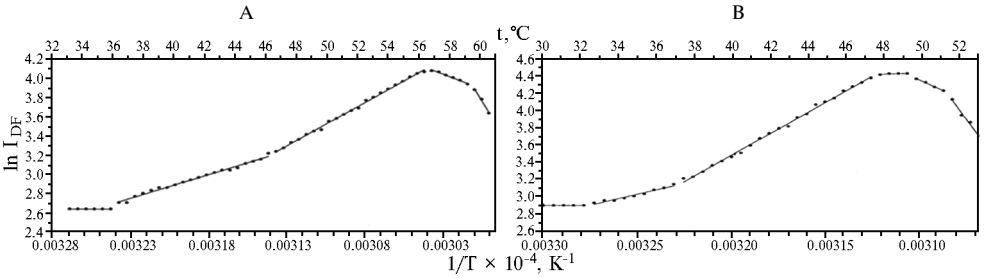

Fig. 4. The Arrhenius plot for the determination of critical temperatures (in Kelvins) **and conformational changes in chloroplasts and thylakoid membranes of the intact above-ear leaf of observed maize (*Zea mays* L.) elite inbred lines of Serbian selection with erect top leaves:** ZPPL 146 (A) and ZPPL 159 (B).

The Arrhenius plot is based on the linearisation of the DF temperature dependence of observed maize inbreds. Critical temperatures (phase transition temperatures) at which conformational changes occur in chloroplasts and the thylakoid membrane are determined by the application of the Arrhenius plot. Results of the Arrhenius plot application to observed new maize inbred lines are

presented in Figure 4 (A, B).

Detailed studies of the thermal processes of DF, and especially of the analysis of the experimental thermal curve, encompassed not only the temperature dependence and the Arrhenius plot, but also the estimation of values of activation energies (Ea) for critical temperatures (phase transition temperatures) in chloroplasts and the thylakoid membranes of the observed maize inbreds with erect top leaves: ZPPL 146 (A) and ZPPL 159 (B). Obtained results are shown in Table 3.

3. Changes in activation energies (Ea expressed in kJ/mol) **and critical temperatures t_{pt}** (phase transition temperatures expressed in °C) **in the thylakoid membrane of the intact above-ear leaf of observed maize (*Zea mays* L.) elite inbred lines of Serbian selection with erect top leaves**

ZPPL 146		ZPPL 159	
Ea	t_{pt}	Ea	t_{pt}
–	34.5	–	32.5
41.00	46.0	42.10	37.0
74.86	56.5	101.20	47.5
50.70	59.5	6.20	49.0
225.50	–	81.10	51.0
		255.00	–

N o t e. Dashes mean no calculation for initial and final values for delayed fluorescence.

Thus, theoretical data and the results of biophysical experimental investigation of pigments and thylakoid membranes in leaves, in particular, the delayed fluorescence of chlorophyll and phase transition in chloroplasts and thylakoid membranes) showed high adaptive potential in studied inbred lines at heat and drought conditions.

The same high-yielding and high-quality features were characteristic of maize hybrids ZP 633, ZP 735 and ZP 737 produced from the inbreed lines ZPPL 146 and ZPPL 159 that was shown in the field trials in different territories of the south-east Europe (Table 4). In these, the results also evidenced of functional relationship between yielding and specific character and adaptive potential of photosynthetic complex in ZP 633, ZP 735 and ZP 737 plants and their morphology which is in accordance with a model of effective photosynthesis. Their important agronomic and morphological traits of ZP 633, ZP 735 and ZP 737 hybrids are presented in Tables 4 and 5.

4. Agronomic traits of observed high-quality maize (*Zea mays* L.) hybrids in field trials

Trait, territory of growing	ZP 633	ZP 735	ZP 737
Hybrid designation	SC	SC	SC
FAO maturity group	550-650	750-850	750-850
Plant height, sm	250	280	290
Ear height, sm	120	130	135
1000-kernel weight, g	380	370	370
Kernel type	Semi-dent	Dent	Dent
Sowing density of silage hybrid, $\times 10^3$ plants · ha^{-1}	60-70	70-75	70-75
Leaf position on plant	Semi-erect to erect	Semi-erect to erect	Semi-erect to erect
Tolerance to drought	Good	Good	Good
Tolerance to diseases	Good	Good	Good
Leaf appearance at harvest	Stay-green	Stay-green	Stay-green
Hybrid silage yield, t · ha^{-1}	60-65	70-80	70-80
Hybrid grain yield, t · ha^{-1}	7.819[a]	8.108[b]	12.732[b]
Hybrid growing regions' altitude, m	300-400	250-400	250-400

N o t e. SC — Single Cross. Hybrid yield achieved in 30 locations in Serbia in the 2008-2011 period (a) and in 6 locations in Greece in the 2006-2009 period (b).

According to data presented in Tables 4 and 5, observed hybrids belong to long-season hybrids with modern architecture and the stay-green trait. Moreover, more than 50 % of grain of these hybrids is in the silage mass, which is

very important for silage quality. Also, the embryo content in grain amounts to above 10 %, which is especially important for quality of nutritive values of hybrids in nutrition of people (especially of children and the elderly) but also in nutrition of domestic animals, as well as for industrial processing of semi- and final products.

5. Ear morphological traits with a grain structure in observed high-quality maize (*Zea mays* L.) hybrids of Serbian selection

Parameter	ZP 633	ZP 735	ZP 737
Grain moisture, %	18	19	20
Ear length, sm	22	25	25
Ear weight,g	252.30	286.42	226.70
Rows per ear	16	18	18
Kernel number	700	800	850
Масса зерна на початке, g	228.36	248.35	200.40
Rate per ear, %:			
grain pericarp	5.32	6.55	4.60
grain embryo	11.28	12.06	10.70
grain endosperm	83.40	81.39	84.70

Note, the maize hybrids ZP 633, ZP 735 and ZP 737 are mainly indented for grain and silage production under agroecological conditions of south-eastern Europe. They are mostly used in the human diet and in nutrition of domestic animals, and to a significantly smaller extent in industrial processing and in the production of semi- and final products.

Studied maize elite inbred lines ZPPL 146 and ZPPL 159 have been broadly used in breeding for the last 5-6 years in Serbia. Moreover, they are widely used in Greece, Macedonia, and in part in Turkey and for south of Bulgaria. For these reasons, relevant observations of their total traits, performances and parameters are presented in Table 6.

6. Summary of significant breeding and seed production traits of effectively photosynthesizing maize (*Zea mays* L.) elite inbred lines of Serbian selection

Trait	ZPPL 146	ZPPL 159
Heterotic origin	BSSS, USA, Zemun Polje	Local population from Argentine (S13) crossed to the inbred PE25-10-1, Zemun Polje
FAO maturity group	650-700	550-600
Grain yield at 14 % moisture, $kg \cdot ha^{-1}$:		
dry land farming[a]	3,500	2,000
irrigation[b]	5,000	3,000
Number of plants at harvest, per ha:		
dry land farming	50,000	50,000
irrigation	60,000	60,000
Stalk properties	Stalk is moderately high with prolific trait. Tassel has elongated central branch with fewer side branches	Stalk is short. Tassel has closed side branches that shed long
Stalk resistance to lodging	Inbreds are resistant to lodging	
Erect position of above ear leaves	< 20.8° (first leaf) < 17.9° (second leaf) < 15.3° (third leaf)	< 21.3° (first leaf) < 18.1° (second leaf) < 15.4° (third leaf)
Stay-green phenotype	Leaf did not remain green until harvest	Leaf remained moderately green until harvest
Stress tolerance	Inbreds are tolerant to drought and high temperatures	
Kernel traits	Semi-dent type, orange kernels, while cob is white	Semi-flint, orange kernels, while cob is red
Grain moisture at harvest, %	20-25	20-25
Dry down rate in the stage of grain maturing	Dry down rate is fast, but hybrids are suited for silage	Dry down rate is not fast, but hybrids are suited for silage
Harvest type	Hand or machine harvest is easy	
Seedling	Inbred emerges well	
Early growth	Early growth is moderate	
Suitability for nutrition of ruminants and nonruminants	Grain is suitable for nutrition of ruminants, nonruminants, human nutrition and for industrial processing	

Continued Table 6

Carotene content in grain	33.2 mg/kg[a] 19.0 μg βCE/g d.m.[b]	31.8 mg/kg[a] 18.1 μg βCE/g d.m.[b]
Suitability for developing silage hybrids	Inbreds are very suitable for developing silage hybrids	
Digestibility of the hybrids developed	Hybrids developed from this inbreds have good digestibility of the whole plant and of milled grain	

N o t e. Carotene content is indicated for dry land ([a]) and irrigation ([b]).

When discussing maize breeding, it must be mentioned that the role of maize as healthy food is still underestimated. Maize is one of the potential energy sources. It contains very little fats, and at the same time, many carbohydrates. One maize ear contains 80-100 calories. Moreover, maize contains many plant fibres and therefore it lowers the levels of blood cholesterol and blood sugar, which lowers the risk of colon cancer [37]. Nevertheless, one should pay attention to allergy attacks as maize is one of the most common allergens. Maize provides vitamins C and B, folic acid and magnesium, which positively affects brain functioning. Phosphorus is essential nutrient for bones, while potassium is necessary for the regular functioning of individual cells in the organism. It is known that excessive levels of some amino acids might cause heart diseases [37, 38]. This condition occurs due to the lack of folic acid in the organs. For these reasons, the use of maize in the diet results in successful protection of the heart. In addition, folic acid is essential for the proper foetal nervous system development, hence maize is recommended for diets of women who intend to become pregnant, as well as pregnant women in the first three months of their pregnancy. In any form maize is very beneficial for normal growth and development and metabolism functions [39, 42]. Beside the stated, the most often medicinal benefits are based on the role of carotenoids. They protect plants from damages caused by photo-induced free radicals. Carotenoids, as precursors to horomones of abscisic acid, give kernels the yellow colour [39, 42]. It is important to point out to a special role of carotenoids as antioxidants in prevention of cardiovascular diseases, cancer and cataract.

As already said, for the last 60 years, a great success has been achieved in maize breeding and the production of high-quality foundation, hybrid and commercial maize seed. Since 1978, thanks to the maize breeding programme the number of plants per area unit has been significantly increasing. This maize breeding programme was referred to as a «plant density» programme and it further directly affected the yield increase of high quality foundation and hybrid maize seed as well as commercial seed [12, 13, 40]. Somewhat later, a programme on the development of maize inbred lines with erect top leaves (inbreds with efficient photosynthesis) was established. In 1998, it was considered that these inbreds were the closest to the proposed efficient photosynthetic model. Almost at the same time, a programme on the development of maize inbred lines rich in pigments and with other chemical properties and extraordinary nutritive values was established [7, 11, 19, 21-22, 37-39].

This study was an attempt to answer the following questions by using different tests and analyses: is there a reliable and dominant trait (one or more) of observed maize inbred lines rich in the pigment complex that would be the basis for the development of new high-quality maize hybrids that would be suitable for food, feed and industrial processing of semi- and final products? The analysis of presented overall results, obtained in the series of experiments, can easily give the positive answer to this question. Consequently, maize elite inbred lines (ZPPL 146 and ZPPL 159) and high-quality hybrids developed from them (ZP 633, ZP 735 and ZP 737) are the best confirmation of the stated. Selected inbred lines and hybrids developed from them are rich in pig-

ments, have significant nutritive values, especially of carotenoids that give kernels their yellow colour [40] that are used in the nutrition of poultry. Carotenoids positively affect health of both, people and animals [22, 37, 38, 41]. This aspect of observed maize inbred lines and hybrids will get priority within the healthy diet of people and animals.

Thus, according to the presented numerous and diverse results on studies of elite inbred lines (ZPPL 146 and ZPPL 159) and high-quality maize hybrids developed from these inbreds (ZP 633, ZP 735 and ZP 737), it can be concluded that ZPPL 146 and ZPPL 159 are rich in carotenoids, yellow pigments, also have significant amounts of other relevant biogenic compounds. Observed inbred lines have erect top leaves and are classified into a group of maize inbreds with significant properties of photosynthetic model and are tolerant to high temperatures. Spectral bands pointing to conformational characteristics of molecules of carotenoids but also other compounds (phosphates, glutens and amides III) were established by the resonance Raman spectroscopy method applied to the leaf of maize inbred lines. Relevant traits, properties and parameters of observed maize inbred lines indicate that the prospects of their use in breeding both for food and forage grain and green biomass for ensilaging. These maize elite inbred lines were used to develop high-quality maize hybrids (ZP 633, ZP 735, ZP 737) that are recognisable for their quality in human nutrition (children and the elderly), which is confirmed by medical observations related to their use in food, feed and industrial processing. Relevant agronomic and morphological traits of maize hybrids are presented. Moreover, results on grain structure and yields of Serbian lines and hybrids obtained in the regions of south-eastern Europe are also displayed.

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FEATURES OF THE PHENOLICS' FORMATION IN SEEDLINGS OF DIFFERENT VARIETIES OF BUCKWHEAT (*Fagopyrum esculentum* Moench)

V.V. KAZANTSEVA[1], E.A. GONCHARUK[1], A.N. FESENKO[2], A.V. SHIROKOVA[3], N.V. ZAGOSKINA[1]

[1]*K.A. Timiryazev Institute of Plant Physiology RAS,* Federal Agency of Scientific Organizations, 35, ul. Botanicheskaya, Moscow, 127276 Russia, e-mail phenolic2012@yandex.ru;
[2]*All-Russian Research Institute of Legumes and Groat Crops,* Federal Agency of Scientific Organizations, 10, korp. 1, ul. Molodezhnaya, pos. Streletskii, Orel Region, Orel Province, 302502 Russia, e-mail fesenko.a.n@rambler.ru;
[3]*N.K. Kol'tsov Institute of Developmental Biology RAS,* Federal Agency of Scientific Organizations, 26, ul. Vavilova, Moscow, 119334 Russia, e-mail glandularia@yahoo.com

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Abstract

A unique feature of higher plants is the capacity to form phenolic compounds, the substances with high antioxidant activity. These secondary metabolites play an important functional role, including cell and tissue protection against stress factors. It is especially important at the initial stages of plant ontogenesis. Buckwheat (*Fagopyrum esculentum* Moench) is the major cereal crop for which the formation of various phenolics, particularly rutin, a biologically active compound of plant origin successfully used in pharmacology, is characteristic. In the young seedlings (at the age of 14 days) of 10 buckwheat varieties mostly bred in Russian research centers during recent decades we studied the morphophysiological parameters and the accumulation of phenolic compounds, including phenylpropanoids and flavonoids, in the hypocotyls and cotyledons. Particularly, the highest level of phenylpropanoids was found in Bol'shevik 4 and Bashkirskaya krasnostebel'naya varieties. Note, it was high and almost equal in the hypocotyls and cotyledons. In the rest varieties the amount of phenylpropanoids in seedlings was 20 to 50 % lower, and in the cotyledons it was 1.5-2.0 times higher compared with the hypocotyls. Accumulation of flavonoids was higher in the Dialog variety, somewhat lower in Bol'shevik 4 and Bashkirskaya krasnostebel'naya varieties (by 10 and 17 %, respectively), and 35 to 40 % lower in the other studied forms. The highest content of anthocyanins was shown in the Bashkirskaya krasnostebel'naya plants, while in other studied buckwheat varieties it was lower. Particularly, in Devyatka, Bol'shevik 4 and Temp plants a decrease was about 50 %, and in Batyr, Dialog, Chatyr tau, Ilishevskaya, Dizain, and Dikul' varieties 70 to 80 % decrease was found. It was shown that recently bred buckwheat varieties have a very rapid growth and development of seedlings, which is important for their better adaptation in the early ontogeny. The most promising varieties, along with Bashkir krasnostebelnaya, are Bolshevik 4, Nine, Dialogue and Tempo. They are characterized by a high capacity for accumulation the phenolic compounds, the important components of the antioxidant defense system in plants. This feature of their metabolism may be a potential criterion for plant resistance to stress factors

Keywords*:* buckwheat, *Fagopyrum esculentum* Moench, varieties, phenolic compounds, phenylpropanoids, flavonoids, anthocyanins.

Buckwheat is the major cereal food crop in the world [1]. Russia, China and some other countries are the world leaders in buckwheat production [2, 3] with the crop area about 2.5 million hectares. In Russia buckwheat is mainly cultivated in Volga region, Central Chernozem region, Bashkortostan, Tatarstan, West and East Siberia. *Fagopyrum esculentum* Moench, a widely grown buckwheat species, is peculiar in high accumulation of fenolics [4, 5]. Buckwheat

leaves are raw material for commercial production of rutin which possesses antioxidative, angioprotective, antibacterial and hepatoprotective activity being an ingredient of a number of medicinal preparations [1].

At that, the buckwheat plants are less competitive compared to other groat crops due to specific biological features such as long flowering, unsynchronized maturation in the crops, sensitivity to low temperatures during early ontogenesis, etc. [6]. So breeding new varieties with tolerance to abiotic and biotic stresses have been widely carried out, when selected plants were mostly estimated morpho-physiologically, with regard to resistance to lodging and pathogens, high yield and grain quality [7, 8] while production of phenolic compounds in buckwheat plants is poorly studied [4, 9].

Phenolics are secondary metabolites produced mainly in higher plants [10, 11]. Phenolic compounds are numerous and extremely different in structure and chemical properties. There are phenylpropanoids, in particular hydroxycinnamic and hydroxybenzoic acids, and flavonoids (flavones, flavonols, anthocyanins, etc.). Phenolic compounds are formed in all plant tissues and their functional role is extremely varied and associated with the processes of photosynthesis, respiration, allelopathy, anti-stress effects, regulation of plant growth and development [10, 12]. The accumulation of these secondary metabolites depends on the plant species, stage of development and growth conditions [11, 13]. The study of the formation of polyphenols is of great practical interest, since they play an important role in the regulation of plant life. Furthermore, they have a high biological and antioxidant activity that makes their practical use successful. Currently, these secondary metabolites of higher plants, including the so-called bioflavonoids, are of great interest throughout the world with regard to their biosynthesis and its regulation, on one side, and the role in plant adaptation to stresses, on the other side [11, 14]. Much attention is also paid to the antioxidant activity of various compounds of phenolic nature and their use in medicine and pharmacology [15-17].

We have studied the morphophysiological characteristics of seedlings in 10 buckwheat varieties bred in the latter years mainly as related to the accumulation of different classes of phenolics, including flavonoids. This approach allows us to determine the peculiarities of their synthesis at early ontogenesis. This aspect is very important because during this period the buckwheat seedlings are exposed to different stresses such as low temperature, low humidity, etc., which leads to their death and, as a consequence, to a decrease in the crop yield. Due to estimation of endogenous levels of polyphenols in this period it is possible to presume a potential stability of plants, which is of practical importance.

Technique. We studied 10 buckwheat (*Fagopyrum esculentum* Moench) middle-ripening varieties bred mostly not long ago, with different heat and drought resistance. The varieties have been included in the State Register of breeding achievements of the Russian Federation. Seed samples were obtained from the collection of All-Russian Research Institute of Legumes and Groat Crops, except Bol'shevik 4 variety from the collection of Kropotovskaya bio-station of N.K. Kol'tsov Institute of Developmental Biology RAS. Seeds were kept in water for 24 hours without lightening, then placed into filter paper rolls and grown in phytotron chamber (K.A. Timiryazev Institute of Plant Physiology RAS) for 14 days at 24 °C with 16 hour photoperiod. In seedlings the stem height, root length, and the weight of cotyledons were evaluated. Water content in tissues was estimated after drying at 70 °C to constant weight.

Phenolic compounds were extracted from cotyledons and hypocotyls with 96 % ethanol. The presence of different classes of phenolic compounds in supernatant was analyzed spectophotometrically. Total soluble phenolic com-

pounds were determined at λ = 725 nm with the Folin and Denis reagent, and flavonoids were assayed at λ = 430 nm using 1 % $AlCl_3$ water solution [18, 19]. Calibration curves for rutin was used in both cases (Chemapol, Czech Republic) with the tested levels expressed as rutin milligram equivalents (ME) per 1 g dry weight. The level of pheniylpropanoids was measured directly in ethanol extracts at λ = 330 nm [20] using calibration curve for caffeic acid (Serva, Germany) with the tested compounds expressed as coffeic acid (ME) per 1 g dry weight. Anthocyanins were extracted from lower parts of hypocotyls using 3 % HCl in ethanol with direct spectophotommetry of the supernatant at λ = 525 nm [21]. Based on the calibration curves for cyaniding (Sigma, USA) the anthocyanin concentration was considered as equal amount of cyaniding (ME) per 1 g dry weight. A spectrophotometer SF 46 (Russia) was used.

Experiments were carried out in 3-fold biological and 3-fold analytical replicates. For obtained data processing the Statistica software were used. The figures show the average values and their standard deviations.

Results. The varieties involved in this investigation (Table 1) are due to attention paid by Russian researchers to theoretical background and practical use of buckwheat genetic systems in breeding programs.

1. Buckwheat (*Fagopyrum esculentum* Moench) Russian bred varieties involved in the study

Originator	Variety	Included in the State Register of the RF
Institute of Developmental Biology RAS, Moscow	Bol'shevik 4	1963
All-Russian Research Institute of Legumes and Groat Crops, Orel	Dikul'	1999
	Dialog	2008
	Dizain	2010
	Temp	2010
Tatar Research Institute of Agriculture, Kazan'	Devyatka	2004
	Chatyr Tau	2005
Bashkir Research Institute of Agriculture, Ufa	Ikishevskaya	2008
	Bashkirskaya krasnostebe'naya	2009

The highest levels of flavonoids is peculiar to Bashkirskaya krasnostebel'naya variety selected from a hybrid population produced by crossing red-flowered Rubra variety mutant and Chernoplodnaya, Ufimskaya and Chishminskaya varieties [8, 22]. As to another varieties, data are rare [4, 9] with estimation conducted mostly at late ontogenesis. The initial stages of buckwheat plant development are extremally poor studied with regard to growth and accumulation of polyphenols.

Data shown in the Table 2 indicates insignificant morphophysiological differences in juvenile plants between the varieties.

2. Morphometric parameters ($X \pm x$) of seedlings in different buckwheat (*Fagopyrum esculentum* Moench) varieties (laboratory tests)

Variety	Height, sm	Root length, sm	Weight of cotyledons, g
Bashkirskaya krasnostebe'naya	10.01±0.55	13.90±0.70	0.067±0.008
Dikul'	11.30±0.41	12.70±0.58	0.062±0.009
Bol'shevik 4	10.70±0.31	13.70±0.67	0.056±0.007
Batyr	11.43±0.25	11.04±0.87	0.062±0.008
Devyatka	13.10±0.38	11.20±0.59	0.063±0.005
Dialog	10.56±0.40	10.01±0.50	0.048±0.009
Dizain	12.30±0.30	11.00±0.83	0.073±0.008
Ilishevskaya	12.70±0.47	9.41±0.76	0.090±0.006
Tenp	12.80±0.33	10.30±0.96	0.054±0.009
Chatyr Tau	11.36±0.36	9.50±0.88	0.049±0.008

N o t e. For detail (origin of the varieties and time when they have been included into State Register of the RF) see Table 1 and text.

The seedlings of Devyatka, Ilishevskaya and Temp varieties were the

highest, and the seedlings of Bashkirskaya krasnostebel'naya and Dialog varieties were shortest. Minimal root length was observed in Ilishevskaya and Chatyr Tau varieties while in Bol'shevik 4 and Bashkirskaya krasnostebel'naya seedlings the root length was maximal. In all variants differences reached 30 %.

Note, as plant height decreased, the tendency to root length increase was traced, being more pronounced in Bashkirskaya krasnostebel'naya, Dikul' and Bol'shevik 4 plants, and, otherwise, the higher plants, the shorter roots were, particularly in Devyatka, Dizain, Ilishevskaya, Temp and Chatyr Tau plants. There were only Batyr and Dialog plants in which these parameters were in fact identical. Besides, in the buckwheat varieties originated in 2008-2010 more intensive growth was characteristic if compared to other varieties. This is important because the buckwheat plants in the early ontogeny are very sensitive to stress factors [3]. Accelerate growth and hence more rapid formation and development of cotyledon leaves allows seedlings to change rapidly the type of supply to autotrophic that provides them better survival and adaptation.

Cotyledon weight was the greatest in the Ilishevskaya seedlings (see Table 2) being lower in other varieties, e.g. by 20-23 % in Bashkirskaya krasnostebel'naya plants, by 28-30 % in Devyatka, Dikul'and Batyr plants, and by 40-53 % in temp, Catyr Tau and dialog varieties. These results are indicative of the differences in the formation and development of the cotyledons in the early ontogenesis of buckwheat seedlings.

An important indicator in evaluating the physiological state of the plant tissues is their water content, which depends on the structure, the age and conditions of cultivation of plants [23]. It is known that the water content of the leaves reaches a maximum at the beginning of the growing season, gradually decreasing toward its end [24]. The water content in cotyledon leaves of most varieties was 90 %, and only in the variety Dikul' it was higher reaching 93 %. For hypocotyls a little larger value (up to 95 %) occurred. This is probably due to the water-retaining capacity of the cells, while on the leaf surface the evaporation occurs due to stomata functions [25]. It should be emphasized that water content in hypocotyls and cotyledons in seedling did not depend on varietal specificity in buckwheat plants.

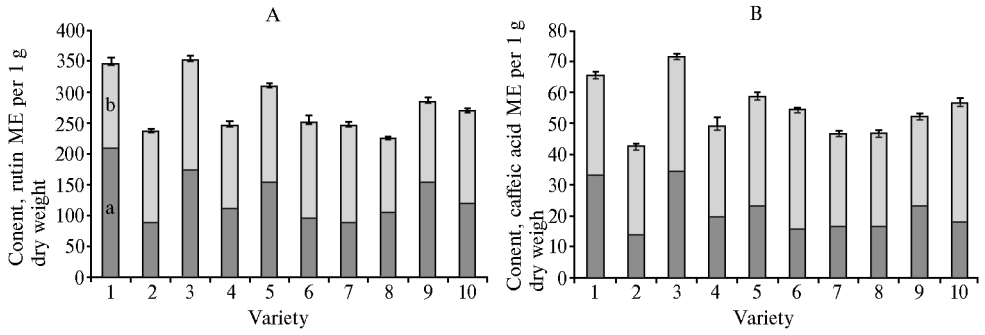

Fig. 1. Total phenolic compounds (A) **and phenylpropanoids** (B) **in hypocotyls** (a) **and cotyledons** (b) **in seedlings of different buckwheat (*Fagopyrum esculentum* Moench) varieties:** 1 — Bashkirskaya krasnostebe'naya; 2 — Dikul'; 3 — Bol'shevik 4; 4 — Batyr; 5 — Devyatka; 6 — Dialog; 7 — Dizain; 8 — Ilishevskaya; 9 — Temp; 10 — Chatyr Tau (laboratory experiments).

As already mentioned, the formation of different classes of phenolic compounds is characteristic for buckwheat [1, 4, 9]. Their total content, which reflects the biosynthetic capacity of plant tissues, showed the greatest indexes in young seedlings of the varieties Bashkirskaya krasnostebel'naya and Bol'sheviks 4 (Fig. 1, A). In the first case, it was due to higher level in hypocotyls compared with leaves, and in this almost 50 % difference occurs. Such a distribution of

phenolic compounds in the tissues of higher plants is rare. Probably, it was formed as a result of breeding varieties Bashkirskaya krasnostebel'naya aimed at making buckwheat with a high capacity for the biosynthesis of secondary metabolites [8]. A similar pattern of phenolic compounds' distribution, but expressed to a much lesser extent, can be traced in seedlings of Temp varieties obtained after repeated and negative mass selection of a combination of Donor KKG × line BO 3-5. In addition, in the State Register of selection achievements in the Russian Federation, the accumulation of phenolic compounds in this variety is not reported, and only its resistance to lodging and drought are mentioned. Thus, the Temp variety deserves attention, and its study should be continued.

In the variety Bilshovyk 4 the level of phenolic compounds was high and almost equal in the hypocotyls and the cotyledons. A similar trend was observed in seedling varieties Devyatka, although the total content of phenolic compounds was lower (by 13 % compared with the variety Bolshevik 4). In all other cases, a higher accumulation of these substances in the cotyledon leaves compared with hypocotyls were observed. The significant accumulation of phenolic compounds in the leaves, especially during the early stages of plant development, was reported [13, 26], that is to be expected, since the biosynthesis of phenolic compounds in the cells of higher plants depends on the chloroplast functions [10, 27, 28].

The major phenolic compounds in higher plants are phenylpropanoids and flavonoids [10, 11]. Phenylpropanoids are biogenetically earlier phenolic substances of phenol metabolism. These are C_6-C_3-compounds which can be accumulated in plant tissues and(or) used in the biosynthesis of flavonoids (C_6-C_3-C_6-compounds).

Phenylpropanoids are widely distributed in higher plants and included in the complex of buckwheat polyphenols [1]. The highest level was recorded in the buckwheat seedling of Bol'shevik 4 and Bashkirskaya krasnostebel'naya varieties (see Fig. 1, B). Reported results were high in hypocotyls and cotyledons being almost the same in value. In other cases, the level of phenylpropanoids in seedlings was 20-50 % lower and in the cotyledons it was 1.5-2.5 times higher than that in hypocotyls.

The main phenolic compounds of above-ground organs of the plants are flavonoids, comprising flavons, flavonols, flavonones, anthocyanidins, etc. [10]. In buckwheat plants the flavonols (rutin, quercetin, kaempferol and morin) and anthocyanins were found [1, 4, 9].

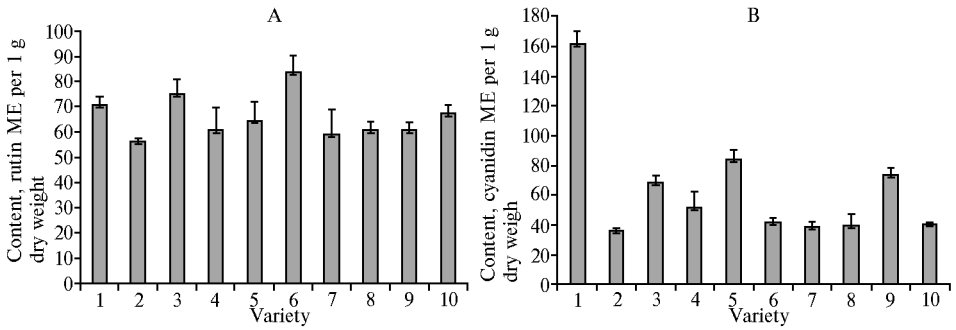

Fig. 2. Flavonoids in cotyledons (A) **and hypocotyls** (B) **in seedlings of different buckwheat (*Fagopyrum esculentum* Moench) varieties:** 1 — Bashkirskaya krasnostebe'naya; 2 — Dikul'; 3 — Bol'shevik 4; 4 — Batyr; 5 — Devyatka; 6 — Dialog; 7 — Dizain; 8 — Ilishevskaya; 9 — Temp; 10 — Chatyr Tau (laboratory experiments).

Accumulation of flavonoids in the cotyledon leaves were higher in buckwheat Dialog variety (Fig. 2, A), being slightly lower in Bol'shevik 4 and

Bashkirskaya krasnostebel'naya varieties (by 10 and 17 %, respectively) and much lower (by 35-40 %) in others.

Anthocyanins (pigments of higher plants) are phenolic substances [1, 10], which not only color organs of plants, but also participate in the protection of their tissues from stress factors (low temperature, heavy metal pollution, drought, etc.) [11]. Synthesis of anthocyanins is characteristic of the initial stages of the seedling development in buckwheat, and mostly for hypocotyl tissue (see Fig. 2, B). The greatest accumulation of anthocyanins was observed in Bashkirskaya krasnostebel'naya plants. It was lower by almost 50 % in Devyatka, Bol'shevik 4 and Temp plants, and by 70-80 % in Batyr, Dialog, Chater Tau, Ilishevskaya, Dizain and Dikul' plants

So, in the early ontogenesis the buckwheat varieties differ in morpho-physiology, distribution of phenolic compounds in the above-ground organs, and in the biosynthetic activity. Along with Bashkirskaya krasnostebel'naya variety, the Bol'shevik 4, Devayka, Dialog and Temp plants also may be deemed accumulating phenolic compounds. The high capacity of phenolic compounds accumulation as the major components of the plant antioxidant protective system can serve as a criterion of high plant resistance to stress factors.

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CHANGES OF ACTIVE COMPOUNDS IN APRICOT FRUITS CAUSED BY STORAGE DEPEND ON CHARACTERISTIC FEATURES OF VARIETIES

L.D. CHALAYA, T.G. PRICHKO

North-Caucasian Zonal Research Institute of Orcharding and Viniculture, Federal Agency of Scientific Organizations, 39, ul. Pobedy, Krasnodar, 350901 Russia, e-mail kubansad@kubannet.ru, prichko@yandex.ru
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Abstract

Apricots (*Armeniaca vulgaris* Lam.) contain a wide variety of nutrients such as sugars and acids, and preventive bioactive compounds such as vitamins, polyphenols and pectins which possess antioxidant activity and affect shelf life of fruits. Like other biological features, these properties are hereditarily determined. Therefore, proper selection of varieties, in addition to the optimal mode of storage, can help to reduce losses in commercial properties and deterioration of the chemical composition of apricots during storage because of rots, physiological diseases, and natural attrition. The article presents a comparative evaluation of the chemical composition and keeping quality of fruits in most common apricot varieties from Krasnodar region that are well adapted to the local conditions of growing. They are the Pineapple, Emerald, Red-Cheeked, Sunny, Amber, ripening in the second ten days of July, and the Krasnodar late, ripening in the third week of July. The differences in many chemical components of fruits peculiar to varieties have been found. An amount of soluble solids in the fruit ranged from 13.5 in Emerald variety to 18.8 % in Pineapple, and the sugars varied from 9.5 to 13.6 %, respectively. Apricot is a high acidic culture with fruits which can contain from 1.4 to 2.0 % organic acids. The high content of pectin at more than 1.0 % was also observed. Pineapple and Sunny varieties accumulated a significant amount of ascorbic acids which was superior to an average value of 14.0 mg/100 g for the central zone of the Krasnodar region. P-active catechins were detected in fruits at 45.6 to 155.9 mg/100 g. The high content of catechins at more than 100.0 mg/100 g was observed in Emerald, Red-Cheeked and Amber varieties. In the Pineapple variety the vitamin PP level of 0.6 mg/100 g was detected. According to L.V. Metlitskii (1976) vitamin PP is a component of many enzymes involved in cellular respiration, metabolism of proteins, and regulation of nervous activity. For full assessment of the biochemical peculiarities of apricots we investigated the content of β-carotene (provitamin A). In fruits with light yellow color of the pulp such as the Pineapple fruits the β-carotene content was 1.66 mg/100 g, in case of intense yellow color, particularly in Red-Cheeked and Krasnodar late varieties, it was 3.05 and 3.52 mg/100 g, respectively. The activity of polygalacturonase (PG) before computation of fruit for storage and during storage was also tested. The PG activity affecting fruit shelf life was the lowest at fruit harvesting. After 5 days of storage no PG activity was found in Sunny and Red-Cheeked varieties, while in Amber variety it increased slightly, indicating the varietal specificity. After 10 days of storage the PG activity increased and reached the maximum value by the day 15 being at peak in Sunny variety. Soluble pectins during storage were spent more intensively than protopectin, and in fruits with lower keeping quality, particularly in the Sunny variety, these processes were more active than in fruits with higher keeping quality, for instance in Amber variety. A 3.7 % decrease in sugars during storage was detected in Sunny and Emerald varieties. The acid level decreased by 10.0 % on average, and vitamin C and P-active catechin content became lower by 15.5 to 20.7 %. In Amber, Krasnodar late and Emerald plants the C vitamin activity remained relatively high. In Krasnodar late and Emerald apricot trees the β-carotene level did not change during storage. In the Sunny and Red-Cheeked varieties it decreased while in Amber increased slightly due to continuing maturation. At the end of storage a natural loss reached 6.8 % in Amber and Emerald varieties and 10.2 % in the Sunny variety. The best marketable quality at 60.0 and 57.4 % rate of fruits of the highest commercial grade were observed in Amber and Krasnodar late varieties. The Sunny variety which annually accumulated ascorbic acid at a high level is recommended as a parental form in breeding for high vitamin C content.

Keywords: apricots, Krasnodar region, varietal characteristics, bioactive substances, storage, pectolytic enzymes.

Apricot (*Armeniaca vulgaris* Lam.) is a fruit crop grown in the southern regions and the Central Chernozem region of the Russian Federation. A biological

feature of apricot is an early entry of flower buds in the flowering phase the timing of which ranged in the conditions of Krasnodar Territory from the first decade of March (1999) to the end of March (2011), that may affect both the value of the crop, and the quality of products. Apricot flowering is often accompanied by recurrent spring frosts or rainy and cloudy weather resulting in the onset of moniliosis (*Monilia fruktigena* Pers.), a dominant crop disease when flower buds are damaged partially or completely [1].

Currently, introduced varieties that are adapted to the conditions of the south of Russia are mainly grown at Kuban production plantations. The most common European varieties with average ripening such as Pineapple, Emerald, Red-Cheeked, Sunny, Amber, and Krasnodar late variety bred in the North Caucasian Regional Research Institute of Horticulture and Viticulture (SKZNIISiV) ripening in the second decade of July [2].

Apricot fruits contain a wide range of nutrient (sugar, acids) and prophylactic (vitamins, polyphenols, pectin) biologically active substances which not only have properties useful for human but also determine storage and ability to resist stress factors during vegetation [3-5]. The favorable effect of natural antioxidants on the quality kept under storage is confirmed by many authors dealing with storage of fruits of different cultures [6, 7]. There is evidence that apricot fruits can be stored no more than 15 days [1, 6]. The main reason for limiting period of storage is the damage by rot and decay from aging, which manifests itself in intensive softening of the fruit pulp, the loss of water and dissolved organic matter spent for breathing [8]. In this, cell turgor is reduced which results in tissue fading, enhancing decomposition processes in cells contained organic substances, in reduced resistance of fruit against microorganisms and reduced shelf life [6, 8].

In world practice, the methods of fruit preparation for storage using antioxidants are widespread, which allows to slow down the metabolic processes in the fruit and increase their resistance to stress factors, physiological diseases, and pathogens [7, 9]. But the potential of the fruit themselves and their capacity for long-term storage are not always taken into account.

The purpose of our research is to study the specialty of chemical composition of the fruit in the apricot varieties from the gene pool of the North Caucasian Regional Research Institute of Horticulture and Viticulture and their impact on the quality of the commodity and biochemical values of fruit during their storage.

Tehnique. We studied the introduced apricot varieties of the Eastern European average ripening sub-group (*Armeniaca vulgaris* Lam.) mostly common in Krasnodar Territory such as Pineapple (origin unknown), Emerald, Red-Cheeked (seedlings of unknown origin), Sunny (obtained by crossing selected hybrid forms with different varieties of apricot), Amber (obtained by sowing seeds from open pollination of the Yerevani variety), and the late-ripening Krasnodar late variety bred in the North Caucasian Regional Research Institute of Horticulture and Viticulture (obtained from seedling D 113). Selection of fruit was held in the Experimental Production Farm (EPF) Tsentral'noe (Krasnodar) in 1999-2011. Many of the studied varieties are the basis for regionalized assortment of varieties and valuable source material for breeding for resistance to frost and diseases.

The study of soluble solids content (SS) was performed using a RL-3 refractometer (Poland); acids were assayed by titration of the prepared sample with 0.1 N NaOH in the presence of phenolphthalein; sugars were analyzed by titration with the mixture of Felling solutions (I and II); ascorbic acid was estimated by titration of the samples with 0.001 N KIO_3; and for P-active compounds

(catechins) measurement a KFK-3-01-ZOMZ photo colorimeter (Russia) was used according to the L.I. Vigorov method [10]. To measure the amount of β-carotene, extraction from fruit was performed with petroleum ether followed by colorimetry using a KFK-4 (Russia); the content of nicotinic acid (vitamin PP) was estimated by the color intensity under the interaction with thiocyanogen bromide and metol using a KFK-3-01-ZOMZ photo colorimeter (Russia). Poly-galacturonase (PG) activity was measured using a KFK-3-01-ZOMZ each 5 days of storage starting from harvesting by the amount of destroyed pectin during enzymatic hydrolysis.

Apricots were stored according to Russian State Standard GOST 50519-93 [11]. All experiments were performed in 3-fold replicates. Mathematical data processing was performed by descriptive statistics and analysis of variance using the Microsoft Excel and Mathcaal 11A software package.

Results. The study of apricots of the Eastern European subgroup showed varietal characteristics on almost all values of the fruit chemical composition. Thus, the amount of soluble solids (SS) ranged from 13.5 (Emerald variety) to 18.8 % (Pineapple variety), and sugar content varied from 9.5 to 13.6 %, respectively (Table 1).

1. Main biochemical characteristics in the fruit of apricot varieties (*Armeniaca vulgaris* Lam.) ($X \pm x$, EPF *Tsentral'noe,* Krasnodar, 1999–2011)

Variety	SS, %	Total sugar,%	Acidity,%	SAR
Pineapple	18.2±1.2	13.6±1.0	1.6±0.40	8.1
Emerald	13.5±0.8	9.5±0.8	2.0±0.45	4.7
Red-Cheeked	15.5±2.4	10.9±0.6	2.0±0.08	5.4
Krasnodar late	15.2±0.8	10.3±1.4	1.4±0.08	7.1
Sunny	18.0±2.2	12.3±1.8	1.7±0.10	7.1
Amber	14.6±0.6	9.8±0.6	1.6±0.08	5.7
Average	15.8±1.3	11.1±1.0	1.7±0.32	6.4

N o t e. SS means soluble solids, SAR is a sugar to acid ratio.

The fruit of the studied varieties were highly acidic as they contained from 1.4 (Krasnodar late) to 2.0 % (Emerald, Red-Cheeked) organic acids including malic acid (up to 90.0 % of total acids) and minor amounts of citric, lactic, and amber acids. These data were partially confirmed by the results of the research performed by E.F.L.J. Anet and T.M. Reynolds [12] who found quinic acid, other than those listed, in apricots.

The sugar to acid ratio (SAR) which characterizes the flavor quality of fruits varied from 4.7 relative units (r.u.) in the Emerald variety, which corresponds to the sour taste, to 8.1 r.u. in the Pineapple variety with sour-sweet fruit.

Apricot contains significant amounts of pectins, mainly in water-soluble form. Therefore, the ratio of soluble pectin to protopectin in most varieties was more than 1.0 r.u. (Table 2).

2. Content (%) of pectin substances in the fruit of different apricot varieties (*Armeniaca vulgaris* Lam.) ($X \pm x$, EPF Tsentral'noe, Krasnodar, 1999-2011)

Variety	Soluble pectin	Protopectin	Total pectin substances	Ratio of pectin to protopectin, r.u.
Pineapple	0.51±0.12	0.51±0.11	1.02±0.12	1.00
Emerald	0.50±0.10	0.48±0.11	0.98±0.10	1.04
Red-Cheeked	0.56±0.14	0.46±0.10	1.02±0.12	1.20
Krasnodar late	0.62±0.06	0.57±0.14	0.99±0.12	1.09
Sunny	0.45±0.06	0.43±0.10	0.88±0.08	1.00
Amber	0.68±0.14	0.65±0.12	1.33±0.12	1.04
Average	0.52±0.12	0.52±0.11	1.04±0.11	1.06

The important role of pectin in the fruit stability against physiological disorders under storage is related to their high water-holding capacity, by which the cell turgor is maintained [6]. We selected the Amber variety in which the

content of pectin was stable regardless of the year of studies [3, 4].

Comparison of our data with the results obtained by Z.I. Kertesz [13] showed that most of the studied apricot varieties from Krasnodar Territory were not inferior to that grown in the United States in the content of total pectin in the fruit (in American varieties, it is an average of 1.03 %).

The vitamin composition is of great importance in antioxidant activity in fruits [5, 7]. The vitamin complex of apricots has been found to contain water- (C, P, PP) and liposoluble (β-carotene with vitamin A activity) compounds of different functional significance (Table 3).

3. Content (mg/100 g) **of vitamins in the fruit in different apricot (*Armeniaca vulgaris* Lam.) varieties** ($X \pm x$, EPF Tsentral'noe, Krasnodar, 1999-2011)

Variety	C	P	PP	β-carotene
Pineapple	17.3±2.50	52.6±4.40	0.55±0.04	1.66±0.04
Emerald	9.0±1.65	155.9±3.82	0.56±0.04	2.15±0.04
Red-Cheeked	11.6±1.10	120.3±4.22	0.43±0.02	3.05±0.05
Krasnodar late	13.5±2.04	62.9±1.20	0.50±0.03	3.52±0.04
Sunny	21.9±3.60	45.6±2.22	0.60±0.04	2.62±0.04
Amber	10.5±1.45	154.4±3.86	0.42±0.01	2.36±0.02
Average	14.0±2.05	98.6±3.26	0.51±0.03	2.56±0.04

Note, the Pineapple and Sunny varieties accumulated a significant amount of ascorbic acid (17.3 and 21.9 mg/100 g, respectively) exceeding the average content (14.0 mg/100 g) typical of the central zone of Krasnodar Territory.

The biological value of fruits can be judged by the accumulation of vitamin P which serves as a vitamin C synergist, increasing the biological effect of the latter. Many phenolic substances including catechins have P-vitamin activity [14-16]. P-active catechins were detected in fruits at 45.6 to 155.9 mg/100 g level. The high content of catechins at more than 100.0 mg/100 g was observed in Emerald, Red-Cheeked and Amber varieties.

One of the important properties of apricot fruits is a relatively high level of vitamin PP which is part of many enzymes involved in cellular respiration, protein metabolism, regulation of nervous activity [17]. Biochemical importance of vitamin PP is due to its use in the synthesis of coenzymes, the nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) that have owns one of the main roles in biological oxidation [6]. Some varieties (Emerald, Pineapple, Sunny) were found to contain over 0.55 mg of vitamin PP per 100 g. This allows attributing apricot to a group of crops with high biochemical parameters, since few of crops contain the same amount of vitamin PP and other vitamins [17].

One of the distinguishing features of apricot is the presence of carotenoid pigments. The studies of H. Brockmann [18] for the first time found that apricot carotenoids consist mainly of β-carotene which has the highest biological activity and gives the fruits their valuable medicinal properties and nice appearance. Depending on the content of β-carotene, the studied fruits had light yellow pulp (Pineapple variety with β-carotene content of 1.66 mg/100 g) or intense yellow pulp (Red-Cheeked and Krasnodar late varieties with β-carotene content of 3.05 and 3.52 mg/100 g, respectively).

Analyzing the chemical composition of the species studied, we can conclude that the fruits of apricot provide the daily intake of vitamins, polyphenols and other components for various groups of the population [19].

Due to the problem of fruit storage, especially of stone fruit storage, to study of the activity of pectolytic enzymes including polygalacturonase (PG) which destroys the 1.4-glycoside bond in the demethoxylated pectin molecule (pectin acids) and influences the shelf life of fruits, is of great importance [6].

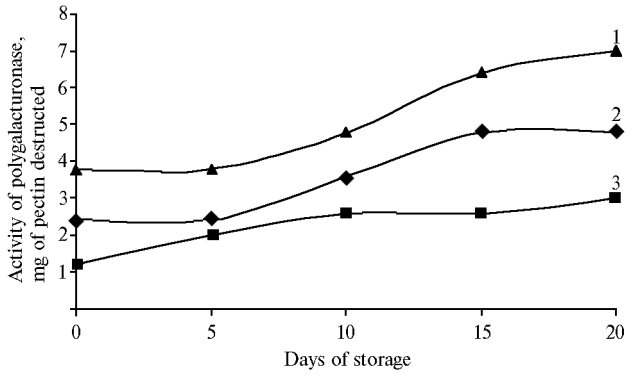

Fig. 1. Dynamics of changes in the activity of polygalacturonase in the fruit during their storage in refrigerating chambers in different varieties of apricot (*Armeniaca vulgaris* Lam.): 1, 2, 3— Sunny, Red-Cheeked, Amber varieties (EPF Tsentral'noe, Krasnodar, 2011).

In the research the lowest PG activity in fruit was demonstrated at harvesting. It was 1.2 mg of destroyed pectin in the Amber variety, 2.4 mg in the Red-Cheeked variety, and 3.8 mg in the Sunny variety. After 5 day storage the PG activity was not found in Sunny and Red-Cheeked varieties, while in Amber variety it increased slightly (Fig. 1). The peak PG activity was found in the Sunny variety with 7.0 mg of destroyed pectin found at the end of the storage, while in the Amber variety the corresponding parameter did not exceed 3.0 mg. This suggests that the effect of enzymes is most active even at low (not more than +2 °C) storage temperature in the varieties the fruits of which contain the minimal amount of pectin.

These data indicate the variety specificity on the proteolytic enzymes' activity which cause disintegration of cell walls.

After 10 day storage the PG activity increased and reached its maximum by the day 15. As a result of PG activity, the total amount of pectin in individual varieties decreased by more than 20.0 % over 20 days of storage (Table 4).

4. Changes in the content (%) of pectin substances in the fruit of different apricot varieties (*Armeniaca vulgaris* Lam.) after 20 days storage in the refrigerator (EPF Tsentral'noe, Krasnodar, 2002-2011)

Variety	Prior to storage	After storage		
		total	soluble pectin	protopectin
Pineapple	0.51±0.12	0.86±0.12	0.46±0.06	0.40±0.05
Emerald	0.50±0.10	0.90±0.12	0.48±0.06	0.42±0.05
Red-Cheeked	0.56±0.14	0.82±0.09	0.50±0.06	0.32±0.04
Krasnodar Later	0.62±0.06	0.85±0.10	0.40±0.05	0.45±0.05
Sunny	0.45±0.06	0.62±0.06	0.32±0.05	0.30±0.03
Amber	0.68±0.14	1.02±0.12	0.52±0.06	0.50±0.04
Average	0.52±0.12	0.84±0.10	0.45±0.05	0.40±0.04

The results showed that soluble pectins during storage were spent more intensively than protopectin, and in fruits with lower keeping quality (the Sunny variety), the amount of pectin substances changed more actively than in fruits with higher keeping quality. As a result, the peel of fruits in the Sunny variety at the end of storage becomes less elastic, flabby, and the pulp was spreadable.

During storage, the changes in the chemical composition of fruit were noted. A 3.7 % decrease in sugars during storage was detected (Sunny and Emerald varieties), the acid level decreased by 10.0 % on average, and vitamin C and P-active catechin content became lower by 15.5 to 20.7 %. In Amber, Krasnodar late and Emerald varieties, the C vitamin activity remained relatively high which confirms that the varietal characteristics are a major factor in determining not only the content but also the stability of ascorbic acid during storage. In the Krasnodar late and Emerald apricot varieties, the β-carotene level did not change during storage; in the Sunny and Red-Cheeked varieties it decreased from 2.62 to 2.54 and from 3.05 to 2.94 mg/100 g, respectively; in the Amber

variety it increased insignificantly due to the fruit ripening.

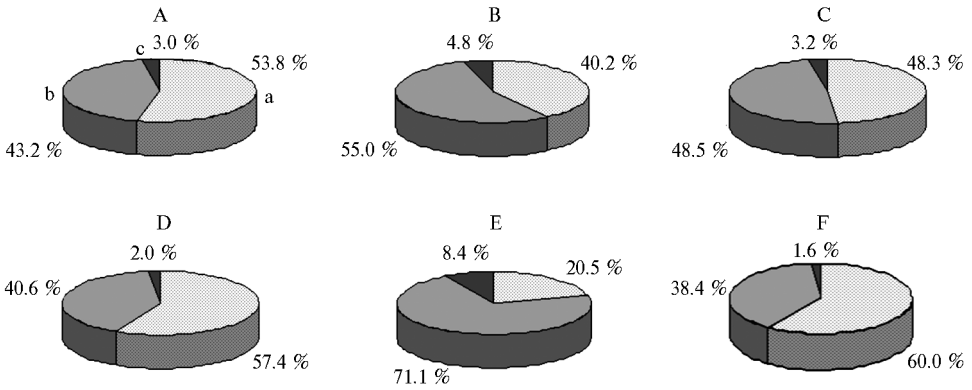

Fig. 2. Quality of the fruits in different apricot varieties (*Armeniaca vulgaris* Lam.) after 20 days storage in the refrigerator: A — Pineapple variety, B — Emerald variety, C — Red-Cheeked variety, D — Krasnodar late variety, E — Sunny variety, F — Amber variety; a — 1st commercial grade, b — 2nd commercial grade, c — rot (EPF Tsentral'noe, Krasnodar, 2002-2011).

The natural loss, as one of the main indicators of preservation, at day 10 of storage was the greatest in the Amber and Sunny varieties (5.0 and 8.4 %, respectively) (Fig. 2). At the end of storage, the natural loss reached 6.8 % in Amber and Emerald varieties and 10.2 % in the Sunny variety. The best marketable quality was observed in the Amber and Krasnodar late varieties, the proportion of rot did not exceed 2.0 %. In Amber and Krasnodar late varieties, the proportion of the 1st commercial grade fruits was 53.8 and 48.3 %, respectively.

Thus, the chemical composition of the fruits of the apricot varieties studied grown in the conditions of the central area of Krasnodar Territory provides the daily rate of consumption of vitamins, polyphenols, and pectin, which determine the therapeutic and prophylactic properties of the food product. The Amber and Krasnodar late varieties stood out among the samples, as the quality of the fruit was maintained in refrigerators for 20 days. It is promoted by the high content of biologically active substances and low activity of polygalacturonase in fruit tissues, and as a result they remain more compact which allows to attribute such products to the 1st commercial grade. The Sunny variety which annually accumulated ascorbic acid at a high level is recommended as a parental form in breeding for high vitamin C content.

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PHENOLIC COMPOUNDS AND FRUCTOSANS IN YACON (*Polymnia sonchifolia* Poepp. & Endl.) CULTIVAR INTRODUCED IN UKRAINE, AND IN OTHER *Asteraceae* PLANTS AS INFLUENCED BY GROWTH CONDITIONS, VIRAL AND PHYTOPHAGE INJURY

M.S. GINS[1], V.K. GINS[1], P.F. KONONKOV[1], A.A. DUNICH[2], A.V. DASHCHENKO[3], L.T. MISHCHENKO[2]

[1]*All-Russian Research Institute of Vegetable Breeding and Seed Production,* Federal Agency of Scientific Organizations, 14, ul. Selektsionnaya, pos. VNIISSOK, Odintsovo Region, Moscow Province, 143080 Russia, e-mail vniissok@mail.ru, anirr@bk.ru;
[2]*Educational and Scientific Centre «Institute of Biology», Taras Shevchenko National University of Kyiv,* 64/13, Volodymyrska Str., Kyiv, 01601 Ukraine, e-mail korenevochka@mail.ru, lmishchenko@ukr.net;
[3]*National University of Life and Environmental Sciences of Ukraine,* Heroyiv Oborony str., 15, Kyiv, 03041 Ukraine, e-mail dannaval@mail.ru
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Abstract

Antioxidants regulate functional activity and reduce the risk of development of diseases. Deficiency of antioxidants leads to sharp decrease in resistance to adverse factors. The vegetable food is the main and most available source of antioxidants for the human. Yacon (*Polymnia sonchifolia* Poepp. & Endl. syn. *Smallanthus sonchifolia*), a new perspective plant (cv. Yudinka originated by All-Russian Research Institute of Vegetable Breeding and Seed Production), was successfully introduced in Ukraine. The content of the biologically active substances (BAS) in plants is known to be influenced by transfer from the places of natural growth in other climatic conditions, and by biotic factors, too. Despite it, only a few papers are devoted to studying of the content of phenols, oligofructans and other BAS in the yacon depending on cultivation and storage conditions, and the data on an influence of pathogens on the BAS in the yacon are absent. Therefore our aim was to determine the level of the main BAS (phenolic compounds and fructosans) in yacon of the Ukrainian introduction (in leaves and root tubers), and also to estimate the influence of biotic factors. The chromatography analysis of ethanol extracts from yacon leaves and root tubers showed the phenolic compounds among which the derivatives of hydroxycinnamic acids (HCA) prevailed. Using the high-efficiency liquid chromatography, it was shown that in the ethanol extract of dry yacon root tubers the derivatives of HCA are mainly presented by a not identified peak (X1) with keeping time of 11.45 min, and also chlorogenic and caffeic acids were found. Main phenolic component of the fresh root tuber ethanol extracts was a substance with keeping time of 12.21 min (X2) which was absent in the dry root tubers and also was a derivative of caffeic acid. The content of chlorogenic and caffeic acids in fresh yacon root tubers was higher compared to dry ones, thus there are labile HCA derivatives changed when drying. We also studied other *Asteraceae* species (*Echinacea purpurea*, *Arctium lappa*, *Helianthus tuberosus*, *Taraxacum officinale*, *Dahlia* Cav.), and, unlike yacon, found one dominant peak of caffeic acid derivatives. It was cichoric acid in coneflower and dandelion leaves, chlorogenic acid in girasol leaves, and not identified substance (X3) coinciding in keeping time with cichoric acid in burdock leaves. In yacon leaves the accumulation of HCA derivatives was higher. Majority (not less than 18) peaks on the chromatogram had a HCA specific UF-spectrum. Three components with keeping time of 7.1-7.6 min could be identified as flavonols with regard to UF-spectra. According to direct spectrophotometry of ethanol extracts, the total amount of hydroxycinnamic acids varied from 2.8 to 4.3 % (as chlorogenic acid per absolutely dry weight) depending on position of leaves on the plant. In root tubers the fructosans level changed from 36 to 45 % depending on the region of cultivation and weather conditions. A comparative assay showed that in the root tubers of yacon multiplied by cutting the fructosans level was 4.98 % higher than if the cutting was not used. Note, the fructosans level in the yacon root tubers was higher compared to girasol and dahlias tubers which are known as their effective sources with 6.2 % and 3.03 % level, respectively. A decrease in the content of main BAS in root tubers of the yacon plants influenced by biotic agents such as viruses and phytophages was also found. Particularly, in infected and damaged yacon plants the low-molecular fructosans, the sum of fructose and total fructosans, and the inulin accumulation were 8.9 %, 13.9 % and 5 % lower, respectively. Thus, the data obtained by us showed that yacon is the perspective source of fructosans

and antioxidants which can be used in manufacturing bioactive preparations.

Keywords: yacon, phenolic compounds, fructosans, inulin, hydroxycinnamic acid, biotic factors.

The character of food determines human health and lifespan largely. In addition to basic nutrients (proteins, fats and carbohydrates) as well as vitamins, trace elements and major mineral elements, a large group of chemical compounds with antioxidant properties is essential for humans [1]. Biologically active substances (BAS), including antioxidants regulate body functions and reduce the risk of some diseases. Deficiency of antioxidants and a breach in antioxidant protection result in a sharp decrease in the resistance to adverse factors. The vegetable food, including alternative and medicinal forms, is the main and most available source of antioxidants for humans [2].

The introduction of plants with high content of bioactive substances is actual worldwide and is associated with a lack of these compounds in the diet of the population in many countries. Estimation and selection of plants with high content of antioxidants, as well as the study of their structure are necessary for the creation of foods fortified with micronutrients and food nutrients [3]. Thus, stachys, Chinese artichoke (*Stachys sieboldii* Mig.), has been introduced in the Nonchernozem Zone of the Russian Federation, and the composition and content of biologically active substances and antioxidants in its nodules have been studied. In addition to sucrose, a complex of BAS and antioxidants has been found in the nodules, such as vitamin C, phenolic compounds including flavonoids, natural glycosides [4]. This useful plant has also been introduced in Ukraine and Bulgaria [5].

Alternative sources of antioxidants include yacon, or sharp-leaved polymnia (*Polymnia sonchifolia* Poepp. & Endl. syn. *Smallan-thus sonchifolia*), perennial plant of the *Asteraceae* family [6]. The main area of yacon is the middle latitudes of South America. It has already been introduced in United States, New Zealand, South Europe, Iran, Japan, Moldova, the Czech Republic, and Uzbekistan. Studies on the introduction of yacon in the CIS started at the All-Russian Research Institute of Vegetable Breeding and Seed Production (VNIISSOK) have also been continued [7-9].

Yacon is considered a valuable medicinal plant due to the high proportion of inulin, a polysaccharide which is easily absorbed in the body and serves as a substitute for sucrose in diabetics dietary, in the root tubers. Scientists from many countries have demonstrated the hypoglycemic characteristics of yacon [10-13]. Mainly, the medicinal effect of the underground part of the plant has been studied, although the leaf extracts have been shown to reduce the blood sugar level [14-17]. Due to the content of chlorogenic, caffeic acids and other phenolic compounds, its leaves also possess antioxidant properties [18-21].

The yacon Yudinka variety bred in VNIISSOK has been introduced in Ukraine [22-24]. The content of BAS may change in plants when they are transfered from the places of their natural growth in other climatic conditions. However, just a few papers deal with the study of the content of phenols, oligofructans and other BAS in yacon and of the cultivation and storage conditions for the plant.

We quantified the accumulation of the main biologically active substances in yacon variety introduced in Ukrain depending on abiotic and biotic factors (soil and climatic conditions of the region, breeding technology, pathogens and pests), and compared these data with the results of the analysis of other *Asteraceae* members.

Technique. Yacon variety Yudinka (originator is the All-Russian Research Institute of Vegetable Breeding and Seed Production) and purple coneflower (*Echinacea purpurea*), burdock (*Arctium lappa*), girasol (*Helianthus tuberosus*), dandelion (Taraxacum officinale) and dahlia (Dahlia Cav.) were studied (research of 2011-2013, Ukraine). Yacon was grown at the experimental plot of the ESC Institute of Biology of the Taras Shevchenko National University (Kyiv) in Kyiv, Poltava, and Chernigiv regions, the rest of the samples were grown in Kyiv and Poltava regions.

Yacon was propagated by stem cuttings [27]. Stems with one pair of leaves were placed in peat pots with soil, covered with plastic caps and watered abundantly. Then they were moved into greenhouses with the air temperature of +18...+20 °C and illumination of 6 thousand Lx (light period duration of 16 h). In the first variant of experiments on laboratory optimization of yacon reproduction and growing in the field, stem cuttings were used, the 2.5 months old yacon was planted in open ground; in the second variant yacon was not grafted, its rhizomes with regrown stems were planted in the field after 2.5 months of growth in the laboratory. Plants were dug in October (before freezing), and leaves and root tubers were collected for the study.

Liquid chromatography was performed (Agilent 1100, Agilent Technologies, USA) with a diode matrix detector and Agilent Zorbax Eclipse XDB-C18 column (150×4.6 mm, sorbent particle size of 5 μm) (Agilent Technologies, USA). Gradient elution system with acetonitrile and phosphoric acid was used. Chlorogenic acid based on anhydrous matter was used as the reference substance. The presence of phenolic compounds was determined in the ethanol extract of leaves, dry and fresh whole root tubers and in their peel. Phenolic compounds in ethanol extracts of the leaves of purple coneflower, burdock, girasol, and dandelion were analyzed as well. Separated substances were identified by comparing the retention time of the peaks in the chromatograms of samples and reference compounds (λ = 325 nm was an operating wavelength).

The amount of fructosans based on dry matter and 5′-hydroxy methylfurfural were determined spectrophotometrically by standard methods at λ = 285 nm using a UV 1600 spectrophotometer (Mapada, China) [28)]. To quantify fructosan fractions, the Selivanov color reaction was used (heating fructose with hydrochloric acid to produce hydroxy methylfurfural that gives a cherry-red color with resorcinol, the intensity of which is determined by spectrophotometry) [29]. To separate high and low molecular weight fructosans, a technique based on their solubility in 96 % ethanol was used. The amount of fructosans were calculated as fructose in aqueous and ethanol extracts of yacon root tubers. The results of water extraction showed the content of fructose and total fructozans, the difference between the values for the water and ethanol extracts showed the inulin content. In addition to yacon, fructosans were determined in the plants of artichoke and dahlia. The total amount of hydroxycinnamic acids (HCA) in the leaves of the 2nd tier and the leaves of the 1st to 4th tiers (an average sample) were estimated by direct spectrophotometry of ethanol extracts (λ = 325 nm; UV 1600, Mapada, China) and expressed based on chlorogenic acid and dry matter [30].

The data were processed by the normal variant distribution parametric criteria, and standard deviation was calculated by the standard method using the Microsoft Excel package.

Results. Figure 1 shows yacon of different ages and the yielded root tu-

bers (healthy and affected by viral infection) [31].

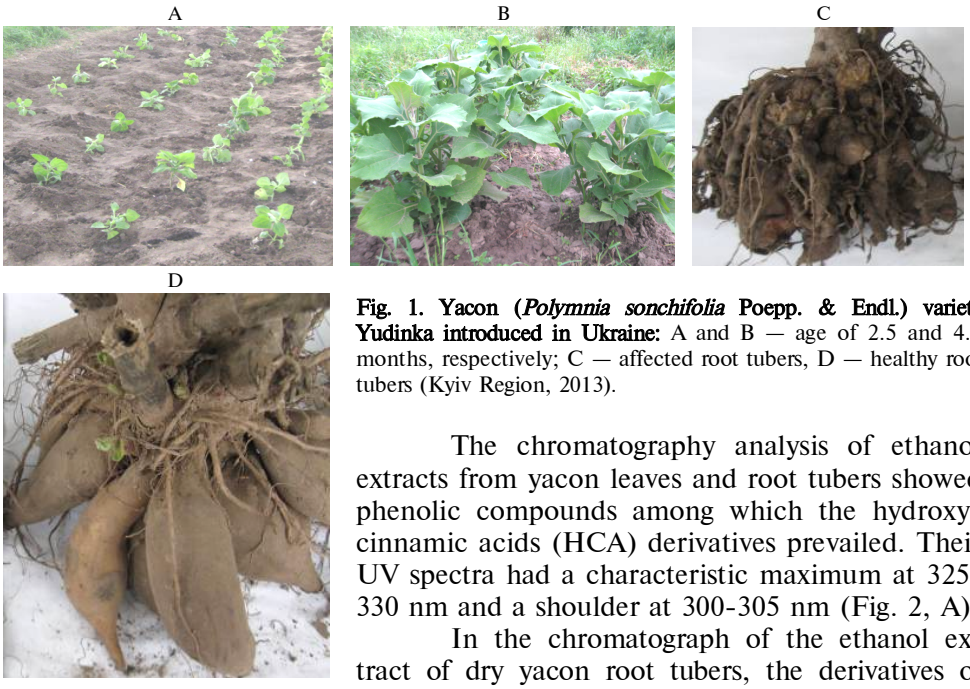

Fig. 1. Yacon (*Polymnia sonchifolia* Poepp. & Endl.) variety Yudinka introduced in Ukraine: A and B — age of 2.5 and 4.5 months, respectively; C — affected root tubers, D — healthy root tubers (Kyiv Region, 2013).

The chromatography analysis of ethanol extracts from yacon leaves and root tubers showed phenolic compounds among which the hydroxycinnamic acids (HCA) derivatives prevailed. Their UV spectra had a characteristic maximum at 325-330 nm and a shoulder at 300-305 nm (Fig. 2, A).

In the chromatograph of the ethanol extract of dry yacon root tubers, the derivatives of HCA were presented by a main not identified peak (X1) with the retention time of 11.45 min. Chlorogenic and caffeic acids were found as well (see Fig. 2, B).

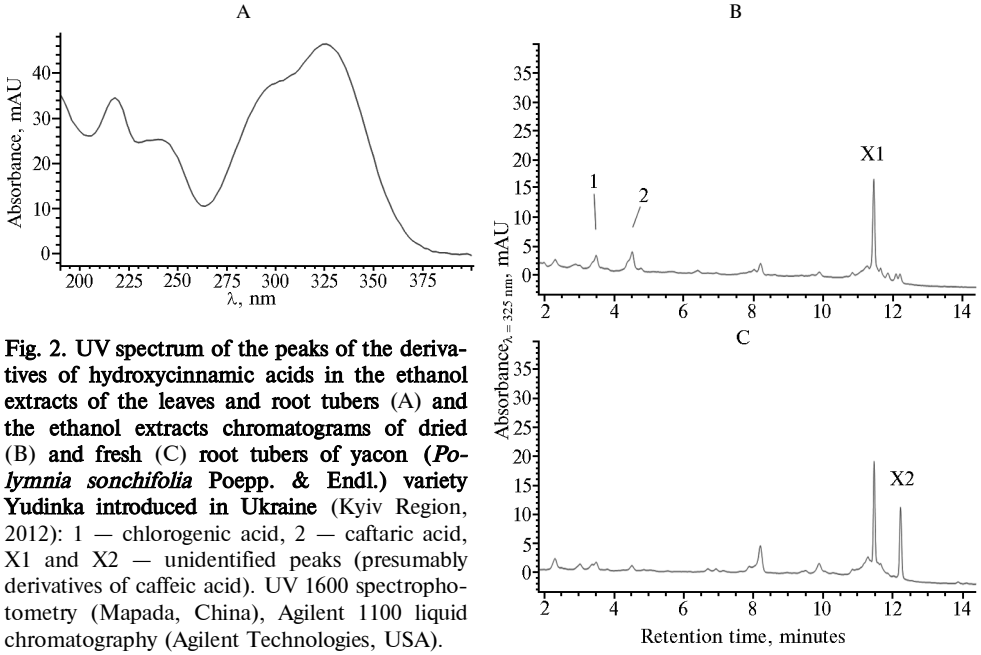

Fig. 2. UV spectrum of the peaks of the derivatives of hydroxycinnamic acids in the ethanol extracts of the leaves and root tubers (A) **and the ethanol extracts chromatograms of dried** (B) **and fresh** (C) **root tubers of yacon (*Polymnia sonchifolia* Poepp. & Endl.) variety Yudinka introduced in Ukraine** (Kyiv Region, 2012): 1 — chlorogenic acid, 2 — caftaric acid, X1 and X2 — unidentified peaks (presumably derivatives of caffeic acid). UV 1600 spectrophotometry (Mapada, China), Agilent 1100 liquid chromatography (Agilent Technologies, USA).

The main phenolic component of the ethanol extract of fresh root tubers was a substance with the retention time of 12.21 min (X2) which was not found in dried root tubers and was also a derivative of caffeic acid (see Fig. 2, C). Similar results indicating the presence of chlorogenic, caffeic and other acids, as well as quercetin and two flavonoids in yacon root tubers have been described in publications [32-35]. The analysis of chromatograms of ethanol ex-

tracts of whole root tubers and its peel demonstrated no qualitative and quantitative differences in HCA.

In yacon leaves, the quantity of HCA derivatives was greater. Depending on the area of cultivation they contained from 2.78 to 4.30 % HCA which indicates the prospect of using not only root tubers but also the aerial part as medicinal raw material. The majority of peaks (not less than 18) in the chromatograms had a UV spectrum specific of HCA. Three components with retention time of 7.1-7.6 min could be identified as flavonols with regard to UF-spectra.

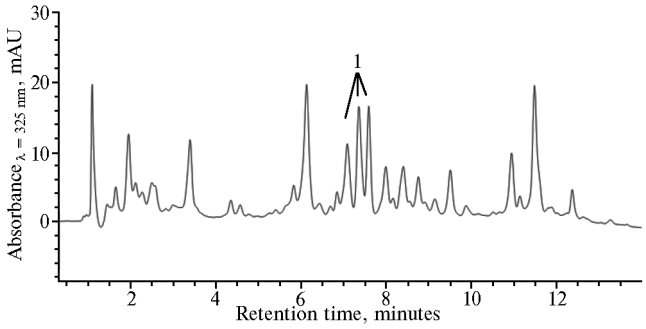

Fig. 3. Chromatogram of ethanol extract of dry leaves of yacon (*Polymnia sonchifolia* Poepp. & Endl.) variety Yudinka introduced in Ukraine (Kyiv Region, 2012): 1 — flavonols. Liquid chromatography (Agilent Technologies, USA).

Our data are consistent with the results of Czech scientists who, in addition to HCA derivatives, found an unknown derivative of chlorogenic acid and one unidentified flavonoid in the leaves of yacon [32].

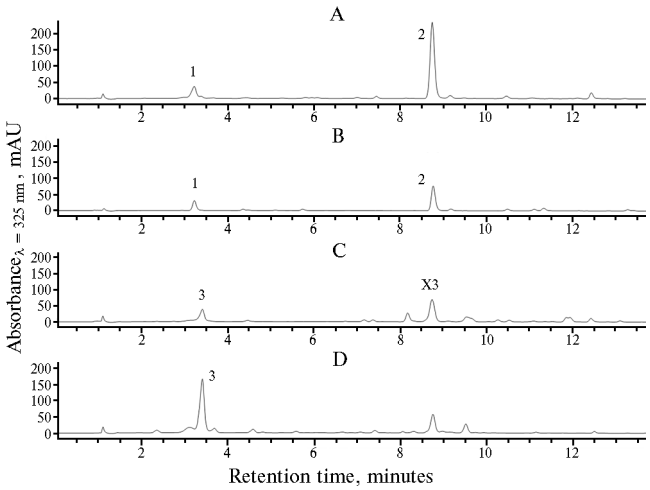

Fig. 4. Chromatograms of ethanol extracts of leaves of various representatives of the *Asteraceae* family (Kyiv Region, 2012): A — dandelion (*Taraxacum officinale*), B — purple coneflower (*Echinacea purpurea*), C — burdock (*Arctium lappa*), D — girasol (*Helianthus tuberosus*); 1 — caftaric acid, 2 — chicory acid, 3 — chlorogenic acid, X3 — unidentified substance. Liquid chromatography (Agilent Technologies, USA).

In HCA derivatives in other plants studied, unlike yacon, a dominant peak of one of caffeic acid derivatives was found. In coneflower and dandelion leaves it was cichoric acid, in girasol leaves it was chlorogenic acid, in burdock leaves it was an unidentified substance (X3) similar to cichoric acid in its retention time.

In yacon, in the 2nd top tier leaves, HCA content was higher ($p < 0.01$) than that of the average for the sample of the 1st to 4th tier. It was greater by 0.38 % in Kyiv Region, and by 0.58 % in Poltava Region (Table). This tendency was found in girasol, particularly, BAS level in the 2nd tier leaves compared to the 1st to 4th tier was greater. These results may be due to redistribution of substances in younger leaves during the plant growth. These data will be useful in the production of high quality drugs.

Other authors demonstrated that yacon root tubers contain from 25 to 83 % of carbohydrates depending on the conditions and the region [25, 36, 37]. In our study, the amount of fructosans in yacon was 44.7 % in Kyiv Region, 44.0 % in Chernigiv Region, and 36.1 % in the Institute experimental plot (Kyiv).

Total amount of hydroxycinnamic acids (HCA based on chlorogenic acid and absolute dry matter) **in different tier leaves in yacon (*Polymnia sonchifolia* Poepp. & Endl.) and other representatives of the *Asteraceae* family in growing regions** (Ukraine, 2011)

Plant species	Location	Tier	HCA content ($X\pm\sigma$), %
Yacon	Kyiv Region	1st-4th	3.92±0.03
Yacon	Poltava Region	1st-4th	2.78±0.03
Girasol	Kyiv Region	1st-4th	4.97±0.04
Girasol	Poltava Region	1st-4th	5.29±0.03
Yacon	Kyiv Region	2nd top	4.30±0.03
Yacon	Poltava Region	2nd top	3.36±0.02
Girasol	Kyiv Region	2nd top	8.84±0.05
Girasol	Poltava Region	2nd top	7.47±0.04
Burdock	Poltava Region	1st-4th	3.78±0.02
Dandelion	Poltava Region	All leaves	4.75±0.03

Note. Please refer for taxonomic species name to section «Technique».

In the experiments on the optimization of yacon reproduction techniques (2013), the fructosans content in root tubers of plants grown from cuttings was 4.98 % greater when compared to non-grafted plants (56.20±0.30 % versus 51.22±0.32 %). Note, it was greater in yacon than in girasol (47.51±0.31 %) and dahlia (50.68±0.28 %) for which higher accumulation of these substances is typical. This means that yacon can be considered a no less promising source of fructosans.

During the yacon growing season in the field (2013), symptoms of viral diseases that affect the number and size of the root tubers were observed (see Fig. 1, C). Leaf damage by caterpillars and the presence of other pests in the soil was found [31]. In the affected root tubers compared to healthy ones, the content of low-molecular fructosans was lower (40.3±0.60 and 49.2±0.76 %, respectively), the level of fructose and total fructosans decreased as well (51.10±0.77 and 65.0±0.98 %), like the amount of inulin (10.8±0.16 and 15.8±0.22 %). This demonstrates the declining quality of medicinal plants. Similar results were obtained by us and by foreign authors for other medicinal plants [38-42].

Thus, yacon root tubers and leaves (compared to other *Asteraceae* memebers studied herein) can be regarded as a promising material for the production of herbal remedies and dietary supplements with antioxidant and hypoglycemic properties due to their high content of phenolic compounds and fructosans, inulin in particular. Due to a considerable reduction of the main active ingredients in yacon root tubers exposed to viral infections and pests, the methods of crop protection should be used timely.

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60 YEAR BRED CONVEYOR OF APPLE VARIETIES, THEIR RESISTANCE TO SCAB AND BIOCHEMICAL CHARACTERISTICS OF FRUITS

E.N. SEDOV, M.A. MAKARKINA, G.A. SEDYSHEVA, Z.M. SEROVA

All-Russian Research Institute of Breeding Fruit Crops, Federal Agency of Scientific Organizations, d. Zhilina, Orel Region, Orel Province, 302530 Russia, e-mail info@vniispk.ru

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Abstract

The results of apple breeding at the All Russian Research Institute of Fruit Crop Breeding (VNIISPK) for 60 years (1953-2013) are given. The basic sections of breeding are shown (the development of scab immune triploid apple varieties with higher contents of nutrient and biologically active substances in fruit). The biochemical characteristics of fruits in 47 apple varieties released from VNIISPK and included into the State Register are given. The development of the varieties with different dates of maturing and length of storage life allows providing the apple calendar of home apple consumption during the whole year round by the way of purposeful selection of varieties. First in Russia and in the world a series of triploid apple varieties have been produced from crossing between plants with different ploidy. These varieties ensure more regular fruiting and high fruit marketability. The VNIISPK varieties are widely introduced in the production and amateur orchards in a number of regions of Russia and Belarus; they are passing testing in the Ukraine, Kazakhstan and other countries.

Keywords: apple, varieties, breeding, scab immunity, dates of fruit maturing, biochemical composition of fruit, triploidy.

Apple breeding is focused on the improvement of assortment and producing varieties with high yield, different dates of ripening, and suitable for intensive horticulture. Successful practical results are undoubtedly based on fundamental study of plant genetics, physiology, biochemistry, etc. [1]. Apple varieties immune or high tolerant to scab are required, in fact, in all Russia regions. In the view to this, genetic and immunological research of plant tolerance to scab have been conducted in All-Russian Research Institute of Breeding Fruit Crops (VNIISPK). We improved the technique of scab experimental infection, selected virulent and aggressive scab biotypes, developed crossing programs. Studies were governed by and carried out with V.V. Zhdanov [1]. Recently, the digenic varieties $V_f + V_r$ and $V_r + V_m$, and the varieties with oligo-polygenic complex scab resistance are mainly produced.

Based on knowledge about the formation of male and female gametes one can plan crossings required to obtain enough hybrids for further selection. Triploids were shown to be best produced in crosses like 2×/4× and 4×/2× (♀/♂). Due to these crosses a set of first Russian triploid apple varieties has been produced. These varieties are generally characterized by more regular yielding year after year, the high fruit marketability, an increased self-fertility, thus being quite promising in practical use. For the polyploidy-based breeding program, the cytoembryological study is essential allowing us to control the peculiarities of generative system in the parent plants, and estimate ploidy in the hybrids when the parent forms with different ploidy were crossed.

For many years the hardiness, a resistance to diseases, improved chemical compositions, small height, the compact and columnar habit, polyploidy and

self-fertility remained a priority in our breeding. In breeding research we used common techniques [2-5].

Genotypic characteristics and main parameters of tolerance, production and fruit quality in the apple varieties originated from All-Russian Research Institute of Breeding Fruit Crops for 60 year period (those included into State Register of the Russian Federation are shown herein)

Variety	Ploidy and scab resistance	Fruit weight, g	Sugars, %	Total acidity %	Sugar to acidity index	Ascorbic acid, mg/100 g	P-active substances, mg/100 g
Summer varieties							
Avgusta	3×	160	10.5	0.73	14.4	9.8	422
Daryena	3×	170	10.3	0.76	13.6	8.8	422
Zhelannoe		140	10.6	0.61	17.4	4.4	384
Maskovskoe	3×, V_f	200	10.7	0.71	15.1	17.5	318
Orlinka		140	10.4	0.73	14.2	7.4	314
Orlovim	V_m	130	10.2	0.77	13.2	8.8	299
Osipovskoe	3×	130	12.1	0.49	24.7	8.1	263
Radost' Nadezhdy		150	10.0	0.64	15.6	4.7	474
Rannee aloe		130	9.5	0.78	12.2	9.4	313
Yubilar	3×, V_f	130	9.4	0.86	10.9	11.5	387
Yavlochnii Spas	3×, V_f	210	10.4	0.70	14.9	9.4	402
Melba (control)		125	9.9	0.71	13.9	11.2	389
Papirovka (control)		130	9.0	0.75	12.0	15.1	259
Autumn varieties							
Zaryanka	V_m	130	10.1	0.79	12.8	18.0	419
Orlovskii pioner	V_m	140	10.0	0.87	11.5	14.8	514
Orlovskoe polosatoe		150	10.3	0.81	12.7	8.5	261
Pamyat' Isaeva	V_m	150	10.4	0.56	18.6	6.6	325
Slavyanin	V_m	150	10.5	0.93	11.3	11.4	360
Solnyshko	V_f	140	9.8	0.84	11.7	7.7	424
Osennee polosatoe (control)		140	9.8	0.59	16.6	9.0	248
Winter varieties							
Aleksandr Boiko	3×, V_f	200	10.7	0.51	21.0	4.4	351
Afrodira	V_f	130	10.4	0.48	21.7	6.8	464
Bezhin lug	3×	150	9.3	0.55	16.9	7.4	436
Bolotovskoe	V_f	150	10.4	0.40	26.0	11.1	477
Ben'yaminovskoe	V_f	130	9.7	0.62	15.6	4.8	235
Veteran		130	10.3	0.71	14.5	19.4	229
Zdorov'e	V_f	140	9.6	0.88	10.9	7.8	449
Ivanovskoe	V_f	150	11.8	0.85	13.9	19.5	432
Imrus	V_f	140	9.6	0.77	12.5	9.3	433
Kandil' orlovskii	V_f	120	10.2	0.56	18.2	7.2	558
Kulikovskoe		125	10.2	0.53	19.2	15.3	317
Kurnakovskoe	V_f	130	10.8	0.73	14.8	11.3	380
Morozovskoe		160	8.7	1.04	8.4	8.0	299
Nizkorosloe	3×	130	10.6	0.35	30.3	18.0	293
Olimpiiskoe		130	10.9	0.77	14.2	15.4	280
Orlik		120	10.8	0.43	25.1	8.5	222
Orlovskaya zarya		135	10.3	0.63	16.3	15.0	334
Orlovskii partizan	3×	190	11.8	0.41	28.8	7.7	426
Orlovskoe poles'e	V_f	140	10.0	0.85	11.8	6.9	438
Pamyati Khitrovo	V_f	170	10.6	0.89	11.9	3.5	480
Pamyat' voinu		140	10.6	0.51	20.8	7.1	182
Pamyat' Simakinu	3×	160	9.5	0.90	10.6	8.7	474
Patriot	3×, V_m	240	11.9	0.46	25.9	9.0	449
Pepin orlovskii		140	10.2	0.59	17.3	15.3	241
Pozhdestvenskoe	3×, V_f	140	9.9	0.57	17.4	4.1	368
Svezhest'	V_f	140	10.1	0.80	12.7	12.5	377
Sinap orlovskii		150	9.9	0.56	17.7	13.4	205
Start	V_f	140	10.9	0.57	19.1	11.0	404
Stroevskoe	V_f	120	10.4	0.61	17.0	7.0	396
Yubilei Moskvy	V_f	120	9.6	0.67	14.3	5.6	352
Antonovka obyknovennaya (control)		140	8,7	0.99	8.8	14.5	340
Severnyi sinap (control)		120	9.0	0.58	15.5	13.9	137

N o t e. 3× marks triploids, V_f, V_m indicate the presence of immunity gene.

Regular studies on apple breeding have been started in 1953 by E.N. Se-

dov. Until 1955 all crosses have been carried out in I.V. Michurin Research Institute of Horticulture (Michurinsk, Tambove Province), then at the Orel Experimental Station, the ancestor of VNIISPK. In this, main contemporary approaches are being used [1]. For 60 year period (1953-2013) a total of 4.8 million flowers have been pollinated with 853 thousand seedlings grown and 187 thousand ones replanted into breeding gardens of which 10.6 thousand ones are being grown. Moreover, 171 elite seedlings have been selected. A total of 74 varieties were submitted to state testing, and 47 varieties of different ripening were included into the State Register (Table). The apple varieties produced in VNIISPK are widely used in commercial plantations, by farmers and in private gardens in Russia and Belorussia, and also are tested in Ukraine, Kazakhstan, etc.

Of 47 varieties originated from VNIISPK, there are 11 ones of summer ripening, 6 ones of autumn ripening, and 30 ones of winter ripening (see Table). Under the climate of Orel Province, the ripening in the summer varieties occurs both in first half of August (e.g., Rannee aloe, Maslovskoe and Yablochnii Spas varieties) and in second half of August (e.g., Orlovim, Zhelannoe, Radost' Nadezhdy, etc.). Of autumn varieties, the Orlovskii pioneer is the early ripening one with the yield harvesting in second half of August and fruits stored until October the end. Later ripening is characteristic of other varieties of this group. In Orlovskoe polosatoe variety the ripening occurs in September the beginning, and its fruits are easy stored till December the end, and in Solnyshko variety it is September 15-20 and December, respectively. In Afrodita, Orlovskoe poles'e, Rozhdestvenskoe, Bolotvskoe varieties fruits are harvested in the middle of September and stored in a refrigerator till January the end. The longest storage is peculiar to Veteran variety (till March the middle), Kulikovskoe (till March the end), Sinap orlovskii (till May) and Svezhest' (till May and beyond) [1]. Thus, a broad diversity of apple varieties with different ripening and storage time can be offered as an «apple conveyer» to provide people with fresh fruits nearly year-round.

Selection for scab immunity is being conducted in VNIISPK since 1976 E.N. Sedov et al., 1983; V.V. Zhdanov et al., 1991). A total of 2.4 million flowers have been pollinated with 464.6 thousand seedlings grown. A total of 20 scab-immune apple varieties were produced, including Maslovskoe and Yablochnii Spas varieties of summer ripening [6]. These varieties are characterized by a short time of fruit maturation and the high marketability, at that, the high level of ascorbic acid (17.5 mg/100 g) is peculiar to Maslovskoe variety. The ripening of another scab-immune variety, Solnyshko, occurs in late autumn. Solnysko plants are characterized by high yield and attractable appearance. The best scab-immune apple winter varieties are Bolotovskoe, Ven'yaminovskoe, Imrus, Kandil' orlovskii, Rozhdestvenskoe, and Svezhest'. In these varieties, short fruit ripening, high yield and marketability are peculiar. Kandil' orlovskii variety is also peculiar in specific cone-like shape of fruits with high level of P-active substances, and in Rozhdestvenskoe variety the fruits are especially delicious.

Biochemical study indicated a high sugar level in fruits (above 10.6 %) in Maslovskoe, Aleksandr Boiko, Kurnakovskoe, Orlik, Olimpiiskoe, Start, Orlovskii partisan, Ivanovskoe, Patriot and Osipovskoe varieties [7-9]. Ascorbic acid content in Maslovskoe (17.5 mg/100 g), Zaryanka (18.0 mg/100 g), Nizkorosloe (18.0 mg/100 g), Veteran (19.4 mg/100 g) and Ivanovskoe (19.5 mg/100 g) varieties is superior to that in other varieties, and high accumulation of P-active substances (over 450 mg/100 g) is characteristic of Orlovskoe poles'e, Pamyati Khitrovo, Pamyat' Semakinu, Radost' Nadezhdy varieties. High marketability and fruit weight is characteristic of the triploid varieties Patriot (240 g), Yablochnii Spas (210 g), Aleksandr Boiko (200 g), Maslovskoe (200 g), Orlovskii partizan

(190 g), Daryena (170 g) [10]. The triploid scab-immune apple varieties are recommended for commercial use, particularly, summer varieties Maslovskoe, Yablochnii Spas and winter varieties Aleksandr Boiko and Rozhdestvenskoe.

Thus, due to 60 year research, a total of 74 apple varieties are produced of which 47 are included into State Register of breeding achievement allowed for practical use in the Russian Federation. These varieties differ in ripening characteristics thus allowing us to construct a fresh apple «conveyor» for year-round consumption. Long-term study of the biochemical composition of fruits makes it possible to choose for this «conveyor» the varieties with a high content of nutrients and bioactive substances. Among the varieties, a total of 20 scab-immune ones and 12 triploid ones with the high marketability have been produced. These varieties are widely used in commercial and private gardens in different Russian regions and in Belorussia, being also tested in Ukraine, Kazakhstan, etc.

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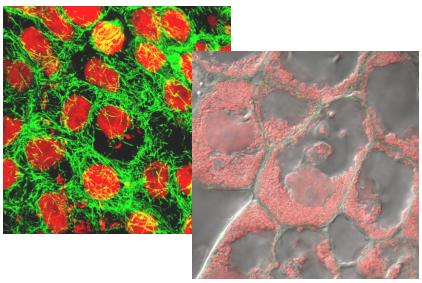
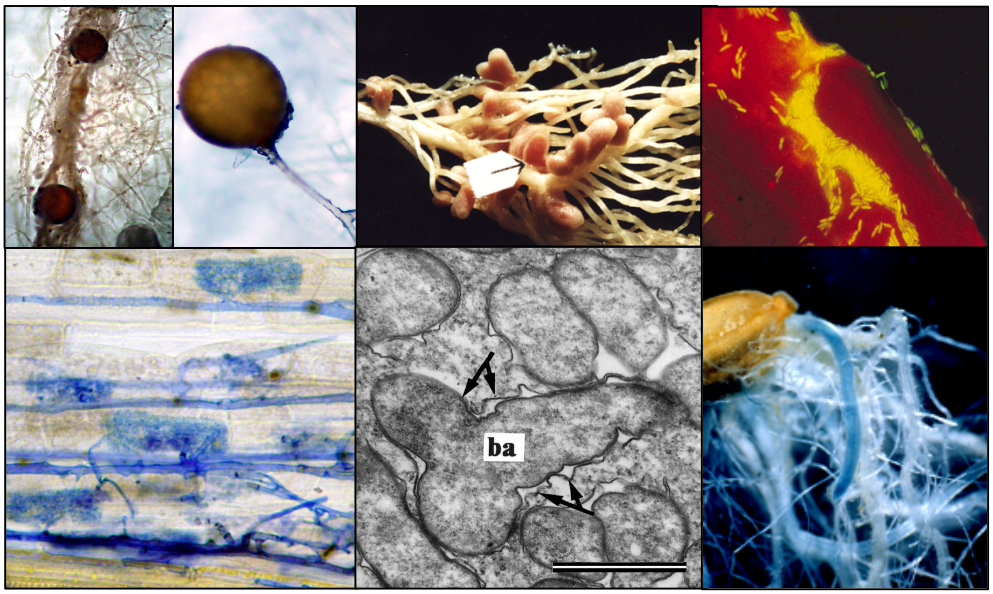
85 Anniversary of ARRIAM

Sergei P. Kostychev (1877-1931), the founder and first director



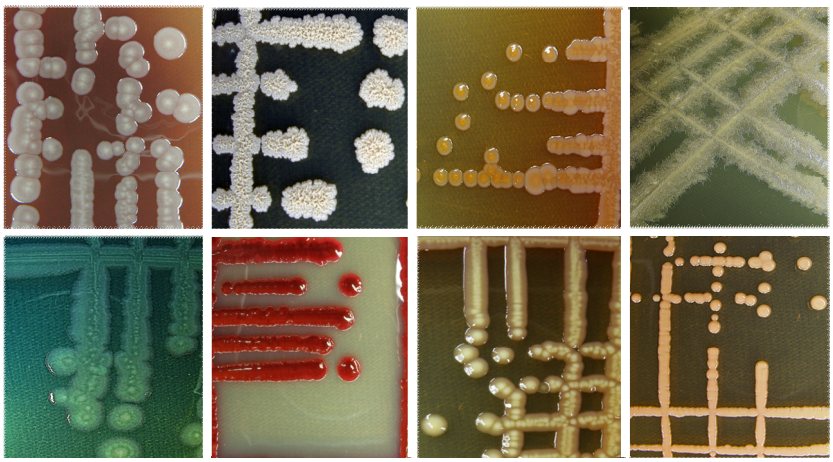
Plant–microbe symbioses



Arbuscular mycorrhiza

Rhizobia–legume symbiosis (ba – bacteroid)

Bacteria associated with plant roots



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MICROORGANISMS AND GLOBAL CLIMATE CHANGE

M.M. LEVITIN

All-Russian Research Institute of Plant Protection, Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail mark_levitin@rambler.ru

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Abstract

Today, a global climate change is speeding up (Intergovernmental Panel on Climate Change — IPCC, Switzerland) that limits leaving organisms' adaptation to the environment. As a result, the distribution of phytopathogenic fungi may obviously change. Particularly, in 1985 a new disease of wheat yellow leaf spot appeared in the European south of Russia (Krasnodar Region) (E.F. Granina et al., 1989). In 2005-2007 the causal agent of yellow leaf spot *Pyrenophora tritici-repentis* was found on wheat in North-Western Region of Russia. On some cultivars the disease severity reached 70 %, and pathogens become more virulent and viable. Despite the North Caucasus and the Far East were specific areas for *Fusarium graminearum* in Russia, since 2003 *F. graminearum* appeared on the territory of the Russian North-West. The average disease severity on cereals was 93.3 % in 2007 and 87.3 % in 2008. Recently *F. graminearum* predominates on cereals in the Netherlands (J. Arts et al., 2003), GB (P. Jennings et al., 2004), North Germany (T. Miedaner et al., 2008) and Finland (T. Yli-Mattila et al., 2010). In the south of Russia, *Septoria tritici* predominates among species causing wheat glum blotch, and in the North-West it is *Stagonospora nodorum.* In 2003-2005, *S. tritici* became the main wheat pathogen in the North-Western Russia. On susceptible spring wheat cultivars the disease was found in 51 to 100 % plants, with a severity of 8 to 30 %. These observations suggest that global warming of climate leads to an expansion of thermophilic fungi species, and pathogens begin to spread from the south to the north. *Pythium, Rhizoctonia, Sclerotinia* soil fungi are influenced by climatic factors. They form the overwintering structures that protect them from external influences. Increasing the temperature can lead to a decrease of the latent period and to increase of pathogens aggressiveness. Temperature can influence the function of the parasites virulence genes and resistance genes in plants. Thus, it is necessary to control the emergence of new plant diseases, improve protective measures, and develop cultivars with high adaptability.

Keywords: climate change, phytopathogenic fungi, soil microorganisms, bioecology of microorganisms, environment conditions.

Various aspects of the problem of global climate change, including its possible catastrophic impact on agriculture are discussed at all levels in recent years. According to experts, Russia not get over 40 million tons of the full amount of crop production in grain equivalent annually. Climate change can result in the extinction of 30-40 % of plant and animal species, degradation of key ecosystems, reduced agricultural productivity and thus to the aggravation of the problem of food safety [1].

The climate change will undoubtedly affect the abundance of harmful and beneficial organisms, their biological and ecological characteristics, relationships with plants. Information about these changes will be critical for creating science-based systems of integrated plant protection and improvement of soil fertility.

The spread of plant diseases under global warming. In 1985, a new disease of wheat yellow leaf spot caused by the *Pyrenophora tritici-repentis* (Died.) Drechsler fungus was identified in the Krasnodar Territory [2]. In 1992-1993 the disease was identified in the Stavropol Territory, though it did

not occur in the northern latitudes. But in 2005-2007, yellow leaf spot was found on wheat in Leningrad, Pskov, and Novgorod regions [3]. On some varieties of spring and winter wheat, the disease severity reached 70 % [4]. Population analysis demonstrated that «southern» populations are more diverse in their racial composition than «northern» ones, but the latter are more virulent to differentiator varieties [5]. The age of a population is known to affects its structure. Apparently, the *P. tritici-repentis* pathogen in the new environmental conditions has not yet accumulated enough mutations to ensure the intraspecies diversity, and is fixed in the new niche by increasing its virulent and aggressive properties. In addition, the life cycle of the parasite includes a sexual and an asexual stages. The presence of a sexual stage creates additional opportunities for the preservation of the fungus in the new environmental conditions and for the increase in genetic diversity, which allows the fungus to remain in non-standard conditions in the uncharacteristic assortment of wheat.

Fusariosis of cereals is among the most dangerous diseases of plants. In the late 1980s and early 1990s, the strongest epiphytotic of wheat head blight outbreak occurred in the North Caucasus. The main causative agent of the disease was the fungus *Fusarium graminearum* Schwabe. Historically, its main habitat in Russia is the North Caucasus and the Far East. This fungus was found in the cereal crops in the Central Chernozem region and central Russia with a low frequency. However, since 2003, *F. graminearum* appeared in complex of pathogens that cause fusariosis of cereal crops cultivated in the North-West Russia [6]. It was originally identified in Leningrad Region, but in 2007 it appeared in Vologda, Novgorod, and Kirov regions, and in 2008 in Kaliningrad and Pskov regions. The average disease severity on cereals was 93.3 % in 2007 and 87.3 % in 2008. The number of samples with *Fusarium* infection in the north of chernozem Soil Zone averaged 93.3 % in 2007 and 87.3 % in 2008 [7], which can be explained by the global warming and the changes observed in the composition of the atmosphere. This is proved by the publication of our colleagues from the Nordic countries. In the recent years, *F. graminearum* predominates on cereals in the Netherlands [8], Great Britain [9], North Germany [10], and Finland [11].

With the ambient temperature changes, changes in species dominance can occur. The situation described for the North of Italy can be an example [12]. In this area, fungus *Fusarium verticillioides* (Sacc.) Nirenberg was predominant on corn. The optimum for the growth of the above species is 25-30 °C. The summer of 2003-2004 was dry and hot. Fungus *Aspergillus flavus* Link ex Gray tolerant to the temperature of 35 °C became predominant. Temperature above 30 °C enhanced the production of aflatoxins. As for the production of tricothene mycotoxins, their accumulation in wheat grains was shown to increase at the temperature above 32 °C [12].

Septoriosis is another harmful disease of wheat. There are two main causative agents. These are *Stagonospora nodorum* (Berk.) Castell. et Germano [teleomorph *Phaeosphaeria nodorum* (E. Mull.) Hedjiar.] which causes wheat leave and ear septoriosis, and *Septoria tritici* Roberge ex Desm. [teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schroet.], wheat leave septoriosis pathogen. The *Stagonospora nodorum* species is widespread, but it is predominant and most harmful in the North-West and Volga-Vyatka region, as well as in the Baltic countries. The *Septoria tritici* species is predominant and causes great harm in the southern regions, such as the North Caucasus, the Lower Volga, in the south-east of Ukraine, and in Moldova [13]. Since 2007, the south species of *S. tritici* became the pathogen in the North-West [14]. Infestation of spring wheat in the phase of

milky-wax ripeness was 51-100 % depending on the variety, and the severity of the disease was from 8 to 30 %.

A new pathogen of *Ramularia collo-cygni* was described on barley in 2011 in the Krasnodar Territory [15]. This thermophilic species is distributed mainly in the southern countries, but in 2013 it was found in the north of Russia in Arkhangelsk Region in the vicinity of Kotlas.

All these observations suggest that global warming results in the expansion of the area of thermophilic fungi species, and southern diseases begin to spread to the north.

Climate and soil organisms. Extreme weather events such as long periods of drought and heavy rainfalls have a strong impact on the metabolic activity of microbes. This can lead to a change in the balance of soil nutrients and even increase the nitrogen oxide emissions. Global climate change may affect the structure of the soil microbiota, directly or indirectly. The direct effect is manifested, for example, by an increase in the soil temperature which usually stimulates the activity of soil microorganisms. Hot and dry spring and summer weather is favorable for the development of soil fungi such as root rot pathogens. The warming of the soil is known to increases the frequency of horizontal gene transfer among bacteria. An increase in temperature from 20 to 30 °C has been experimentally shown [16] to increase 10-fold the frequency of conjugation and gene transfer between *Escherichia coli* и *Rhizobium meliloti*. Indirect effects of climate change on microorganisms are manifested through the effect on the physiological and biochemical processes in plants. For example, it is known that an increase in CO_2 concentration decreases the concentration of nitrates in the soil and at the same time increases mycorrhiza saturation by 47 %, and the number of nitrogen-fixing bacteria increases as well [17].

Impact of climate change on the bio-ecology of microorganisms. Each organism has its optimum temperature for growth and development. Typically, in the majority of fungi, this optimum is in the range of 24-28 °C with a minimum of 4-8 °C and the maximum allowable temperature of 30-35 °C. In some species, the maximum exceeds 40 °C, e.g., for the spores of *Ustilago avenae* Pers. it is 50-53 °C [18]. Apparently, heat-resistant species should be paid particular attention when predicting phytosanitary situation due to climate warming.

Fluctuations of temperature sensitivity may occur within the same genus. For example, wheat rust pathogens differ in their requirements for temperature. In particular, yellow rust develops at the temperature of 2-15 °C, brown rust needs 10-30 °C and for stem rust it is 15-35 °C [19]. The spores of *Tilletia asperifolioides* G.W. Fisch., *T. bromi-tectorum* J. Urries, *T. caries* (DC) Tul., *T. contraversa* Kuehn, *T. elymi* Diet et Holw., *T. fusca* Eliss et Everh., *T. guyotiana* Hariot, *T. holci* (West.) DeToni, *T. scrobiculata* G.W. Fisch. germinate at 5 °C, whereas it is 10 °C for *T. asperifola* Eliss et Everh. spores and 15 °C for *T. pallida* G.W. Fisch. Spores [20]. It should also be considered that in different stages (conidia sporulation, mycelial growth, formation of overwintering structures) fungi may have different requirements for temperature. Fungus *Cronartium fusiforme* Hedgc. et N.R. Hunt is an example, with its ascospores germinating at the minimum temperature of 11 °C, optimum of 21 °C and maximum of 29 °C, urediniospores germinating at the minimum temperature of 8 °C and maximum of 29 °C, and teliospores germinating at 15 °C and 26 °C, respectively [21].

Obviously, for the prediction of the potential development of a disease in the new environmental conditions, particular attention should be paid to the detailed study of the temperature requirements in each pathogen stage. This is

also important because different scenarios for climate warming are expected. Some experts believe that winters will be warmer, while others suggest that it will be summers, and the warming will affect the northern latitudes greater [22]. Moderately warm winters may contribute to the survival of the fungal genera *Alternaria*, *Cercospora*, *Colletotrichum*, *Phomopsis*, *Septoria*, *Venturia.* Higher winter temperature contributes to the preservation of the stem rust pathogen *Puccinia graminis* Pers. and enhances the further development of the disease on *Festuca arundinaceae* and *Lolium perenne* [23]. Soil fungi belonging to the *Pythium, Rhizoctonia, Sclerotinia* genera, etc. will be affected by climatic factors to a lesser extent, as they form the overwintering structures that protect them from external influences. According to researchers, the warmer summer contributes to the development of the *Podosphaera*, *Sphaerotheca*, *Uncinula* and *Ustilago* species [24].

Temperature increases may result in a decrease of the latent period such as in a wheat septoriosis pathogen *S. tritici* [25]. When this pathogen was cultured in different temperature conditions, a dependency of the pycnidia formation frequency on the incubation temperature was found [26]. Thus, pycnidia were formed on the varieties of Katepwa, Kyle, and AC Melita at day 11 after inoculation and incubation at 22 °C during the day and at 15 °C at night and at incubation day 19 at 15 °C during the day and at 11 °C at night. Observations of the potato late blight in Finland have shown that while at the beginning of the 1990s it appeared in 80-90 days after planting, starting from 1998 it appeared on the day 40 to 50 after planting [27]. With increasing temperature, the leaf infestation increases concurrently which indicates the growing pathogen aggressiveness. Rust fungi behave similarly, i.e., temperature increase often results in an increase of aggressiveness [28].

Climate and interrelations of microorganisms with plants. Undoubtedly, climate change will affect the host and parasite interrelations. Under the impact of high temperatures, plants habit can change, tissues are destroyed, and organs die. At higher temperatures, the changes in the RNA metabolism and protein synthesis, as well as enzyme activity are possible [29]. Plants are known to be more susceptible to diseases after stress [30]. This will undoubtedly affect their resistance against parasites and pathogens. Research shows that, for example, wheat and oats become more susceptible to pathogens with an increase in temperature, but some types of cereal become more resistant with increasing temperature [31]. The temperature can seriously affect the effectiveness of resistance genes. Thus, the temperature above 20 °C may cause the loss of resistance to stem rust in oat varieties with genes *Pg3* and *Pg4* [32]. Conversely, the lignification of cell walls is enhanced at higher temperatures, thereby the resistance against fungal pathogens increases. Rape resistance to stem cancer is observed at a temperature of 15 °C and is not manifested at 25 °C [33]. The varieties with durable resistance to pathogens are less dependent on the environmental factors [34]. The temperature also affects the functioning of virulence genes in parasites [35].

Some protective measures in the conditions of climate warming. First of all, constant monitoring of the emergence of new plant diseases is required. Particular attention should be paid to pathogen species that may be found in the more northern regions. To control the soil microbial communities, metagenomic studies are necessary. To predict the species preservation in the new environmental conditions and the development of diseases, a detailed study of the biological and ecological characteristics of pathogens is required. As noted above, even the fungi belonging to the same genus differ in their requirements to the temperature. Based on the biological and ecological

features of pathogens, their development due to climate warming can be predicted. However, it should be kept in mind that climate change may be different in different areas of the same country. The result of protective measures, of course, depends on the effects of climatic factors. Extreme temperature, winds, rainfall, etc. may affect the phytotoxicity of the fungicides, the dynamics of their content in the soil, precipitation on the leaves, leaf uptake and degradation of the drug. Our study in several areas of the country has proven that climate and weather conditions had a significant impact on the effectiveness of pesticides. Long experience in the North Ossetia showed that treatment with pesticides in the years with normal weather conditions increased the yield of Partizanka variety wheat by 4.3-5.0 %. The yield was reduced by 8.5-13.7 % in the year with excessive moisture after treatment with pesticides [36]. These findings suggest the need to consider not only the degree of development of pests, but also climatic features. In breeding programs, more attention should be paid to the unification of small resistance genes and genes controlling race nonspecific resistance in varieties, which will provide long-term disease resistance in the changing environmental conditions. New approaches to the breeding of resistant varieties, including marker (marker-assisted selection) and transgenic selection should be used more comprehensively [37, 38].

Thus, humanity has entered the era of global climate change. The challenges of agro-ecosystems conservation in the new environmental conditions are on the agenda. Special attention should be paid to the breeding of resistant varieties, in particular to the creation of varieties with a broader ability to adapt to the changing environmental conditions. One of the factors of maintaining agro-ecosystem stability is the science-based system of integrated plant protection. Climate change on the planet is of international importance, so it would be timely to establish an international network of observing the spread of plant diseases, the microorganisms in the soil environment and to ensure the continuous exchange of information between countries.

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BIODIVERSITY OF ENDOPHYTIC BACTERIA AS A PROMISING BIOTECHNOLOGICAL RESOURCE

V.K. CHEBOTAR', A.V. SHCHERBAKOV, E.N. SHCHERBAKOVA, S.N. MASLENNIKOVA, A.N. ZAPLATKIN, N.V. MAL'FANOVA

All-Russian Research Institute for Agricultural Microbiology, Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail vladchebotar@rambler.ru

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Abstract

Endophytic called bacteria are those colonizing the internal tissues of plants without causing disease and not rendering negative influence on its development. There are great prospects for search, selection and study of new species of endophytic bacteria, improving the development of plants, with the aim of creating new microbial preparations for adaptive crop production. Since bacterial endophytes colonize the same ecological niches in the plant as phytopathogenic microorganisms, they are a promising agent for biocontrol of phytopathogens. Classical studies of biodiversity of endophytic bacteria based on a characterization of isolates obtained from internal plant tissues after surface sterilization. Endophytic bacteria are able to improve phosphorus nutrition of plants, to produce IAA and siderophores. It is shown that endophytic bacteria are capable of producing vitamins, have a number of additional properties necessary for the improvement of plant development, such as: regulation of osmotic pressure, regulation of stomata, modification of root development of plants, regulation of nitrogen nutrition of plants. Endophytic bacteria are able to reduce or prevent the negative effects of pathogenic microorganisms on plants. Inoculation of plants by endophytic bacteria is able to significantly reduce the harm caused to plants by pathogenic fungi, bacteria, viruses, insects and nematodes. Unique strains of endophytic bacteria can be used directly for inoculation of seeds or seedlings, reducing, thus, the influence of biotic and abiotic factors on the plant, due to the active colonization of internal tissues of plants and subsequent positive biochemical and physiological effect on the plant. While in endosphere, endophytes have a significant advantage over organisms that live in the rhizosphere and phyllosphere due to the stable pH, humidity, flow of nutrients and lack of competition from a large number of microorganisms. For the inoculation of plants with endophytic bacteria do not require large amounts of inoculum, taking into account high specificity of such plant-microbe symbiosis and competitiveness of endophytic bacteria. This technique can be very attractive for biotechnological productions, seeking the replacement of traditional chemical pesticides.

Keywords: endophytic bacteria, biodiversity, plant-microbial interaction, plant growth promotion biocontrol, secondary metabolites, the genome of bacteria, microbial preparations.

Associations of plants with beneficial microorganisms attract the attention of scientists not only as an object for the study of the fundamental bases of interaction of different organisms, but also in terms of their possible use in the practice of environmentally oriented crop production. Besides rhizosphere microorganisms [1-3], the so called endophytic bacteria are known that colonize the plant tissues without causing diseases and do not render the negative effect on the plant [4, 5]. Any of the 300,000 species of plants that exist in the world is the host for one or more species of endophytic bacteria [6]. However, currently, only a few of plant species have been sufficiently studied with respect to the presence of endophytes.

So, the search of new species of endophytic bacteria that have beneficial effect on the development of plants opens the prospects for the development of effective microbial preparations for adaptive crop production [7]. Bacterial endophytes colonize the same ecological niches in the plant as phytopathogenic microorganisms, so they are a promising agent for the biocontrol of phytopathogens [3].

Several studies have shown that endophytic bacteria are capable of inhibiting the development of pathogenic microorganisms [8, 9] and nematodes [10-12] by the synthesis of biologically active compounds. The study of the biodiversity of these bacteria will make it possible to isolate and identify the substances for the new drugs against human, plant and animal diseases [6]. Also, some strains of endophytic bacteria can be used for phytoremediation, i.e. for the cleaning of technologically contaminated areas by creating special plant-bacterial systems [7, 13-18].

The endophyte niche in a plant can be taken only by those bacteria that penetrate plant tissues. Usually they colonize intercellular spaces and can be isolated from all plant parts, including seeds. Endophytic bacteria have been found in monocotyledonous and dicotyledonous plants, both woody (oak *Quercus* L., pear *Pyrus* L.) and herbaceous (sugar beet *Beta vulgaris* L., corn *Zea mays* L.) [19]. Classical studies were based on the characterization of isolates obtained from internal plant tissues after surface sterilization [20, 21]. Detailed lists of bacterial endophytes, including Gram-positive and Gram-negative species are given in several reviews [22, 23].

The study of endophyte microbial communities that inhabit stems, roots and tubers of crops by sequence analysis of the 16S RNA gene, fatty acids profile and utilization of various carbon sources, revealed that they are represented by the genera *Cellulomonas*, *Clavibacter*, *Curtobacterium*, *Pseudomonas* and *Microbacterium* [24]. The study of the aerial part of *Crocus albiflorus* revealed the diversity of bacterial endophyte communities, both previously known and unknown to science [25]. The high density of endophytic bacteria was observed in seedlings of poplar (*Populus* L.), spruce (*Picea* A. Dietr.) and larch (*Larix* Mill.) grown from tissue culture [26]. Based on the sequence analysis of the 16S RNA gene, the most of these isolates were classified as belonging to the *Paenibacillus* genus. Other endophytic bacteria of the genera *Methylobacterium, Stenotrophomonas* or *Bacillus* were found only in some poplar, spruce and larch tissue cultures. The *Paenibacillus* species that are close to *P. humicus* are accumulated in tissues in vitro without the apparent adverse effect on plants. Poplar microsprings inoculated with an endophytic strain of *Paenibacillus* sp. 22 had significantly more roots per spring, and such roots were longer compared to control roots after 3 weeks of culture [26].

In China (Hebei Province), the diversity of endophytic bacteria of rice was studied using the sequence analysis of the 16S RNA gene (*Oryza sativa* L.) [27]. The presence of phylae of bacteria belonging to the alpha, beta, gamma, delta and epsilon subclasses of *Proteobacteria*, *Cytophaga*, *Flexibacter*, *Deinococcus-Thermus*, *Acidobacteria* and archean has been shown. *Betaproteobacteria* were the dominant group (27.08 % of all isolates), in which the *Stenotrophomonas* genus was predominant. Over 14 % of the isolates belonged to the uncultivated bacterial species [27]. In India, the variety of endophytic bacteria was investigated in the stalks of maize (*Zea mays* L.) grown in the tropics [28]. Endophytes were found throughout the growing season. Their abundance was 1.36×10^5-6.12×10^5 CFU/g of raw biomass. Identification of bacterial isolates performed by chromatographic analysis of fatty acid profiles demonstrated the dominance of *Bacillus pumilus*, *B. subtilis*, *Pseudomonas aeruginosa* and *P. fluorescens*.

A total of 853 strains of endophytes were isolated in the study of four crop species, corn *Zea mays* L., sorghum *Sorghum* Moench, soybean *Glycine max* (L.) Merr. and wheat *Triticum* L., as well as a wide range of wild cereals and legumes. About half of them belonged to the Gram-positive, and the rest were the Gram-negative bacteria. The analysis of the profile of fatty acids showed that the isolates of endophytes belonged to 15 genera, *Agrobacterium*, *Bacillus*, *Bradyr-*

hizobium, *Cellulomonas*, *Clavibacter*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Microbacterium*, *Micrococcus*, *Pseudomonas*, *Rothia*, *Xanthomonas. Bacillus*, *Corynebacterium* and *Microbacterium* were predominant. Moreover, endophytic bacteria of the *Cellulomonas*, *Clavibacter*, *Curtobacterium* and *Microbacterium* genera isolated from both the cultural and wild plants were of the highest colonization activity in maize and sorghum [29].

Modern method of imaging of the microorganisms based on auto fluorescent proteins [30] help to detect and count the microorganisms in situ on the surface and within a plant [30-32]. One of these marker systems is represented by a green fluorescent protein (GFP), which proved to be very useful in the monitoring the colonization of internal plant tissues by pseudomonas [31, 32]. Bacterial cells with the *gfp* gene under a constitutive promoter integrated into the chromosome can be readily identified using epifluorescent microscopy or a confocal laser scanning microscope [15, 33]. The colonization of internal plant tissues by endophytic bacteria can also be visualized using a β-glucuronidase (GUS) reporter system. Thus, a GUS-labeled *Herbaspirillum seropedicae* Z67 strain was used to inoculate rice seedlings. At this, the most intense staining was observed in coleoptiles, roots and at the junction of lateral roots to the main root [34]. Subsequently, *Herbaspirillum seropedicae* colonizes intercellular space, aerenchyma and cortical cells, and some bacterial cells can permeate the stele and further into the vascular tissue.

Visible anatomical differentiation of the partners is not characteristic of the plant associations with endophytic bacteria. However, the molecular mechanisms described for the legume-Rhizobium and arbuscular-mycorrhizal symbioses may be involved in their development [35].

There is evidence indicating the existence of certain specific interactions in the system of «endophytic bacteria—host plant». The *Azoarcus* sp. obligate nitrogen-fixing endophyte (strain BH 72) has been shown to induce the defense mechanisms of the host plant, making the colonization of rice with other endophytic bacteria difficult [36]. As a result of maize seedlings inoculation by dominant strains of stem endophytes *Bacillus pumilus, B. subtilis, Pseudomonas aeruginosa*, and *P. fluorescens*, greatest density of endophytic bacteria in seedlings was observed in the variant with *B. subtilis* [37].

Investigations of the growth-stimulating activity of endophytic bacteria have been conducted [37]. Unlike the biological control strains of rhizosphere bacteria, they do not inhibit the growth of pathogenic microorganisms but stimulate plant growth by improving their mineral nutrition. Endophytic bacteria are able to improve phosphorus nutrition of plants [38, 39], to produce indolyl acetic acid [40], siderophores [41], and vitamins [42]. In addition, they have been shown to take part in the regulation of osmotic pressure, regulation of stomata, modification of root development, and regulation of nitrogen nutrition. In recent years, growth-stimulating endophytes have been actively used for reforestation and phytoremediation of technologically contaminated soils [7].

Endophytic bacteria are able to reduce or prevent the negative effects of pathogenic microorganisms on plants [18, 44, 45]. Inoculation of plants by endophytic bacteria is able to significantly reduce the harm caused to plants by pathogenic fungi, bacteria, viruses, insects and nematodes [22, 45-47]. Certain species of endophytic bacteria are assumed to trigger the defense mechanism of plants known as induced systemic resistance (ISR) which is similar to systemic acquired resistance (SAR) [7, 48]. Consequently, bacterial endophytes are promising for the development of environmentally friendly methods of microbiological control of plant diseases.

Many endophytes are the representatives of well-known soil bacteria of the genera *Pseudomonas, Bacillus* and *Burkholderia* [22] serving as producers of bacterial secondary metabolites (antibiotics, anti-cancer substances, volatile organic compounds, fungicidal, insecticidal and immunosuppressive agents). However, endophytic bacteria are still insufficiently used as sources of biologically active substances [7].

Unique strains of endophytic bacteria can be used directly for inoculation of seeds or seedlings, reducing the effect of biotic and abiotic factors on the plant, due to the active colonization of internal tissues of plants and subsequent positive biochemical and physiological effect on the plant. While in endosphere, endophytes have a significant advantage over organisms that live in the rhizosphere and phyllosphere due to the stable pH, humidity, flow of nutrients and lack of competition from a large number of microorganisms [42]. It is very important that endophytes are not accidental bacteria occupying the endosphere niche. Most likely, they are chosen by the very plant as most compatible and capable of providing it with the substances required for the protection against stress factors. The energy spent by the plant to produce the biomass of endophytic bacteria, is compensated by the improved development ofthe physiological state of the host.

The study of endophytes in the internal tissues of poplar showed that they are promising for the creation of microbial preparations used for phytoremediation of fields contaminated with toluene, volatile hydrocarbons and heavy metals [7, 15, 17].

Inoculation of plants with endophytic bacteria does not require large amounts of inoculum, taking into account high specificity of such plant-microbe symbiosis and the competitiveness of endophytic bacteria. This technique can be very attractive for biotechnological production, seeking for the replacement of traditional chemical pesticides. The future use of combinations of endophytes with commercial pesticides for the treatment of seeds or seedlings may result in a synergistic effect against one or more pathogens. Chemical pesticides can produce a transient inhibitory effect on phytopathogenic microorganisms, while the biological agents affect phytopathogens adversely throughout the growing season.

Thus, in nature, plants develop in close interaction with endophytic bacteria that are able to increase crop yields, promote soil phytoremediation, inhibit the development of pathogens, fix atmospheric nitrogen and produce biologically active substances. Using endofit-plant interactions can enhance the development of crops and reduce costs on the production of food and technical agricultural production. Understanding the mechanisms that ensure endophytic bacteria the ability to interact with plants and to positively affect their development will enable the better use of the biotechnological potential of these microorganisms.

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PHYLOGENETIC ANALYSIS OF *Rhizobium* STRAINS, ISOLATED FROM NODULES OF *Vavilovia formosa* (Stev.) Fed.

A.K. KIMEKLIS[1], V.I. SAFRONOVA[1], I.G. KUZNETSOVA[1], A.L. SAZANOVA[1], A.A. BELIMOV[1], A.G. PINAEV[1], E.P. CHIZHEVSKAYA[1], A.R. PUKHAEV[2], K.P. POPOV[3], E.E. ANDRONOV[1], N.A. PROVOROV[1]

[1]*All-Russian Research Institute for Agricultural Microbiology,* Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail kimeklis@gmail.com;
[2]*Gorsky State Agrarian University,* 37, ul. Kirova, Vladikavkaz, Republic of North Ossetia-Alania, 362040 Russia;
[3]*North-Ossetian Nature Reserve,* 1, ul. Ch. Basievoi, Alagir, Republic of North Ossetia-Alania, 363240 Russia
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Abstract

Among the 5 genera of tribe *Fabeae*, *Vavilovia* Fed. is the least studied consisting of the only species *Vavilovia formosa* (Stev.) Fed. Vavilovia's area of growth is limited by the highlands of Central and Eastern Caucasus, with only several known populations on the territories of Armenia, Dagestan, Norht Ossetia, Azerbaijan, Iran, Iraq, Siria, Turkey, and by the environmental conditions. The only phylogenetic research of rhizobia isolated from the nodules of vavilovia from the Nortn Ossetian population demonstrated significance of both slow-growing and fast-growing microsymbionts' specific and genetic diversity. It was shown that all of the fast-growing isolates, belonging to *Rhizobium leguminosarum* species, carry *nodX* gene in their genomes. Three expeditions to the regions of Armenia, Dagestan and North Ossetia succeeded in finding and collecting plants of *Vavilovia formosa* (Stev.) Fed. with its nodules, from which later rhizobia isolates were obtained. We have chosen nineteen fast-growing isolates, derived from ten plants' nodules, to identify their species affiliation, to trace geographical isolation and also to try to track down its genetic differences from rhizobia, which nodulate other plants of tribe *Fabeae*. To make this we sequenced ITS (internally transcribed spacer) fragment and *nodA* gene, and made screening of the isolates for the presence of *nodX* gene, which controls rhizobia host specificity. Obtained sequences were used to calculate genetic distances between groups of rhizobia, i.e. different regions isolates (Armenia, Dagestan, North Ossetia) and isolates from different plant hosts (vavilovia, pea, clover). Results of ITS sequencing showed that all strains involved in the analysis belong to *R. leguminosarum* (bv. *viciae*) species. ITS-dendrogram shows relatively high heterogeneity of isolates, but on *nodA*-dendrogram they form a very compact group. Difference in the structure of these dendrograms allows to assume that *nodA* gene, chained with the genes of host specificity, can be easily transferred within the populations of *R. leguminosarum*, providing unlimited combinations of specificity to vavilovia with different variants of bacterial chromosome. Comparison of genetic distances based on ITS-sequences for the isolates in this study shows tendency to geographic isolation between them. Data on *nodA*-based genetic distances along with the presence of *nodX* gene in the genomes of all *R. leguminosarum* strains in this study point out the presence of its host specificity within biovar *viciae*. It seems that correlation between strain origin and the genetic structure of *nodA* reflects presence of highly specific interactions among each group of *R. leguminosarum* strains with their plant-hosts, whereas correlations with the structure of ITS-loci reflect rhizobia adaptation to soil environment.

Keywords: *Vavilovia formosa*, *Rhizobium leguminosarum*, legume-rhizobia symbiosis.

Tribe *Fabeae* (син. *Vicieae*) is among most representative ones in the family *Fabaceae* Endl. It comprises over 300 species, and, however, its taxonomy is still being revised significantly. At that, many species of the tribe are of practical use in agriculture. Most ancient cultivated plants such as pea (*Pisum sativum* L.) and tare (*Vicia sativa* L.) are the representatives of this tribe [1]. Of five genera of the tribe, namely *Lathyrus* L., *Vicia* L., *Lens* Mill., *Pisum* L. and *Vavilovia* Fed., the last one which consists of a single species *Vavilovia formosa* (Stev.) Fed. remains least studied. Its areal covers the highlands of Central and

Eastern Caucasus, and only a few populations are also known in Armenia [2], Dagestan and North Ossetia [3], Azerbaijan [4], Iran, Iraq, Syria and Turkey [5], and its vegetation is limited to conditions the environment [6], therefore, the plant itself and its microsymbionts, remained unknown for a long time.

Recently, based on phylogenetically important genes *matK*, *trnL-F* and *trnS-G*, and ITS (internally transcribed spacer) fragment, the evidence was reported that the vavilovia is taxonomically close to genera *Pisum* and *Lathyrus*, being, nevertheless, clustered separately within tribe *Fabeae* [7]. The only reported phylogenetic study of rhizobia isolates from vavilovia nodules from North Ossetian population showed a fairly broad species abundance and genetic diversity of both fast-growing and slow-growing microsymbionts [8].

In the same study it was also found that all fast-growing isolates of *R. leguminosarum* possessed the gene *nodX* peculiar to the symbionts of *Pisum sativum* cultivar Afghanistan [9]. All known plants of tribe *Fabeae* are of the same cross inoculation group and can produce nodules with the bacteria of the abovementioned species, though symbiosis formation is mostly indifferent to the presence of *nodX* gene. Nevertheless, in the *P. sativum* cv. *Afghanistan*, or Afghan pea, there is $sym2^A$ allele encoding receptor specific to the Nod-factor decorated with extra acetyl group bound under the *nodX* gene control [10].

At present, the studies of *nodX* gene role in individual interaction between a legume plant and rhizobia are fast developed [11]. Data summarized hereinabove, are enough to consider *Vavilovia formosa* and its microsymbiont a promising model in revealing mechanisms of specificity evolution in the legume-rhizobia symbiosis.

Due to our successful expeditions to Caucasian territories, the samples of *Vavilovia formosa* have been collected, and rhizobia from the plant nodules were isolated.

In this research, the fast-growing rhizobia isolates from *Vavilovia formosa* nodules were taxonomically attributed based on analysis of ITS region sequences, and their host specificity was studied by phylogenetic analysis of *nodA* and *nodX* genes. Additionally, the genetic distances in rhizobia isolated from different host plants of distinct locations were compared with regard to ITS and *nodA* sequences to estimate the environmentally important polymorphic parameters in studied isolates.

Technique. Nodulated vavilovia plants were collected in 2012-2013 in North Ossetinian Reserve (North Ossetia, Alagirskii Region), Armenia and Dagestan at more than 1,500 m above see level. Of total, 19 rhizobia strains isolated from 10 plants of three different populations were studied.

For rhizobia isolation we used common procedures [12]. From each nodule a strain was isolated and further grown on the media № 79 [13] at 28 °C. All isolated have been deposited to the ARRIAM Russian collection of agricultural microorganisms and stored at Station of Low Temperature Automated Storage of Biological Samples at –80 °C (Liconic Instruments, Lichtenstein) [14]. Data are available online at http://www.arriam.spb.ru).

Bacterial DNA was isolated according to standard protocol [15], and 1,000-1,300 bp ITS fragment (intergenic transcribed spacer) was amplified using primers FGPS1490-72 5'-TGCGGCTGGATCCCCTCCTT-3' and FGPL132′-38 5′-CCGGGTTTCCCCATTCGG-3′ [16, 17]. The *nodA* gene 66 bp fragment was analyzed with primers nodA-1 5′-GCRGTGGAARNTRN-NCTGGGAAA-3′ and nodA-2 5′-GGNCCGTCRTCRAASGTCARGTA-3′ [18]. The PCR mixture (25 μl) contained 150 μM dNTPs (Helicon, Russia), 1 U Taq-polymerase (Eurogen, Russia), 10 pM of each primers and 10-20 ng of purified DNA as matrix. Amplification was performed at a T100 amplifier (Bio-Rad, USA) as follows: ini-

tial denaturation at 95 °C for 2 minutes; 35 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 50 °C with FGPS1490-72/FGPL132′-38 primers or at 49 °C with nodA-1/nodA-2 primers for 30 seconds, elongation at 72 °C for 1 minute; final elongation at 72 °C for 3 minutes.. PCR fragments were separated electroforetically in 1 % agarose gel (Amresco, USA) with 0,5× TAE, with DNA molecular weigh marker 100 bp + 1,5 Kb + 3 Kb (Sibenzyme, Russia). PCR products were purified as described [19]. ITS region and *nodA* gene were sequenced on a ABI PRISM 3500xl genetic analyzer (Applied Biosystems, USA), with UGENE program (Unipro, Russia) for primary data processing [20]. The similarity was found by BLAST analysis using GeneBank database (http://www.ncbi.nlm.nih.gov/). The obtained data were used to design two dendrograms by joint neighbor method (MEGA v. 5.0) [21]. Evolution distances were evaluated by p-distance technique. A bootstrap analysis for 1,000 random samples was used for reliable clustering.

The *nodX* gene in isolated was detected in PCR with primers oMP199-F 5′-CCATGGGACCATCCAATGAAC-3′ and oMP196-R 5′-TTAAGCGACGGAAAGCCTTC-3′ [22]. The PCR mix composition and protocol was the same as described hereinabove except annealing at 53 °C. *R. leguminosarum* bv. *viciae* A1 strain [22] producing nodules with Afghan pea plants was a positive control.

Genetic distances were calculated for ITS fragments and *nodA* genes for each taxonomic and geographically distant group of rhizobia the same as between these groups. A genetic distance as estimated on ITS region and *nodA* gene sequences was compared in two distinct groups of microorganisma. The first one comprised *R. leguminosarum* bv. *viciae* (pea rhizobia), *R. leguminosarum* bv. *trifolii* (clover rhizobia) and tested isolated with correlation to host specificity, and the second one consisted of vavilovia isolated as correlated to geografic origin. We compared genetic distances both within and between the groups. The p-distance parameter estimated using MEGA v. 5.0 [21].

Results. ITS fragments were amplified and sequenced, partially or completely, in all tested isolated, and in Dagestanian isolates there were at least two different copies of ribosomal operon with ITS fragments of different size. In the dendrogram based on ITS sequencing (Fig. 1) these sequences are marker as l (long) and s (short).

In the Table 1 we indicated the size of amplified fragments and their homology to ITS sequence of typical strains of *R. leguminosarum* species of which TOM strain possesses *nodX* gene. Genomes of these strains are sequenced and available online in the NCBI database. Note, with regard to ITS, strains isolated from vavilovia nodules are similar to *viciae* biovar rather than to *trifolii* biovar, so far as an average similarity coefficient was 83±0.9 % in strain 3841, 89±0.8 % in strain TOM and only 80±1.0 % in strain WSM2304.

1. Origin of strains isolated from vavilovia *Vavilovia formosa* (Stev.) Fed. nodules and similarity of their ITS regions to referent strains sequences deposited in GenBank

Strain	Plant №	ITS fragment size, np	Similarity, %		
			Rhizobium leguminosarum bv. *viciae* 3841	*Rhizobium leguminosarum* bv. *viciae* TOM	*Rhizobium leguminosarum* bv. *trifolii* WSM2304
North Ossetia					
Vaf-01	1	565	96	86	90
Vaf-09		613	75	90	75
Vaf-10	3	1206	83	95	81
Vaf-12		1184	85	89	88
Vaf-23	4	565	99	88	100
Vaf-25		649	80	99	72
Vaf-26	5	1113	91	94	91

Continued Table 1

		Armenia			
Vaf-45	7	1199	82	93	80
		1010	78	90	79
Vaf-72		1199	82	93	80
		1010	78	90	79
Vaf-46	8	1199	82	93	80
		1010	78	90	79
Vaf-51		1199	82	93	80
		1010	78	90	79
		Dagestan			
VD1/1k	1D	1105	92	86	87
VD1/9m		1287	86	94	75
VD3/2(1)	3D	1032	82	83	77
VD3/7k		1032	82	83	77
VD6/12k	6D	1032	82	83	77
VD6/13m		1032	82	83	77
VD7/1	7D	1032	82	83	77
VD7/20m		1032	82	83	77

ITS dendrogram showed that all tested strains was attributed to family *Rhizobiaceae* and clustered into two groups of bacterial species with different types of ribosomal operon (the clusters I and II). Moreover, strains were grouped with no regard to taxonomic attribution or geographical prevalence. *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, and *R. etli*, the same as different vaviolovia isolates from Armenia and Dagestan were representatives of both groups. It probably could be due to only few chromosome types providing adaptation to environmental conditions and viability in bacteria.

Rhizobia isolated from vavilovia nodules did not form an individual cluster, and separated into groups demonstrating tendency to geographical isolation in the tested microorganisms. All Armenian strains, the same as 6 of 8 strains from Dagestan, were clustered at high statistical level (> 82 %) in group I. ITS fragments of vavilovia strains from North Ossetia was found only in group II and clustered along with strains from the same territory (Vaf-01 and Vaf-12, Vaf-09 and Vaf-10, Vaf-23 and Vaf-26). Vaf-25 strain was the only exception and grouped at 87 % bootstrap level to strains VD1/9m from Dagestan and *R. leguminosarum* bv. *viciae* TOM with *nodX* gene. Similar clusterization pattern we observed earlier in North Ossetinian isolates [8]. Therefore, it was reasonable to study whether there was *nodX* gene in the isolates from Armenia and Dagestan (Fig. 2), and it was found it all tested isolates.

Importantly, the ITS sequence, being the core part of the genome, allows us to understand only the taxonomic attribution of the stains whereas host specificity can be understood only from studying genes involved in formation of legume-rhizobia symbiosis. These are the genes of *nod* operons [23]. So we studied *nodA* gene. Though *nodA* gene does not control the symbiotic specificity directly, it is linked to genes encoding factors which provide host specificity. This gene is convenient to study due to a single copy in the rhizobia genome [18]. ITS and *nodA* based dendrograms differed sufficiently (Fig. 1, 3). In the *nodA* dendrogram, all isolates clustered with *R. leguminosarum* bv. *viciae* strains at 100 % statistical level. Thus, these isolates may be considered the rhizobia of the pea cross inoculation group. Based on *nodA* gene analysis, the isolates of this cluster were divided into three groups which did not coincide with ITS groups. In this, the isolates from North Ossetia and Dagestan clustered together into groups I and III, while Armenian isolates were found only in group II (see Fig. 3). It should be noted that in the *nodA* dendrogram the strain *R. leguminosarum* bv. *viciae* TOM was most closely related to the studied isolated. The strains from other taxones were not found in all three clusters comprising vavilovia isolates, that may be

consider an additional evidence toward isolation of these strains.

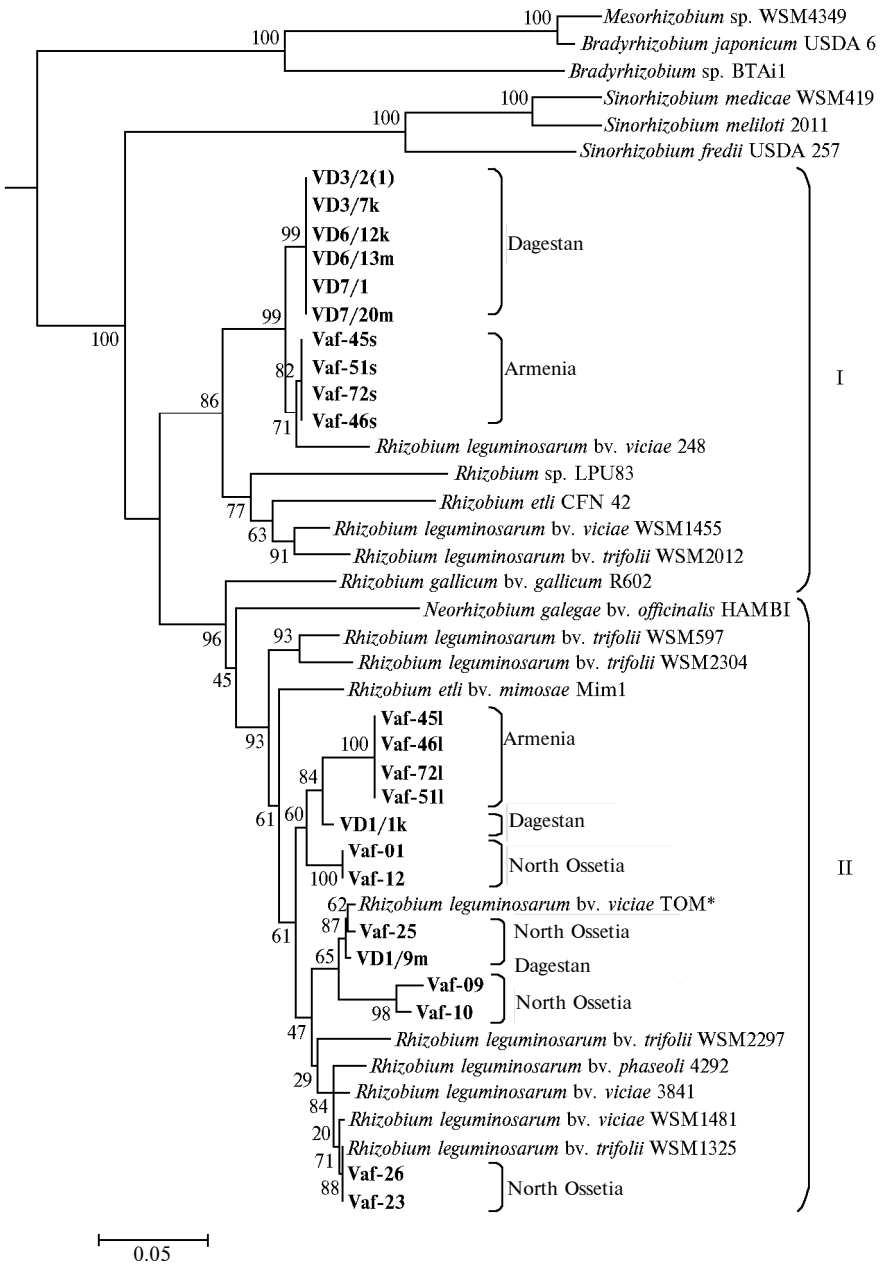

Fig. 1. Dendrogram based on ITS (758 bp) **sequences in rhizobia strains isolated from *Vavilovia formosa* (Stev.) Fed. plants depending on geographical origin.** Strains are grouped by the «neighbor-joining» method. Phylogenetic tree of the isolates reflects their taxononomy. Bootstrap value > 45 % for 1,000 random samples are show. I and II are statistically reliable clusters. Strain with *nodX* gene is marked (∗); s (short) and l (long) are Armenian strains with different copies of ITS fragments. Studied strains are mark bold.

In different groups of rhizobia no relationship with host specificity was traced based on ITS fragment sequencing analysis, since genetic distance within the groups was statistically indistinguishable from that between the groups (Table 2). Thus, more reasonably, there is a tendency to geographical isolation of the vaviolovia microsymbionts involved in our study.

2. Genetic distance on ITS and *nodA* gene sequences in rhizobia isolated from different host plants depending on geographical origin

Strain, origin	Genetic distance	
	ITS	*nodA*
Correlation to host plant		
As compared within group:		
from vavilovia (*Rhizobium leguminosarum* bv. *viciae*)	0.082±0.008	0.036±0.005
from pea (*Rhizobium leguminosarum* bv. *viciae*)	0.115±0.010	0.016±0.003
from clover (*R. leguminosarum* bv. *trifolii*)	0.089±0.008	0.119±0.010
As compared between groups:		
vavilovia—pea	0.096±0.008	0.045±0.006
vavilovia —clover	0.099±0.008	0.252±0.015
pea—clover	0.100±0.008	0.254±0.016
Correlation to geographical origin		
As compared within group:		
Armenia	0.000±0.000	0.001±0.001
Armenia	0.054±0.007	0.040±0.005
North Ossetia	0.050±0.006	0.040±0.005
As compared between groups:		
Armenia — Armenia	0.116±0.012	0.039±0.006
Armenia — North Ossetia	0.060±0.007	0.039±0.006
Armenia — North Ossetia	0.111±0.011	0.034±0.005

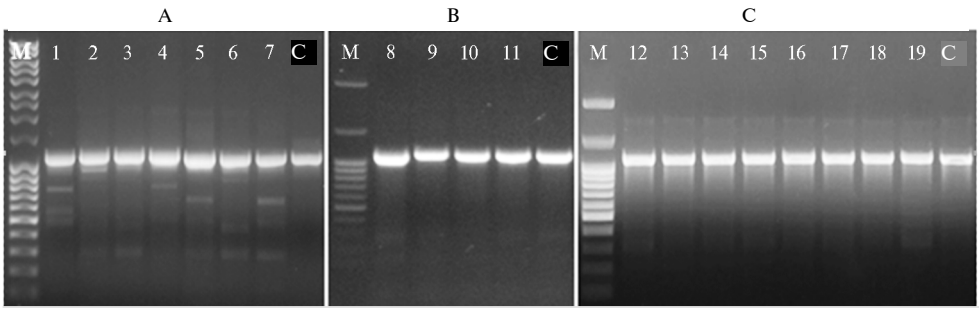

Fig. 2. PCR analysis of rhizobia isolated from *Vavilovia formosa* (Stev.) Fed. in North Ossetia (A)**, Armenia** (B) **and Dagestan** (C) **with primers omP196/omP199**: 1 — Vaf-01, 2 — Vaf-09, 3 — Vaf-10, 4 — Vaf-12, 5 — Vaf-23, 6 — Vaf-25, 7 — Vaf-26, 8 — Vaf-45, 9 — Vaf-72, 10 — Vaf-46, 11 — Vaf-51, 12 — VD1/1k, 13 — VD1/9m, 14 — VD3/2(1), 15 — VD3/7k, 16 — VD6/12k, 17 — VD6/13m, 18 — VD7/1, 19 — VD7/20m. DNA of *Rhizobium leguminosarum* bv. *viciae* A1 was used as control (C). Molecular weight marker (M) is MassRuler (Thermo Scientific, USA) (A) и 100 bp + 1.5 Kb + 3Kb (Sibenzyme, Russia) (B, C).

Nevertheless, in the genetic distance assessment based on the *nodA* gene sequence homology, clear correlation to the host specificity was disclosed though no relationship to the geographical origin of vavilovia plants was found. The *nodA* gene determines binding fatty acid residue to oligochitin chain which is common in the Nod-factor synthesis for all rhizobia, therefore, the observed correlation is probably due to *nodA* linkage to the host specificity genes. In *R. leguminosarum* these genes are grouped as compact clusters of about 20,000 bp in size on the *Sym*-plasmids. The revealed correlation seems to reflect the fact of high specific interactions between each group of *R. leguminosarum* strains and the corresponding host plants.

Because of no similarity in *nodA* and ITS based dendrogram patterns, it is reasonably to assume an easy exchange of the plasmid-located *nod*-genes in *R. leguminosarum* populations which provides free combination of the bacterial genes of host specificity to vavilovia and different types of bacterial chromosomes in biovar *viciae* and, in some cases, in biovar *trifolii*,. This is evidenced by 100 % homology of ITS region in one vavilovia rhizobial isolate of those involved in our investigation and in a representative strain WSM2304, being in line with earlier reported probability of active *Sym*-plasmids transfer between mentioned biovars in nature.

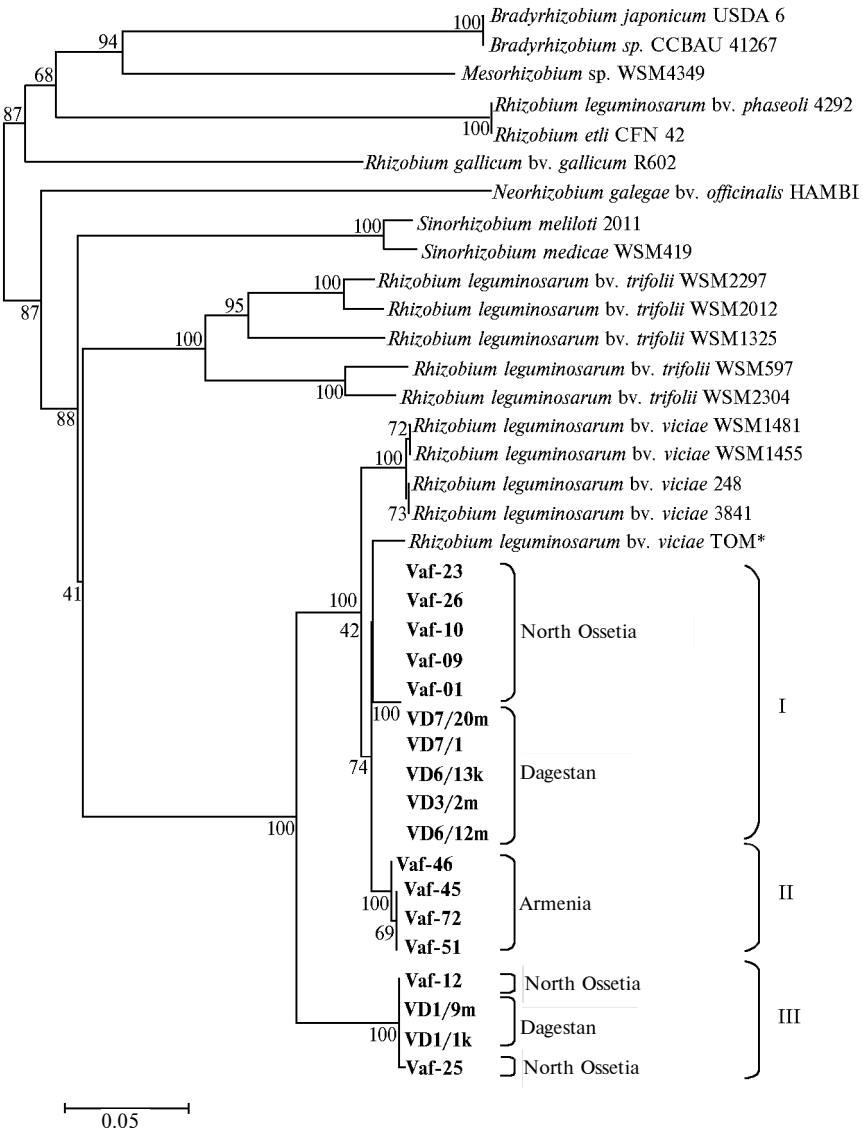

Рис. 3. Dendrogram based on *nodA* (531 bp) **sequence in rhizobia strains isolated from *Vavilovia formosa* (Stev.) Fed. plants depending on geographical origin.** Strains are grouped by the «neighbor-joining» method. Phylogenetic tree of the isolates reflects their taxononomy. Bootstrap value > 45 % for 1,000 random samples are show. I, II and II are statistically reliable clusters. Strain with *nodX* gene is marked (∗). Studied strains are marked bold.

Thus, from ITS sequencing analysis, all rhizobia strains isolated from *Vavilovia formosa* (Stev.) Fed. nodules and involved in our investigation were taxonomically attributed to *Rhizobium leguminosarum*. Moreover, taking into account the phylogenetic analysis of both ITS region and *nodA* gene sequences, it is possible to attribute these strains to the boivar *viciae*. According to the calculated genetic distances, all these isolates form a distinct statistically separated group in which a tendency to geographical isolation and strict host specificity is traced. Grouping *R. leguminosarum* bv. *viciae* strains into clusters separately from other strains, and disclosing Afghan pea nodulation gene *nodX* in all isolates from *Vavilovia formosa* can be basic for further study of these rhizobia group in the view to its possible attribution as an individual taxonomic group of *R. leguminosarum* species.

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APPLICATION OF NEW BIOFERTILIZERS AND BIOLOGICAL PRODUCTS IN THE CULTIVATION OF SPRING WHEAT (*Triticum aestivum* L.) AND POTATO (*Solanum tuberosum* L.)

G.Yu. RABINOVICH, N.G. KOVALEV, Yu.D. SMIRNOVA

All-Russian Research Institute of Reclaimed Lands, Federal Agency of Scientific Organizations, 27, pos. Emmauss, Kalininskii Region, Tver' Province, 170530 Russia, e-mail vniimz@list.ru
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Abstract

Worldwide, the mineral fertilizers, because of their multiple negative effects, become less popular. Therefore, more producers prefer to use biofertilizer and biological preparations for obtaining high crop yield with good quality. Fertilizers fill the soil with additional material, while biologicals contribute to effective mobilization of soil organic matter and biota. At All-Russian Research Institute of Reclaimed Lands (VNIIMZ) the KMN biofertilizer (multi-purpose compost) has been developed. Advantage of the KMN as a base fertilizer lies in its high nutritional value, physiological, ecological and biogenic properties. Also, a novel biological product, the LPB, have been developed. It is characterized by the presence of physiologically relevant amounts of growth factors and energy sources in a combination favorable to the plant. The LPB composition allows to maintain soil fertility and crop productivity. In the present study, we evaluated the effectiveness of the KMN and LPB on the potato (*Solanum tubetosu*m L.) variety Zhukovsky and spring wheat (*Triticum aestivum* L.) variety Irgina. The micro plot tests were conducted in 2011-2013 on the experimental field of VNIIMZ (Tver' Province). With spring wheat, the KMN biofertilizer was used at a dose of 7 t/ha, and NPK dose was 300 kg/ha. Biological product (LPB stock preparation) was diluted with tap water as 1:300, 1:500 and 1:1000 and applied at 0.1 l/m^2 by spraying plants. In control no fertilizers were used. In total, there were 12 combinations of plant treatment. In wheat, a total yield, weight of 1000 grains, and the grain protein content were estimated. With potato, only KMN (4 t/ha) was used as fertilizer. Potato plants were treated with LPB three times (at sprouting, budding and flowering) by means of a hand sprayer. In this, four LPB doses (0.05, 0.1, 0.2 and 0.3 l/m^2) and two dilutions (1:30 and 1:300) of the stock preparation were used. Control potato plants were not treated with LPB. The potato yield and the tuber distribution by size were estimated. The intensity of redox processes in the soil was evaluated by the oxidative-reduction ratio (ORR) as the catalase to dehydrogenase activity rate. Spraying spring wheat with 1:300 LPB solution at 0.1 l/m^2, additionally to KMN application, resulted in the highest yield among all the studied variants (27.5 kg/ha), and it was 15 % higher compared to LPB application together with NPK. The rich harvest was obtained due to larger grains. Mobilizing effect in the soil under spring wheat was higher if no basic fertilizers were used, and also when NPK was used without biopreparation. At the same time, the crop yield with NPK and without fertilizers was generally inferior to that obtained with NPK together with biologicals, when the yield increased due to activity of LPB microflora, and with KMN due to activation of microflora of biofertilizer and bioprparation, particularly at high concentration of the biopreparation (1:300). The highest yield of potatoes (372.1 kg/ha, including 352.1 kg/ha of commercial tubers) was obtained by using LPB (1:300) at 0.1 l/m^2 with KMN as the basic fertilizer. Crop spraying with LPB was enough to supply plants with nutrients at the key growth phases. The KMN role was to supply plants at the early development with available nutrients. Note, the soil after harvesting remained free from chemical pollution and enriched with helpful microflora, contributing to the reproduction and preservation of soil fertility. Therefore, the developed biologicals can be successfully used in crop cultivation.

Keywords: multi-purpose compost, LPB, spring wheat, *Triticum aestivum* L., potato, *Solanum tuberosum* L., productivity, cultivation, agrotechnology.

The global experience of agriculture shows that the crop yield is directly dependent on the amount of fertilizer used. However, a sharp increase in their price and the general deterioration of environmental conditions force the search

for efficient and environmentally friendly ways to increase yields [1, 3] that ensure the preservation of soil structure and the balance of soil organic compounds, trace and mineral elements [4, 5] and facilitate self-regulation of soil ecosystems [6, 7] and that are cheaper at the same time [8, 9]. There are a lot of plant growth regulators that determine the growth and formation of various plant organs, the timing and nature of flowering, and the time of ripening due to biologically active compounds [10]. Many microorganisms from the soil or the root zone of plants are known as agriculturally beneficial and can serve as a basis for the creation of bacterial preparations.

Thus, a series of biological preparations Radiance (developed in EM-Biotech and Novosibirsk State Agrarian University, Russia) includes several dozen species of microorganisms [11]. Albit (Albit Co., Russia) contains purified active substances from soil bacteria *Bacillus megaterium* and *Pseudomonas aureofaciens* [12]. Baykal EM-1 (EM-Center, Russia) is a composition of strains (photosynthetic bacteria, lactic acid producers, nitrogen-fixing bacteria, yeasts and fungi) [13]. Asolen (Institute of Biology, Ufa Scientific Center of RAS, Russia) was developed based on free-living nitrogen-fixing soil bacteria *Azotobacter vinelandii* IB 4 (cell titer from 4×10^9 to 8×10^9 CFU/ml) [14]. Azospirillum-based preparations (*Azospirillum lipoferum, A. brasilense* and *A. amazonense*) have been created (Tamil Nadu Agrarian University, India) [15]. In China, biological preparations based on nitrogen-fixing bacteria and phosphobacteria from bamboo rhizosphere are supposed to be used in liquid form in the composition of bio-organic fertilizers produced using the waste of this plant [16]. Due to the *Azotobacter chroococcum*-based microbial preparation (Punjab Agricultural University, India), carrot yield increased by 15.8 %, and carotene content increased by 30.6 % [17]. A biological preparation of *Bacillus megaterium* used by European reserchers increased the content of nitrogen and phosphorus under the inoculation of lentil beans [18]. Biological preparations provide effective mobilization of soil organic matter and biota which distinguishes them from fertilizers. Biological products are supposed to be used in complex with basic organic fertilizers.

Composting technologies in the production of biological fertilizers are various. For example, poultry manure [19], a mixture of peat, manure, zeolite, sawdust and broth resulted from fish waste processing to flour [20], ground reef corals, excrements of domestic animals, waste water, waste from vegetable fiber mixed in the presence of organic compounds [21] are used. In India, biological fertilizer is produced of cattle manure, composted coconut fiber and green fertilizer in combination with *Azospirillum* and phosphobacteria [22].

The properties and composition of multi-purpose compost (KMN, developed in the All-Russian Research Institute of Reclaimed Lands — VNIIMZ) [23, 24] make it possible to refer it to complete high-quality organic fertilizers (basic fertilizer and dressing). It is used in all crops grown in various regions of Russia and abroad, including potato and cereals. Local and overall KMN application KMN provides an average of double to triple activation of soil microflora and mobilization of biogenic elements (gain up to 25 %) which increases productivity. The value and advantages of KMN are its high nutritional value, physiological, ecological and biogenic properties. The balanced microbiological composition of KMN contributes to the maintenance of soil fertility, and as a consequence, not only the crop productivity increases, but also the quality of products improves.

Also, a radically new enzymatically-extraction technique to create liquid biological products of different classes was proposed in VNIIMZ [26, 27]. The

LPB product which includes nutritional elements and growth factors is one of them. The LPB nutritional value is provided by favorable acidity (pH 7.0-8.0), high content of K_2O (up to 9.5 g/l) and P_2O_5 (10.0 g/l), and a rich composition of trace elements including Mg, Zn, Mn, and Fe. The abundance of agronomically beneficial microflora reaches 10^{12} CFU/ml. Concentration of toxic elements in LPB is significantly lower compared to MAC (maximum allowable concentration), it contains no pathogenic microorganisms and parasites, so its use as a dressing is completely safe for the soil, vegetation and the final products intended for human consumption. LPB can be used to maintain soil fertility and crop productivity. LPB is recommended to be used as a biological growth and development stimulator by soil wetting and spraying, and as a biological product for soil fertilization by background soil wetting combined with the application of the basic fertilizer.

The purpose of this study was to evaluate the effectiveness of the KMN biological fertilizer and LPB preparation in the row and cereal crops (potatoes and spring wheat) for the two main backgrounds of fertilizers.

Technique. The research was held in 2011-2012 on the Zhukovskii potato variety and in 2012-2013 on Irgina spring wheat variety in the experimental field of the All-Russian Research Institute of Reclaimed Lands (Tver' region) in the micro plot tests. Plots were located randomly, separated by protective bands [29].

The experiment in spring wheat included the following variants: 1 — control (no fertilizers and biological products applied); 2, 3, 4 — spraying with LPB (dose of 0.1 l/m^2) diluted with tap water as 1:300, 1:500 and 1:1,000, respectively; 5 — NPK application (3 cwt/ha); 6, 7, 8 — spraying with LPB as 1:300, 1:500, 1:1,000, respectively, with the background of NPK; 9 — KMN application (7 cwt/ha); 10, 11, 12 — spraying with LPB as 1:300, 1:500, 1:1,000, respectively, with the background of KMN. Plot discount area was 9 m^2, experiments were performed in triplicate. The plants were mowed down by hand in the phase of full ripeness. After drying, ears were threshed, grain was cleaned and weighed. Productivity and the weight of 1,000 grains at each plot were estimated in accordance with the procedure [29, 30]. The amount of protein in grain was assessed by the Kjeldahl method (State Standard GOST 51417-99).

The experiment with potato planting was held with the background of KMN at a dose of 4 t/ha. Plot discount area was 7 m^2, experiments were performed in triplicate. The plantings were treated with LPB in triplicate (in the phases of sprouting, budding, and flowering) using hand sprayer. Four LPB doses of 0.05, 0.1, 0.2 and 0.3 l/m^2 and two dilutions of 1:30 and 1:300 of the stock preparation were used. Control areas were not treated with LPB. The crops were harvested by hand, the yields and the fractional composition were determined according to the procedure [30, 31].

Soil samples were aseptically collected from the arable layer (0-20 cm), assays were performed according to the approved standard techniques [32, 33]. The intensity of redox processes in the soil was evaluated by the oxidative-reduction ratio (ORR) as the catalase to dehydrogenase activity rate.

The data were processed using Microsoft Excel 2003.

Results. High yield was observed in spring wheat variety Irgina not treated with the basic fertilizer (control), but sprayed with LPB diluted as 1:500 and 1:1,000 (Fig. 1, A). If NPK was used as the basic fertilizer, the greatest increase in the yield was obtained with the use of LPB diluted as 1: 300. With the combined use of the KMN biofertilizer and the LPB biological product

(1:1,000), a decrease in the wheat yield was observed, but this value increased significantly with the LPB dilution of 1:500.

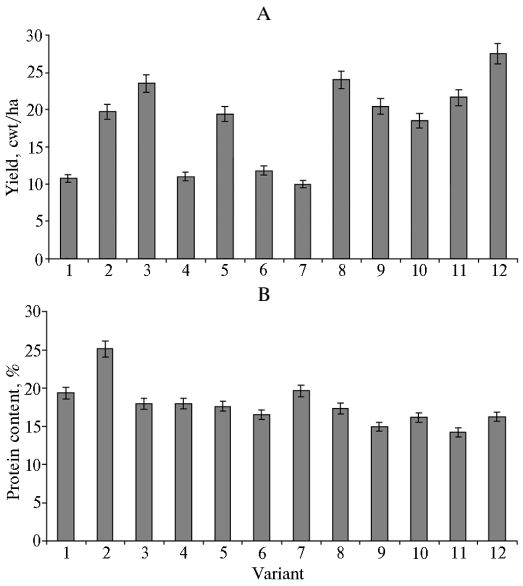

Fig. 1. Yield (A) **and protein content in air-dry substance of the grain** (B) **of spring wheat (*Triticum aestivum* L.) variety Irgina depending on the application of biologicals and fertilizers in wheat crops:** 1 — control, 2 — biological product LPB 1:300, 3 — LPB 1:500, 4 — LPB 1:1,000, 5 — NPK (3 cwt/ha), 6 — NPK + LPB 1:300, 7 — NPK + LPB 1:500, 8 — NPK + LPB 1:1,000, 9 — multi-purpose compost (KMN, 7 cwt/ra), 10 — KMN + LPB 1:300, 11 — KMH + LPB 1:500, 12 — KMN + LPB 1:1,000 (Tver' Region, 2012-2013).

Sparaying with LPB in the variant of 1:300 additionally to KMN provided the maximum yield increase. With respect to the pure control without fertilizers, it was 154 %; to KMN background, it was 35 %, to the variant of NPK background spraying with LPB it was 15 %. Such a significant increase was due to larger grains. Thus, the weight of 1,000 grains increased significantly: it was 33.28 g in the pure control, 45.52 g in the variant of KMN, 63.19 g with KMN + LPB (1:300), 49.08 g with NPK + LPB (1:300).

A variety of factors were involved in the formation of the harvest, including the elemental composition of the soil and the intensity of redox processes defined by the soil microflora and the biologicals used. The ORR value with no fertilizers was almost equivalent to that in the variant of LPB treatment at a dilution of 1:1,000, and decreased at 1:500 and 1:300. The maximum ORR value was obtained with the pure NPK background, the use of the product decreased it approximately as twice. At this, ORR was relatively high in the variant of LPB 1:300. The complementarity between NPK and more concentrated biological can be explained by a significant number of active LPB microflora using the available resources. ORR was not high with KMN applied additionally to LPB and without LPB. It is obvious that the crop was formed due to the elements of nutrition and development of agronomically beneficial microflora, so the intensity of redox processes decreased.

Thus, mobilizing effect in the soil under spring wheat was higher if no basic fertilizers were used (biopreparation at a beneficial dose activated the potential of the very soil), and also when NPK was used without biopreparation (effect of mineral fertilizer). At the same time, the crop yield with NPK and without fertilizers was generally inferior to that obtained with NPK together with biologicals (the yield increased due to activity of LPB microflora) and with KMN (due to activation of microflora of biofertilizer and biopreparation, particularly at its lower dilution of 1:300).

Protein accumulation in wheat was higher in control and with the NPK background (see Fig. 1, B). This value reached its maximum (25.1 %) in the variant of LPB (no fertilizer) at a dilution of 1:1,000. High protein content (19.6 %) was also observed with the use of biopreparation diluted as 1:500 with the NPK background.

Based on the yield data, we can conclude that the use of the novel biological product LPB in cultivation of spring wheat is expedient for all exam-

ined fertilizer backgrounds (both NPK and KMN). The greatest effect was reached by the combined use of KMN and LPB diluted as 1:300 (even compared to the traditional for this crop mineral fertilizer of azophoska). However, despite the decline in yields, formation of higher-quality grain was observed in the variants with no fertilizer.

In the experiment with potatoes, not all LPB concentrations and doses impacted the formation of productivity favorably. The best effect was provided by a more diluted LPB (1:300), at this the doses of 0.05 и 0.1 l/m^2 ensured an approximately the same increase of 7 %, and the doses of 0.2 and 0.3 l/m^2 decreased the yield by 6 and 10 %, respectively, compared to control (KMN, Table).

Potato (*Solanum tuberosum* L.) variety Zhukovsky yield depending on the use of LPB biological preparation and multi-purpose compost (KMN) (Tver' region, 2011-2012)

Variant	Yield, cwt/ha			
	total	versus control	commercial	versus control
KMN (control)	347.5		318.5	
KMN + LPB 1:30 (0.05 l/m^2)	349.7	+2.2	323.0	+4.5
KMN + LPB 1:30 (0.1 l/m^2)	353.4	+5.9	332.4	+13.9
KMN + LPB 1:30 (0.2 l/m^2)	330.9	–16.6	312.7	–5.8
KMN + LPB 1:30 (0.3 l/m^2)	305.6	–41.9	294.6	–23.9
KMN + LPB 1:300 (0.05 l/m^2)	369.3	+21.8	347.2	+28.7
KMN + LPB 1:300 (0.1 l/m^2)	372.1	+24.6	352.1	+33.6
KMN + LPB 1:300 (0.2 l/m^2)	323.5	–24.0	294.7	–23.8
KMN + LPB 1:300 (0.3 l/m^2)	309.7	–37.8	285.1	–33.4
$HCP_{0.5}$	18.6		19.4	

The distribution of potato fractions on the number of tubers per plant in control appeared about the same, and in the best variants of LPB use an increase in the number of larger potatos was observed. The ratio of potato fractions per plant proved that nearly the entire crop consisted of large and medium-sized potatos. The weight of marketable potatos in the variant of LPB spraying at a dose of 0.1 l/m^2 (dilution of 1:300) increased by 33 cwt/ha as compared to control.

The abundance of agronomically beneficial microflora under potatoes during the growing season varied with no regularities, and in the period of tuber formation only there was a clear link between the number of microorganisms and the yield. Thus, LPB diluted as 1:30 had no effect on the abundance of ammonifying microorganisms (Fig. 2). The use of LPB solution diluted as 1:300 resulted in a significant increase of this value. The number of phosphate mobilizing microflora was increased in variants with LPB dosages of 0.05 and 0.1 l/m^2 in both dilutions, which correlated with potato yield.

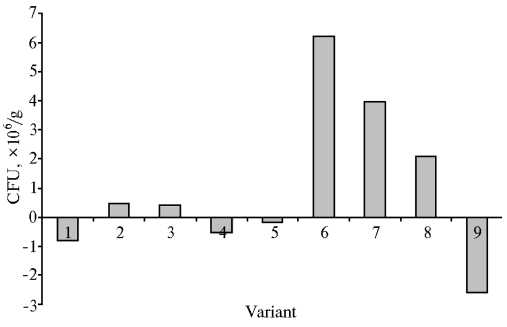

Fig. 2. Changes in the number of ammonifying microorganisms in the soil under potato (*Solanum tuberosum* L.) variety Zhukovsky by the experiment completion relative to the beginning the growing season in the variants of the use of a LPB biological product and multi-purpose compost (KMN): 1 — KMN (control), 2 — KMN + LPB 1:30 (0.05 l/m^2), 3 — KMN + LPB 1:30 (0.1 l/m^2), 4 — KMN + LPB 1:30 (0.2 l/m^2), 5—KMN + LPB 1:30 (0.3 l/m^2), 6 — KMN + LPB 1:300 (0.05 l/m^2), 7 — KMN + LPB 1:300 (0.1 l/m^2), 8 — KMN + LPB 1:300 (0.2 l/m^2), 9 — KMN + LPB 1:300 (0.3 l/m^2) (Tver' region, 2011-2012).

The role of KMN local application with potato planting was to supply plants with available nutrients at their early development. At the time of flowering and early tuber formation, the role of phosphorus and potassium in plant

development increased. At this stage, LPB contributed to the formation of reproductive organs and reserve substances in the crop commercial part.

The results indicate that LPB is advisable to be used as a growth and development promoter in potato variety Zhukovsky. Sowing is to be sprayed with the biological preparation at a concentration of 1:300 and doses of 0.05-0.1 l/m^2. In the direct LPB contact with potato tops, plants' needs in nutrition elements was satisfied, and the microorganisms of the agent attributed to the activation of biochemical reactions in the process of potato ontogenesis. As a result of this combined effect of the biological preparation, a significant increase in the yield was observed, and the number of soil microflora grew as well.

Thus, the combined effect of KMN (multi-purpose compost) and LPB (biological preparation which includes nutritious elements and growth factors) on the yield of spring wheat and potatoes was favorable. The increase was due to the formation of larger grains or enlargement of potato tubers (the proportion of marketable potatoes increased significantly). After harvesting, the soil remained free from chemical pollution and enriched with microflora contributing to the reproduction and preservation of soil fertility. The biologicals developed can replace traditional fertilizers and preparations in agro-technologies of the crops studied.

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SELECTION OF SALT TOLERANT ALFALFA (*Medicago* L.) PLANTS FROM DIFFERENT VARIETIES AND THEIR MORFO BIOLOGICAL AND SYMBIOTIC PROPERTIES ANALYSIS

M.L. ROUMIANTSEVA[1], G.V. STEPANOVA[2], O.N. KURCHAK[1], O.P. ONISHCHUK[1], V.S. MUNTYAN[1], E.A. DZYUBENKO[3], N.I. DZYUBENKO[3], B.V. SIMAROV[1]

[1]*All-Russian Research Institute for Agricultural Microbiology,* Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail mroumiantseva@yandex.ru;
[2]*V.R. Villiams All-Russian Research Institute for Forages,* Federal Agency of Scientific Organizations, 1, Nauchnii Gorodok, Lobnya, Moscow Province, 141055 Russia, e-mail gvstep@yandex.ru;
[3]*N.I. Vavilov Institute of Plant Genetic Resources (VIR),* Federal Agency of Scientific Organizations, 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail elena.dzyubenko@gmail.com
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Abstract

Soils degradation growing in our days is associated with the depletion of their fertility, a result of crop rotation with excessive amounts of mineral fertilizers and chemical plant protection products, as well as it links with widespread worsening climatic conditions and environmental conditions. For this reason, agriculture based on environmentally friendly technologies must be an absolute priority. Legumes can fix atmospheric nitrogen in symbiosis with nodule bacteria and accumulate it in plant biomass. Legumes are unique predecessors for grain crops, as they contribute to the effective restoration of soil fertility by introducing nitrogen into bioavailable form. Pastures based on legumes contribute to the restoration of soils destroyed and excluded from crop rotation, such as desert or saline. In this, the development of pathways to create new productive plant-microbe systems that can grow in adverse conditions, is of great theoretical and practical significance. The objectives of the study was to identify salt-tolerant plants of alfalfa (*Medicago* L.), to obtain plants of the I_1 generation by self-pollination approach and to analyse their morphobiological and symbiotic properties in model experiments. The study was performed on 13 tetraploid and diploid varieties of alfalfa, including commercially valuable varieties Soleustoychivaya and Agniya, both of which were tested without rhizobia inoculation and in symbioses with *Sinorhizobium meliloti* strains. An analysis of the symbiotic activity of alfalfa varieties showed that they were highly responsible to *S. meliloti* Rm2011 strain inoculation and formed an effective symbiosis under saline conditions. Geographically different varieties were evaluated for the homogeneity according to dry matter (DM) accumulation at 75 mM NaCl without inoculation, and at 100 mM NaCl with inoculation by *S. meliloti*. Obtained DM data among the studied cultivars significantly changed only in case of symbiosis that was established with the assistance of the dispersion coefficient (D). Plants of salt tolerant phenotype was obtained for diploid *M. caerulea* and *M. falcata* species, as well as for tetraploid *M. sativa* L. varieties Soleustoychivaya and Agniya in microvegetative experiments done at the All-Russian Research Institute for agriculture microbiology (ARRIAM). Selected salt tolerant plants of both varieties were planted further in greenhouse complex (STC) of V.R. Villiams All-Russian Research Institute for Forages. It was found that salt-tolerant plants of Soleustoychivaya and Agniya are characterized by predominantly purple color of flowers, by a twisted form of bean, by relatively high branching and bushy, by later transition to a period of winter rest according to 3-year vegetation trials in the STC. From seeds obtained from tested plants the I_1 plants were grown, which were studied in microvegetative experiments in ARRIAM. Plants I_1 of both varieties were analyzed by DM, by growth rate of aboveground and underground parts, by number of nodules formed in typical (salt-free) conditions and under salinity. As a result, it was found that generation I_1 plants of both varieties were homogeneous or sufficiently homogeneous according to DM data. Not inoculated generation I_1 plants of Soleustoychivaya variety successfully developed in saline conditions (the average increase of DM was 36.92 % in comparison to plants of initial variety). DM of plants I_1 of Agniya variety was, on the contrary, lower than that of the plants of the initial variety in the saline conditions, being 16.55 % less. The high level of interaction specificity of both varieties of generation I_1 plants with strains CIAM1774 or Rm2011, differing in salt tolerance, was assessed by DM. Thus, under salt stress impact the highest

values of DM was obtained for Soleustoychivaya variety plants in symbiosis with strain Rm2011 characterizing by *S. meliloti* salt tolerance typical degree. However, the symbiotic system on the basis of salt tolerant genotype of Agniya variety with salt-tolerant strain CIAM1774 may also be promising for cultivation in saline soils. It was found that the length of the root system decreased due to symbiosis, and this parameter depends on the specific plant-microbe interactions. It was concluded that the selection of salt-tolerant genotypes of plants and strains with a certain level of salt tolerance is promising in order to create symbiotic systems with enhanced adaptability.

Keywords: rhizobia, alfalfa (*Medicago* L.), effective symbiosis, salt tolerance, root and stem length, plant biomass.

Legumes posess the features that can make them promising to restore the degraded soils [1, 2]. They fix atmospheric nitrogen in symbiosis with nodule bacteria and accumulate it in the fertile layer of soil [3]. The area of soils that need restoration, including saline waterlogged and acidified soils is constantly increasing due to worsening the climatic situation both in the world and in Russia [4-6]. The successful development of such areas often depends on the proper selection of crops [7, 8].

Ecotypes of legumes and endemic legumes growing in different eco-geographic regions can be the source of new varieties with higher adaptive capacity [9]. Genus *Medicago* is represented by species that vary considerably in their adaptability and productivity under different soil and climatic conditions. Alfalfa (*Medicago varia*) is widespread in central Russia but can grow in low- and moderate-saline soils in the desert zone of the Caspian, and yellow alfalfa *M. falcate* can inhabit slightly acidic soils of the Murmansk and Arkhangelsk regions [10, 11]. Identification of alfalfa plants (phenotypes) differing in their adaptivity (stress resistance) is one of the approaches to the creation of varieties and symbiotic systems with high adaptive capacity.

Inoculation of alfalfa with nodule bacteria strains (*Sinorhizobium meliloti*) selected genetically has been shown to enhance alfalfa productivity under salinity [12-15]. Estimation of salt tolerance of rhizobia, plants, and their symbioses can be successfully performed in the laboratory models and microvegetative experiments [1, 12, 16].

Herein, we report new data on the specificity of variety-microbe interaction in the systems of high symbiotic efficiency under salt-free conditions and as influenced by salt stress.

So in our study a salt-tolerance of tetraploid and diploid alfalfa species was evaluated under various nitrogen nutrition to identify contrast phenotypes, obtain the offspring from self-pollination, and compare their morphological, biological and symbiotic parameters.

Technique. Alfalfa (*Medicago* L.) varieties and ecotypes were provided from the VIR collection (N.I. Vavilov All-Russian Research Institute of Plant Genetic Resources) and the V.R. Villiams All-Russian Research Institute for Forages. *Sinorhizobium meliloti* cultures used for inoculation were Rm 2011 test strain typically resistant to 550 mM NaCl, and CIAM1774 strain (AK23 or A1) with salt tolerance to 700 mM NaCl characteristic of 10 % strains in this species [17]. The strains were cultured in TY medium at 28 °C [16].

Under sterile pot experiments, plants were grown without NaCl (a salt-free standard) or in the presence of 75 or 100 mM NaCl (salinity) with 10-fold replication for 28 or 56 days depending on the aim of the experiment, and KNO_3 (3 mM) as the source of nitrogen was added to the soil if needed, as described [12, 16]. Vermiculite or 0.6 % agar with Krasil'nikov-Korenyako mineral medium was a substrate. Plant productivity was evaluated by dry matter (DM) accumulation (%) versus control sample. To determine the germination power (GP), the seeds in Petri dishes were exposed to 28 °C, the percentage of germinated seeds to their total number was calculated at day 3. In pot experiments,

vessels were used filled with 6 kg of fertile soil (pH 6.94), at humus content of 4.41 %, total nitrogen of 0.28 %, and mobile phosphorus and potassium of 560.7 and 432.0 mg/kg, respectively. Plants were grown without additional dressing under natural lighting and temperature. Photoperiodicity of plants was analyzed in natural light and controlled temperature conditions.

Genotypes with different salt tolerance were selected in pot tests at day 56 in the presence of 75 mM NaCl as described [18]. Selected plants were grown salt-free in 0.5 l containers with sterile vermiculite, and after 2 months they were transplanted into containers with soil and grown in MLR-351H phytotron («Sanyo Electric Co.», Japan) to obtaining seeds by forced self-pollination. Forced self-pollination and analysis of morphological and biochemical parameters (2012-2014) were performed as described [19] in the breeding greenhouse complex (BGC, V.R. Villiams All-Russian Research Institute for Forages). I_1 plants of Soleustoichivaya and Agniya varieties grown from seeds (20 pcs. in each of the 8 variants) were studied for homogeneity, productivity and symbiotic effectiveness in pot tests [16].

Statistical analysis was performed using Statistica v. 6.0 software and Microsoft Excel 2013 software package. Analysis of variance and correlation analysis were performed, coefficient of variation (*Cv*) and Student *t*-test were calculated [20, 21].

Results. Alfalfa varieties and ecotypes were represented by the samples from geographically distant regions with arid conditions and/or salinity, rapid changes in daily and seasonal temperature (Table 1). For the study we selected both cultivated alfalfa varieties (*Medicago sativa*, *M. varia*) and populations of wild alfalfa *M. falcata* from the northern and southern regions, one of which, the Aral Sea area, is exposed to extreme salinity (see Table 1). An endemic alfalfa species *M. trautvetteri* grows in the same area. In addition, the populations of *M. caerulea*, an ancient blue-flowering species widespread in the steppe and flooded coastal areas of the Caspian, were analyzed. Salt tolerance was estimated in cultivated alfalfa varieties Soleustoichivaya and Agniya, of which the first one was created based on variety Khivinskaya local by cell technologies under salinity stress [22], and the second one was produced using a combined selection in complex hybrid P211 population obtained from crossing domestic and Canadian varieties (Pasture 88 × North hybrid 69 × Rizoma) [23].

1. Alfalfa (*Medicago* L.) samples of different eco-geographical origin tested for salinity tolerance

Species (ploidy)	Alfalfa variety and sample	Seed collection area	№ according to VIR catalogue
M. falcata (2*n*)	subsp. *Borealis* Grosshm., wild	Pskov region, Russia	k-25557
	subsp. Romanica prod., wild, ecotype	Eastern part of Kazakhstan	k-49669
M. caerulea Less. ex. Ledeb. (2*n*)	Wild, ecotype	Dagestan, Russia	k-12821
	Wild, ecotype	Guriev region, Kazakhstan	k-28915
	Wild, ecotype	Azerbaijan	k-49904
	Wild, ecotype	Stavropol' Territory	k-44044
M. trautvetteri Sumn. (2*n*)	Wild, ecotype	Aktyubinsk region, Kazakhstan	k-36579
M. sativa L. (4*n*)	Tibetskaya variety	Aktyubinsk region, Kazakhstan	k-25782
	Local cultivated variety	Kyrgyzstan	k-6376
		Uzbekistan	k-8913
		Libya	k-39107
M. sativa L. subsp. *sativa* (4n)	Soleustoichivaya variety	Moscow region, Russia	–
M. sativa L. nothosubsp. *varia* (Martyn) (4*n*)	Agniya variety	Moscow region, Russia	–

N o t e. Samples were provided from the VIR collection (N.I. Vavilov All-Russian Research Institute of Plant Genetic Resources, St. Petersburg); dash means that the samples were provided by the V.R. Villiams All-Russian Research Institute for Forages (Moscow Province).

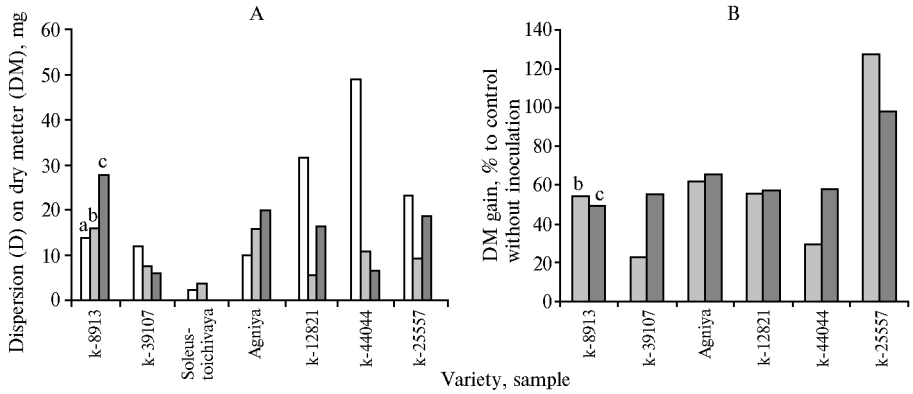

Fig. 1. Dry matter (DM) **dispersion** (A) **and productivity** (B) **in various alfalfa (*Medicago* L.) varieties and ecotypes inoculated with *Sinorhizobium meliloti* strains under salinity stress:** A — 75 mM NaCl, B — 100 mM NaCl; a, and b, c — control (without inoculation), and inoculation with strains Rm2011 and CIAM1774 (pot tests).

Symbiotic efficiency and salt tolerance of different alfalfa species. The variety homogeneity was assessed by the DM in plants grown at 75 mM NaCl which were or were not inoculated with *S. meliloti* strains. In this, the dispersion coefficient (D) was used according to the guidelines [16]. Without inoculation, the D values ranged from 10.70 to 13.70 in Agniya variety and local *M. sativa* varieties. Extremely low (2.11) and conversely high values of D (17.50) were observed in varieties Soleustoichivaya (Fig. 1, A) and Tibetskaya, respectively. No dependence between D values and plant geographic origin was found. For example, similar values were obtained in *M. caerulea* sample pairs from Kazakhstan and Dagestan at an average D of 29.80, and from Azerbaijan and Stavropol' Territory at an average D of 29.80. Contrasting D values were also noted in *M. falcata* populations of Kazakhstan and Pskov region (50.30 and 23.20, respectively). The maximum D value was found in the endemic *M. trautvetteri* species (62.90). Apparently, the low-D varieties are represented by the genotypes with similar tolerance, and in the high-D varieties it varies. The D value was proved to change significantly after inoculation with *S. meliloti* strains (see Fig. 1, A). In some cases, an inverse relationship between the D values with and without inoculation was found (e.g., in *M. caerulea* k-1282 or Agniya variety) (see Fig. 1, A). Thus, changes in dispersion reflect the specificity of plant-microbe interactions.

Plant productivity was evaluated in pot tests by DM in studied alfalfa species and varieties in symbiosis with *S. meliloti* strains at 100 mM NaCl salinity. High gain in DM was noted in a local variety of Uzbekistan (k-8913) and variety Agniya inoculated with Rm2011 or CIAM1774, respectively (an average increase of 57.60 %, see Fig. 1, B). Under saline conditions, these strains formed effective symbioses with *M. caerulea* k-12821 and *M. falcata* k-25557 samples. The efficiency of symbiotic interaction of *M. sativa* k-39107 and *M. caerulea* k-44044 with strain CIAM1774 was found to be twice higher than in the symbiosis of the same host plants with strain Rm2011 (see Fig. 1, B). The local and cultivated alfalfa varieties were concluded to be highly responsive to inoculation and able to form effective symbioses under saline conditions.

Based on the results, it was of interest to identify plant genotypes differing in salt tolerance. The forms contrasting in habitus (i.e., min as low-stem ones, and max as those with a developed high stem) were selected from each of the samples studied. After growing under sterile salt-free conditions, they were moved to phytotron. With forced self-pollination, the seeds from phenotypically different

plants were obtained in the 2nd year in *M. caerulea* and *M. falcata*, and also in *M. sativa* L. varieties Agniya and Soleustoichivaya, but only in Soleustoichivaya plants their number was sufficient for further studying.

Morphological and biochemical parameters in the plants of min- and max-phenotypes. Morphological characteristics are the ones that do not depend on the growth conditions (corolla color, the number of flowers per inflorescence, bean form) but are used to determine alfalfa variety types. In plants of Soleustoichivaya (So) variety of contrasting phenotypes (i.e., So-max and So-min) there was a corolla color from lilac to deep lilac typical of alfalfa (*M. sativa* L.) blue hybrid variety type. At the same time, in the contrasting phenotypes (Ag-max and Ag-min) of the Agniya (Ag) variety the color of flowers varied. It was lilac in max-forms and showed a dominance of purple alfalfa genes, whereas in min-forms a spectrum of shades from yellow-lilac to yellow indicated their genetic relation to northern alfalfa (*M. borealis* L.) which was among the parental forms of the initial variety [23].

2. Morphological and biochemical parameters and productivity in phenotypically contrast alfalfa *M. sativa* L. varieties Agniya and Soleustoichivaya (pot tests, breeding greenhouse complex, 2012-2014)

Parameter	Plant phenotypes		t_{act} max/min
	max	min	
Morphological features:			
twists per bean, pcs.	2.7	2.5	–
flowers per inflorescence, pcs.	25	18	–
stems, pcs./plant	71	48	1.42
branches, pcs./plant	253	124	2.24
stems with flowers, pcs./plant	12	6	1.84
Content per dry matter, %:			
raw ash	9.21	8.72	0.94
phosphorus	0.39	0.39	–
potassium	1.41	1.39	0.06
Dry matter, g/plant	151	134	0.88
Seeds, g/plant	12.70	11.50	0.21

N o t e. Max- anf min-phenotype plants are contrasting in habitus (min means low-stem, max means a developed high stem); t_{act} is the actual value of *t*-test (95 % confidence interval; theoretical value of *t*-test t_{05} = 2.15). Dashes mean no significant differences between the plants of max and min phenotypes.

In perennial alfalfa species, beans are of the spirally twisted form, the number of twists being species-specific (e.g., 0.5-1.0 in sickle alfalfa, 3.5-4.0 in purple alfalfa). This parameter was 2.1 in the Agniya variety which is one twist less compared to the Soleustoichivaya plants ($t_{act} = 2.46 > t_{05}$). At the same time, we have not identified significant differences for the above trait between the samples of plants with different phenotypes (Table 2), however, a tendency to the increased bean twistedness was observed in the plants of salinity tolerant (max) phenotype.

Samples of plants of these two varieties significantly differed in the number of flowers per inflorescence and in their tilling capacity ($t_{act} > t_{01}$), but the max and min samples were not statistically different ($t_{act} < t_{05}$). However, the salt-tolerant plants tended to increase the average number of flowers per inflorescence and of stems per plant (see Table 2).

The analysis of average number of branches per plant showed that in max phenotype it was greater as twice ($t_{act} = 2.24 > t_{05}$, see Table 2). Similar were obtained in the analysis of the number of stems with flowers per plant ($t_{act} = 1.84$; $t_{05} = 2.15$; see Table 2). Consequently, the plants of salt tolerant phenotype tended to early ripening.

Analysis of photoperiodicity under natural light and controlled temperature showed that the photoperiod reduction (10 hours or less, decade III of October in the Central Region of the Russian Federation) resulted in marcescence

and plant transition to the resting stage. This process is quantified by the DM content. In max-phenotype plants, this value was 11.30 % higher in the marcescence period (data as of October 20, 2014, see Table 2). Upon exiting winter dormancy and with the photoperiod increase to 8 hours, early re-growth was observed in max-forms; when the photoperiod was 1 hour more, re-growth was found in min-phenotypes ($t_{act\ max}$ = 2.36; $t_{act\ min}$ = 1.97; t_{05} = 2.15). The studied samples differed in the raw ash content (see Table 2). Thus, these data support the fact that saline tolerant plants can longer retain activity in the shortened photoperiod.

As the plants of contrasting phenotypes were grown on fertile soil with a neutral pH (pH 6.94), it made it impossible to identify clear differences between the study groups. However, summarizing the findings, we can conclude that the plants of saline tolerant phenotype identified in the varieties studied, are characterized by predominantly lilac colored flowers, more twisted bean form, relatively high branching and tillering, a short-day photoperiodic response and increased DM ash content.

Homogeneity and productivity in the I_1 generation. The main features of I_1 plants grown from seeds of forced self-pollinated contrasting phenotypes were studied in 8 variants, of 20 pcs. in each.

Evaluation of homogeneity of I_1 seeds in both varieties by GP versus the seeds of initial forms (control) revealed no significant differences (average GP value of 95 %). I_1 plants without inoculation were estimated by the DM value using a coefficient of variation (*Cv*). *Cv* values in both studied alfalfa varieties, Soleustoichivaya (So-I_1) and Agniya (Ag-I_1), were significantly lower compared to that in respective controls (Fig. 2, A). But *Cv* changed significantly in individual phenotype groups depending on the growing conditions. Particularly, this value was higher with no salinity in Ag-I_1-max2 and under salinity in So-I_1-min2 (see Fig. 2, A). According to our data, phenotypically non-similar So-I_1 and Ag-I_1 plants should be considered as homogeneous (*Cv* < 17 %) [21], and the above two groups as a fairly homogeneous (*Cv* in the range of 17-33 %) [21].

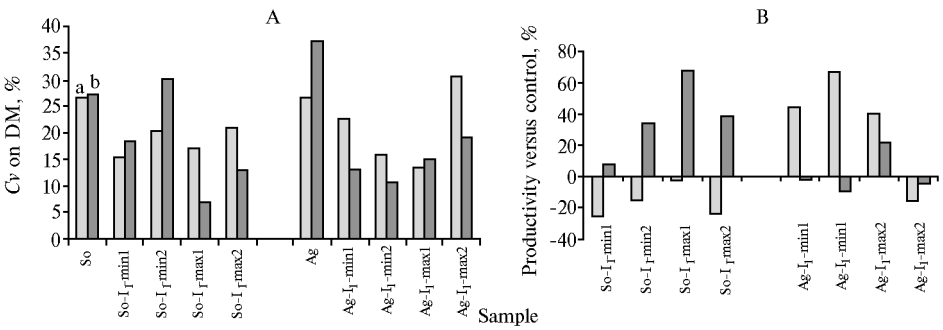

Fig. 2. Homogeneity by *Cv* (A) **and productivity** (B) **by dry matter** (DM) **in I_1 plants of phenotypically contrasting groups in alfalfa (*M. sativa* L.) varieties Agniya** (Ag) **and Soleustoichivaya** (So) in **normal conditions and under saline stress:** a — control (salt-free), b — 75 mM NaCl; min and max — low-stem and high-stem forms. Inoculation with *Sinorhizobium meliloti* and mineral nitrogen were not used (pot tests).

Productivity by DM in So-I_1 and Ag-I_1 plants of contrasting phenotypes (no inoculation) in the absence of salinity and under salinity were compared with the parameters found in initial plants of relevant varieties (Ag and So) reported under similar conditions (control, see Fig. 2, B). The min- and max-Ag-I_1 plants varied considerably in their productivity when NaCl was not added. Thus, the genotypes of Ag-I_1-min and Ag-I_1-max plants were either considerably superior (average of 50.50 %) in DM to control plants, or did not differ signifi-

cantly from them. The plants of only one (Ag-I_1-max1) of the four phenotypic groups were developing better compared to control (DM increase of 22.10 %) under salinity (see Fig. 2, B). So-I_1 plants, on the contrary, were successfully developing in saline conditions (DM increase from 33.85 to 67.69 %), with the exception of the So-I_1-min1 in which the DM gain versus control did not exceed 7.69 %. Productivity was an average of 16.55 % lower versus control in all phenotypic So-I_1 groups with no salinity. Adding mineral nitrogen into the substrate promoted an increase in the DM value in all So-I_1 and Ag-I_1 phenotype groups of an average of 1.90 times regardless of salinity (data not shown).

Thus, the phenotypically non-similar Ag-I_1 and So-I_1 plant groups characterized as homogeneous were, however, significantly different in their productivity. So-I_1 groups appeared to be more expressed halophytes than the plants of the initial variety. The observed fact is due to the phenotypic segregation in the Soleustoichivaya variety produced from a callus culture using medium with 1.98 M NaCl [5]. Ag-I_1 plants did not differ in their salinity tolerance from control, however, a possibility of the use of the Agniya variety to search for stress-resistant genotypes has been shown. Therefore, further analysis will be performed in phenotypically different Ag-I_1 and So-I_1 groups.

Symbiotic systems based on I_1 plants. The effectiveness of Ag-I_1 plants in symbiosis with strain CIAM1774 or Rm2011 was similar under salinity and with NaCl-free substrate (average DM gain of 60.05 % and 135.05, respectively) (Fig. 3, A). It also did not differ much in the case of So-I_1 plants' symbiosis with the same strains in the salt-free variants (an average of 43.80 %), but under salinity the gain with Rm2011 was 23.0 % greater compared to CIAM1774 (see Fig. 3, B). Thus, a triple increase in dry matter in inoculated Ag-I_1 plants was observed under normal conditions, and a double increase was found in So-I_1 under salinity stress.

Consequently, symbiosis of alfalfa Soleustoichivaya variety with the strain of typical salinity tolerance (Rm2011) is highly effective under the salinity stress. Nevertheless, the symbiotic system on the basis of salt tolerant genotype of Agniya variety with salt-tolerant strain CIAM1774 may also be promising.

Stems were found to develop better in case of substrates without NaCl in the Ag-I_1 group inoculated with strain CIAM1774 (12.04 % length increase versus the variant of strain Rm1021, and 29.62 % compared to non-inoculated control) (see Fig. 3, C, D). Conversely, the stem length in So-I_1 plants was 9.78 % greater with Rm2011, than with CIAM1774. However, under salinity stress, the stem length in Ag-I_1 plants with symbiotrophic nutrition was close to or slightly less (by 5.48 %) than in control. At the same time, under salinity stress the stem length increased by 38.85 % in So-I_1 plants in the symbiosis with Rm2011 and by 7.05 % only in the symbiosis with CIAM1774 (see Fig. 3, C, D).

Thus, in the absence of salinity stress, the height of the aerial part of Ag-I_1 and So-I_1 plants depend on the inoculating strain, whereas no significant differences in DM were revealed (see Fig. 3, C, D). At that, in symbiosis with Rm2011 or CIAM1774, respectively, the Ag-I_1 and So-I_1 plants were low. Similar differences were observed under salinity for So-I_1. The plants inoculated with CIAM1774 were low, in case of Rm2011 they were high, while no significant differences were found in the respective DM values.

Intensive development of the stem and biomass accumulation depends on the process of nitrogen fixation which is due to the nodules formed on the roots [10]. The number of nodules in Ag-I_1 and So-I_1 plants inoculated with CIAM1774 under salinity stress-free conditions was 2 times greater compared to Rm2011 versus control plants inoculated with the same strain (29.11 and 10.27 %, respectively). Under salinity, this parameter was 3.02 and 1.34 times

greater in Ag-I_1 and So-I_1 plants inoculated with Rm2011 compared to CIAM1774. Our findings suggest that plant-microbe interactions may vary considerably under normal conditions and under salinity.

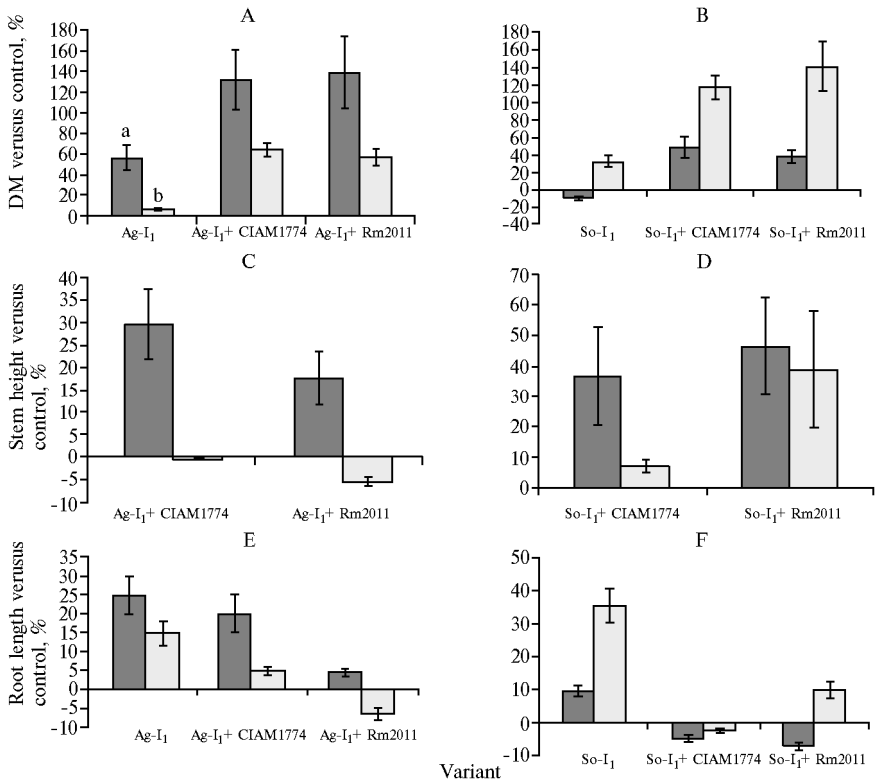

Fig. 3. Changes in the dry matter (DM) (A, B) **content, stem** (C, D) **and root** (E, F) **length in I_1 alfalfa (*M. sativa* L.) varieties Agniya** (A, C, E) **and Soleustoichivaya** (B, D, F) **at symbiotrophic nutrition in normal conditions** (a) **and under saline stress** (b)**:** Ag and So — Agniya and Soleustoyichivaya varieties, respectively; CIAM1774 and Rm2011 — *Sinorhizobium meliloti* strains used for inoculation; A, B, E, F — versus respective non-inoculated or inoculated Ag or So plants, C, D — versus non-inoculated Ag-I_1 or So-I_1 plants. Salinity stress with 75 mM NaCl (pot experiments).

The development of the roots which is an important characteristic of successful development of plants was also eveluated [24]. The root length in non-inoculated I_1 plants of both varieties was significantly greater versus control plants under normal conditions (see Fig. 3, E, F). For example, this value in Ag-I_1 was 2.57 times higher than in So-I_1, whereas under salinity, a 35.48 % root elongation was observed in So-I_1, which is more than 2 times greater compared to the corresponding value in Ag-I_1 (see Fig. 3, E, F). Consequently, the root development in alfalfa under salinity stress can serve as a feature of the variety.

With I_1 plants inoculation, the root length appeared to change significantly. Thus, a similar decrease in this value in Ag-I_1 and So-I_1 was observed in their symbiosis with Rm2011 (an average of 20.62 %) both in normal conditions and at salinity. In Ag-I_1 plants inoculated with strain CIAM1774, the reduction in root length was not significant if NaCl was not added into the substrate, and amounted to 10.43 % under the salinity stress versus the corresponding control plants. Inoculation of So-I_1 plants with the same strain resulted in the 14.44 and 37.88 % root length reduction (versus control), respectively, in normal conditions and under salinity. Consequently, the salt-tolerant strain of CIAM1774 affected the length of the roots in So-I_1 plants specifically, which resulted in a decrease of this value versus that of Ag-I_1 by 2.87

and 3.76 times, respectively, in normal conditions and under the salinity stress. Thus, the effect of strain inoculum on the development of the root system of the host plant was established, but the change in the root length did not correlate with the productivity of plants both normally and under salinity ($r = 0.3$ and $r = 0.5$, respectively).

In conclusion, as a result of selection and analysis of plant groups contrastly differing in salt tolerance, principally new data were obtained about the level of specificity of microbe-variety systems with high symbiotic efficiency under standard conditions and under salinity stress. Symbiotic systems derived from salinity tolerant generation I_1 are characterized by increased productivity, active nodule formation, and longer plant stems. A decrease in the length of the root system was first demonstrted under symbiotrophic nutrition which may indicate both an increase the absorptive capacity of the roots (G.V. Stepanov, personal communication), and the activation of the processes of nitrogen transfer from the roots to the aerial parts [25]. This biometric indicator has been found to depend on the specificity of plant-microbe interactions. Interestingly, the strain of salinity tolerant phenotype had a more significant impact on the development of the underground part of the plant in a salinity tolerant phenotype which was especially evident under the salinity stress. This can be explained by the fact that under the interaction of micro- and macrosymbiont with increased salinity tolerance, optimal conditions are formed for the metabolic activity of bacteroides, the internal environment of which (even in standard conditions) is hyperosmotic [26]. The identified combinations of host plants and strain inoculum under the salinity stress suggest that an optimum balance of nitrogen and carbon is maintained in the above systems. It is obvious that the study of metabolic characteristics of symbiosystems formed based on the stress resistant micro- and macrosymbionts is a significant step towards the directed construction of symbiotic systems with a given adaptive potential.

So, our study has demonstrated the prospects of the analysis of stress resistance in both plant and microbial components resulting in the possibility of constructing directed symbiotic systems with a given adaptive potential.

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MECHANISM AND ACTIVITY SPECTRUM OF MICROBIOLOGICAL PREPARATION BATSIKOL WITH PHYTOPROTECTIVE ACTION

S.D. GRISHECHKINA

All-Russian Research Institute for Agricultural Microbiology, Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail svetagrishechkina@mail.ru

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Abstract

Various groups of agents are involved in biological crop protection to control pests and diseases. Of them, *Bacillus* genus possessing activity against harmful insects and phytopathogens is most promising and widely used. In this, the biologicals based on *Bacillus thuringiensis* (Bt) dominate. More than 70 varieties of Bt have been identified. These bacteria can survive for a long time after treatment. Preparations based on three Bt serovars (A, B, C) are mostly used for insects' biocontrol. Serovar A Bt subspecies can form crystal endotoxins which are active against *Lepidoptera*; serovar B Bt subspecies attack the larvae of mosquitoes and black flies, and phytophagous *Diptera*; and serovar C Bt subspecies are active against *Coleoptera* beetles. A new serovar F (fungi) of this bacillus was identified. Physiological and biochemical properties of *Bacillus thuringiensis* provide the assimilation of nutrient substrates and antibiosis against biocenosis partners. Batsikol, the biological preparation based on *B. thuringiensis* var. *darmstadiensis* (H_{10}) with entomopathogenic action, was created at All-Russian Research Institute of Agricultural Microbiology (St. Petersburg). Batsikol contains components of culture liquid, spores, insecticidal and fungicidal exo- and endotoxins, due to which it possesses multifunctional properties. The article presents the mechanisms of entomopathogenic and antifungal action of microbial preparations based on Bt. Results of testing Batsikol effectiveness against various pests and diseases in field trials and vegetation experiments are shown. Liquid form of biological product was used in the study (spore titer of 3.5×10^9/ml). Field and vegetation tests were carried out in 1994-2013 in different regions of Russia (Leningrad, Novosibirsk, Volgograd region, North Ossetia, Stavropol and Primorsky regions). Batsikol was sprayed against phytophagous pests on vegetating plants. The efficacy against pests varied from 50 to 100 %. Different modes of application against phytopathogen were tested according to the type of parasitism and environmental characteristics of fungi (i.e., spraying, irrigation, seed treatment). In field experiments the efficacy of spraying strawberry plants against gray mold was 60-74 %. Soil watering was used against Fusarium wilt on tomatoes and flax with efficacy of 74-87 % and 34-42 %, respectively. When seeds were treated prior to sowing the efficacy was 66-71 % in case of soaking barley seeds against root rot, and 40-45 % while soaking potato tubers against damping-off. Based on the tests conducted with Batsikol in different regions of Russia, the spectrum of its activities against wide range of phytophagous pests and pathogenic fungi was revealed on different crops. The obtained data expand the understanding of Bt biology and, in particular, the action spectrum against various pests and diseases dangerous for many cultivated plants. Presented materials allow considering *Bacillus thuringiensis* as the basis of microbiological preparations with a multifunctional activity. The obtained data will allow expanding the scope of its application, and it will help to improve ecological situation.

Keywords: *Bacillus thuringiensis,* Batsikol, phytophagous insects, phytophathogenic fungi, biological efficiency.

Various groups of agents are involved in biological crop protection to control pests and diseases. Currently, much attention is paid to the bacteria with phytoprotective function. Of them, the *Bacillus* genus is most promising and widely used for creation of biological preparations. They possess activity against harmful phytophague insects and phytopathogens [1-14]. The biologicals based on *Bacillus thuringiensis* (Bt) are predominant. In world practice, Bt is used as a

safe insecticidal agent, its share in the market of biopesticides is about 90-95 %.

High adaptive capacity of aerobic spore-forming *thuringiensis* bacteria under various extreme conditions determine their widespread in nature. The greatest number of bacilli is isolated from the soil, they are often found in water and extracted from sick and dead insects. Over 70 Bt varieties greatly effective against *Lepidoptera*, *Coleoptera*, *Diptera* and *Hymenoptera* phytophages have been identified. Bt bacteria can survive for a long time after treatment of plants. They have high selectivity of action, are effective against target objects, safe for human, warm blooded animals and beneficial organisms [15]. Antifeedant, teratogenic and dereproductive features provide their high biological effectiveness. Bt-based products are processible in manufacture and use.

Preparations based on three Bt serovars are mostly used for insect biocontrol in agrocenoses. Bt subspecies of serovar A form endotoxin crystals that are most active against lepidopterous insects (*Lepidoptera*). They are producers of drugs such as Bitoxybacillin (Bt var. *thuringiensis*), Dendrobacillin (Bt var. *dendrolimus*), Entobacterin (Bt var. *galleria*), Lepidocide (Russia), DIPel (USA), Bactospeine (Bt var. *kurstaki*) (France), etc. Over 70 insect species are sensitive to this serovar. Bt subspecies of serovar B (Bt var. *israelensis*) are used as producers of larvicidal biologics, such as Bactoculicide, Bacticide (Russia), Bactimos (France), Teknar (Switzerland), Vectobac (USA), etc., that attack the larvae of mosquitoes and black flies, and phytophagous mosquitoes (rice and champignon mosquitoes) (*Diptera*). Bt subspecies of serovar C (Bt var. *tenebrionis,* Bt var. *darmstadiensis*) are active against beetles (*Coleoptera*), are used as producers of Decimide, Colorado, Batsikol (Russia), Novodor (Denmark), and other biological preparations [2]. A new serovar F (fungi) [1] of this bacillus has been identified.

Batsikol, an entomopathogenic biological preparation based on *B. thuringiensis* var. *darmstadiensis* (H_{10}) containing the components of liquid culture, spores, insecticidal and fungicidal exo- and endotoxins, due to which it possesses multifunctional properties, was created at All-Russian Research Institute of Agricultural Microbiology (ARRIAM). The following Batsikol forms are available: dry powder, paste and liquid form.

Batsikol is similar to Bitoxybacillin (BTB) in its purpose and effectiveness. The latter possesses insecticidal effect and is active against a wide range of pests phytophages. It is recommended against cabbage moth (*Mamestra brassicae* L.), large and small whites (*Pieris brassicae*, *P rapae* L.) caterpillars in cabbage; meadow moth (*Loxostege sticticalis* L.) caterpillars in beet, alfalfa, sunflower, carrot, and cabbage crops; grape berry moth (*Polychrosis botrana* Schiff.) in grapes; corn earworm (*Heliothis zea* F.), turnip moth (*Scotia segetum* Schiff.), and lappet moth (*Laphigma exigua* Hb.) caterpillars in cotton plants; apple ermine moth and small ermine moth (*Yponomeuta malinellus* Zell., *Y. padellus* L.), brown oak (*Aporia crataegi* L.), fall webworm (*Hyphantria cunea* Drury) caterpillars in fruit trees and berry plants; brown oak tortrix (*Archips crataegana* Hb.), gypsy moth and lackey moth (*Ocneria dispar* L. and *Malacosoma neustria* L.), winter moth (*Operophtera brumata* Cl.), mottled umber (*Erannis defoliaria* L.), brown-tail moths (*Euproctis chrysorrhoea* L., *E. karghalica* M.) in fruit and woody plants, Colorado beetle larvae (*Leptinotarsa decemlineata* Say) in potatoes, tomatoes, aubergine; spider mite (*Tetranychus urticae* Koch) in cucumbers in greenhouses, small gooseberry sawfly (*Pristiphora pallipes* Lep.), common gooseberry sawfly (*Pteronidea ribesii* Scop.) in currants, gooseberries and other pests [2]. In addition, BTB is active against jewel beetle (*Agrilus ribesi* Schaefer) [16]. Extensive BTB testing conducted in various regions of the Russian Federation and the CIS (Krasnodar and Stavropol' Territories, North Ossetia, Lenin-

grad region, Republic of Crimea, Transcarpathia, Belarus, Lithuania) showed its high efficiency (from 80 to 100 %) [2].

Both pathogen and insect characteristics should be taken into account for the successful use of biologics for protection measures. In this regard, it is important to know the mechanism of their interrelations as well as of their relations with the environment. Like other bacilli, *B. thuringiensis* possesses physiological and biochemical features to ensure the absorption of nutrient substrates and antibiosis against biocenosis partners. Bacteria of the genus *Bacillus* are characterized by polyenzymatic features. Various enzymes of the class of hydrolases are found in them which results in simultaneous activities against pest insects and pathogenic fungi [1, 17-19].

Bt effect on pests results from its toxicity to insects, entomopathogenic and metatoxic effects due to the presence of crystals of endotoxin, exotoxin, phospholipase C and spores. This set of virulence factors is active against different insect species in varying degrees and in varying combinations. Bacteria cause diseases that are accompanied by septicemia, the severe lesions, in which the hemolymph and its phagocytic and non-specific immune mechanisms are no longer able to suppress the proliferation of microorganisms that penetrate it continuously. Cells of infected tissues are broken and the large quantity of parasitic bacteria enter hemolymph thus causing septicemia. Bacteria penetrate the intestinal epithelium where they proliferate rapidly and cause the insect death [2].

The mechanism of Bt antifungal action is linked to a number of factors. Bacteria produce and excrete lytic enzymes into the external medium, particularly protease and chitinase that lyse the cell walls of phytopathogenic fungi [20-24]. With lysis, the content of fungal hyphae becomes the source of power and energy for the bacilli. Moreover, bacilli can produce antibiotics that have a depressing effect against fungi [25].

Recently, much attention is given to the research related to the formation of cyclic lipopeptide antibiotics by the *Bacillus* bacteria; these antibiotics are responsible for the antagonistic effect [26-32].

Effect of *B. subtilis* on *Fusarium oxysporum* is explained by the combined effect of mycolitic enzymes and antibiotic substances [33]. Some authors suggest a link between the antibiotic activity and δ-endotoxin; perhaps, the *B. thuringiensis* var. *thuringiensis* antifungal effect is due to the disconnection of oxidative phosphorylation and respiration processes in the target objects [34].

Significant crop losses due to pests and diseases combined with the need for environmentally friendly products make the use of microbiological preparations extremely important. However, compared to chemical pesticides, their assortment is smaller, therefore it is necessary to create and use the new formulation.

Our research made it possible to estimate the spectrum of action and to identify the effectiveness of the Batsikol biopreparation developed in ARRIAM against mass pests, especially phytophage coleopteran pests (*Coleoptera*) [35], and against the phytopathogens that cause plant diseases [36], which extends the possibilities of its application.

In this paper, we summarized the results of the study of Batsikol efficiency against pests and phytopathogens in a number of crops in various areas and at different cultivation technologies.

Technique. Field and pot tests were performed in 1994-2013 in different regions of Russia. Liquid form of insecticidal biological preparation Batsikol (ARRIAM) was used in the study (spore titer of 3.5×10^9/ml). Experiments were performed in 3 replicates.

Batsikol activity against Colorado potato beetle (*Leptinotarsa decemlineata* Say) was studied in potatoes (Nevsky, Lugovskoi, Elizaveta varieties) at

the farms of Leningrad and Novosibirsk regions. Potato planting area of 200 m^2 infected with Colorado potato beetle was sprayed with the preparation at the rate of 12-15 l/ha and working fluid flow of 400 l/ha. Counting was performed with 25 plants selected along the area diagonal prior to treatment and at post-treatment days 5 to 10.

The plants of the strawberry variety Tsarskoselskaya were sprayed during budding in Leningrad Region in the plots of 100 m^2 area. The rate of the preparation use was 15 l/ha. Buds infected with strawberry blossom weevil (*Anthonomus rubi* Hbst.) were counted prior to the treatment and at post-treatment days 10 and 20 in 25 plants. Berries infected with grey mould were evaluated at the day 20.

Preparation efficiency (12 l/ha) against cruciferous flea beetles (*Phyllotreta*) and cabbage leaf beetle (*Phaedon cochleariae* F.) was studied in rutabaga, rape, mustard, and cabbage in Leningrad, Novosibirsk, and Volgograd regions in the plots of 50 m^2 area. Counting was performed prior to treatment and at the post-treatment days 5 and 10; 20 plants were examined, and the percentage of occupancy was calculated.

Carrot was treated (15 l/ha) against carrot psyllid (*Trioza apicalis* Frst.) at the plots of 10 m^2 (counts prior to the treatment and at the post-treatment day 10 in 20 plants).

Raspberry plants were treated with batsikol (15 l/ha) against raspberry mite (*Eriophyes gracillis* Nal.). Mites were counted before and after treatment in the samples of 10-30 leaves.

Flower crops were sprayed (20 l/ha) against thrips (*Thysanoptera*) in greenhouses. Percentage of occupied plants was calculated prior to treatment and at day 10 after treatment.

Batsikol activity (20 l/ha) was tested in buckwheat Emerald variety in Primorsky Territory (Ussuriisk) against buckwheat weevil (*Rhinoncus sibiricus* Faust). Vegetating plants were treated. Leaf infection in the phase of germination and stem infection during flowering and before harvesting was registered.

In estimating Batsikol efficacy against harmful fungi, application techniques appropriate to the parasitism type and environmental pathogen characteristics were used. Vegetating plants of strawberry Tsarskoselskaya variety were treated with Batsikol (15 l/ha) against gray mold (*Botrytis cinerea*), the distribution of which was assessed by the number of infected berries. Soil infected with pathogen (*Fusarium oxysporum*) was watered with the preparation at a rate of 100 ml/kg against fusarium wilt of tomatoes and flax. The efficacy of pre-sowing treatment was studied in flax against fusarium wilt, in barley against Helminthosporium root blight (*Bipolaris sorokiniana*) and in potatoes against rhizoctoniosis (*Rhizoctonia solani*). Seeds were soaked in the preparation for 3 hours. Artificial infections were created in accordance with the guidelines [37, 38].

Proportion (P) and severity (R) of the disease was evaluated according to the formula [39]: $P = A \times 100/N$, where P is a proportion of infected plants, %, A is the number of infected plants, N is the total number of plants in the samples; $R = \Sigma(a \times в)/N \times K$, where R is severity of the disease, %; $\Sigma(a \times b)$ is a sum of diseased plants number (a) multiplied by the corresponding score of disease development (b); N is a total number of recorded plants; K means the highest infection scale score.

Fusarium wilt infection was recorded in flax by N.I. Loshakova et. al scale [40], in tomatoes by S.D. Grishechkina et. al [36]. Prevalence of barley plants with root rot was evaluated on the VIZR scale (All-Russian Institute of Plant Protection, St. Petersburg) [41]. Rhizoctoniosis infection in potato tubers was recorded according to the guidelines [42].

Results. Batsikol effect on Colorado potato beetle has been identified earlier. Treatment of potatoes in Stavropol' and Krasnodar territories, in Volgograd and Leningrad regions and in North Ossetia demonstrated high (to 96-100 %) activity against this pest (2).

Batsikol showed its activity against dangerous mass pests, such as cruciferous fleas of *Phyllotreta* genus [43], oriental mustard leaf beetle *Colaphellus hoefti* Men., flea beetle *Phyllotreta vittula* Redt., pollen beetle *Meligethes aeneus* F., cabbage leaf beetle *Phaedon cochleariae* F., elm leaf beetle *Xanthogaleruca luteola* Müller [44], cereal leaf beetle *Oulema melanopus* L., shield bugs (*Eurydema*), scale insects (*Diaspididae*) [2], strawberry blossom weevil *Anthonomus rubi* Hbst. [45], buckwheat weevil *Rhinoncus sibiricus* Faust [46], carrot psyllid *Trioza apicalis* Frst., raspberry mite *Eriophyes gracilis* Nal. [47], and thrips *Thysanoptera* [48] (Table).

Biological efficacy of the *Bacillus thuringiensis* var. *darmstadiensis*-based liquid Batsikol form (H_{10}) developed by the All-Russian Research Institute of Agricultural Microbiology (St. Petersburg) **against pest phytophages in various crops** (1994-2013)

Pest	Crop	Test sites	Efficacy, %
Colorado potato beetle *Leptinotarsa decemlineata* Say	Potato	Novosibirsk and Leningrad Regions	96-100
Cruciferous flea beetles of genus *Phyllotreta*	Cabbage	Novosibirsk and Leningrad Regions	92-96
Oriental mustard leaf beetle *Colaphellus hoefti* Men.	Mustard	Volgograd Region	98
Flea beetle *Phyllotreta vittula* Redt.	Spring cereals	Stavropol Territory	89
Cereal leaf beetle *Oulema melanopus* L.	Cereals	Stavropol Territory	82-100
Pollen beetle *Meligethes aeneus* F.	Rape	Volgograd Region	75-80
Cabbage leaf beetle *Phaedon cochleariae* F.	Cabbage	Volgograd Region	82.5-98.9
Elm leaf beetle *Xanthogaleruca luteola* Müller	Cabbage	Volgograd Region	86-90
Strawberry blossom weevil *Anthonomus rubi* Hbst.	Strawberry	Leningrad Region	75-80
Buckwheat weevil *Rhinoncus sibiricus* Faust	Buckwheat	Primorsky Territory	56
Carrot psyllid *Trioza apicalis* Frst.	Carrot	Leningrad Region	47.2
Raspberry mite *Eriophyes gracillis* Nal.	Raspberry	Leningrad Region	96.3
Thrips *Thysanoptera*	Flower crops	Leningrad Region	80-90

In the study of the product activity against strawberry blossom weevil, its effect on the gray mold pathogen *Botrytis cinerea* Pers. was found in strawberries. Earlier, we observed antifungal Batsikol activity against a number of pathogenic fungi in in vitro experiments. With supplementing medium with the product at the concentration of 10 %, we observed 100 % inhibition of the growth of fungi *Botrytis cinerea* Pers. colonies, 80 % inhibition in *Pythium* sp., 70 % in *Bipolaris sorokiniana* (Sacc.) Shoemaker, 52 % in *Verticillium dahliae* Kleb., 42 % in *Rhizoctonia solani* Kuhn, 51 % in *Fusarium avenaceum* (Fr.) Sacc., 43 % in *F. oxysporum* Schlecht., and 26% in *F. solani* App. et Wr. [49].

The product activity against a number of phytopathogens was confirmed in field and pot experiments. The efficacy of spraying strawberry plants against gray mold was 60-74 % [44]; with soil watering against Fusarium wilt it was 74-87 % in tomatoes [50] and 34-42 % in flax [51]; efficacy was 66-71 % in case of soaking barley seeds against root rot [52] and 40-45 % with the treatment of potato tubers against rhizoctoniosis [53].

Thus, our results expand the understanding of *Bacillus thuringiensis* biological features and, in particular, its action spectrum against various pests and dangerous pathogens for many cultivated plants. Along with insecticidal activity, Batsikol possesses antifungal activity as well that has a technological perspective. We found its activity against strawberry blossom and buckwheat weevils, cruciferous flea beetles, carrot psyllid, raspberry mite, thrips, and pathogens *Botrytis cinerea* Pers., *Pythiim* sp., *Bipolaris sorokiniana* (Sacc.) Shoemaker, *Verticillium dahliae* Kleb., *Rhizoctonia solani* Kuhn, and *Fusarium avenaceum* (Fr.) Sacc.,

F. oxysporum Schlecht, *F. solani* App. et Wr. Batsikol inclusion in the list of microbiological agents can expand the range of biological plant protection products and their scope, which will contribute to the production of ecologically pure food and to the environment.

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THE MAIN PESTS MICROBIOLOGICAL CONTROL IN VEGETABLE, BACCATE CROPS AND POTATO IN LENINGRAD PROVINCE

S.A. DOBROKHOTOV[1], A.I. ANISIMOV[1], S.D. GRISHECHKINA[2], L.G. DANILOV[3], G.R. LEDNEV[3], K.N. FURSOV[1]

[1]*Saint Petersburg State Agrarian University*, 2, Peterburgskoe sh., St. Petersburg, 196601 Russia, e-mail anisimov_anatoly@mail.ru;
[2]*All-Russian Research Institute for Agricultural Microbiology,* Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia;
[3]*All-Russian Research Institute of Plant Protection,* Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia
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Abstract

The using of microbiological preparations for plant protection steadily extends in the world. A short list of microbiological preparations that are authorized for applying on the territory of the Russian Federation for insect pest control on crops is presented in the State Catalog of pesticides and agrochemicals. However, the biological preparations allowed for berry crops is very limited in number, and for pine strawberry no one is indicated in the Russian Federation State Catalog. In the represented work the capability of microbiological and other ecologically friendly preparations to control main pests on vegetable (cabbage, carrot, swede), baccate (blackberry, red raspberry, strawberry) crops and potato are considered under the conditions of Leningrad Province. Experiments were carried out in 2005-2014. In the research, we specified norms, terms, frequency, and rate of treatment with microbiological preparations. The tested preparations are created on the basis of different *Bacillus thuringiensis* Berliner strains (Bitocsibacillin, Lepidocid, Batsikol), entomopathogenic eelworm *Steinernema carpocapsae* Weiser (Nemabakt), also the laboratory sample based on entomopathogenic fungi *Metarhizium anisoplia* Metchn. was used. Some agrotechnical methods for insect pests control were investigated too. We studied the effect of various terms of planting, field isolation and distribution of the insect pests on the large territories, and the number on insect pests as influenced by nutrient input during plant growth. In each experiment there was a control variant (without application of any preparations). A chemical or biochemical preparation allowed for use in the territory of the Russian Federation was mostly used as a standard for comparison. The biological efficiency (BE) of the investigated preparations was estimated. It was found out that rather often the microbiological preparations were inferior to the chemical standards by BE. However, Bitocsibacillin and Lepidocid developed and manufactured in the Russian Federation can provide the 90-95 % BE against the cabbage white butterfly. The BE of these preparations against cabbage moth ranged from 60 % to 80 %. The biological efficiency of Batsicol against cruciferous tiddlywinks was 60-80 % when double treatments were used. The BE reached 100 % in control of Colorado beetle larvae with Bitocsibacillin and Batsikol. BE of both Nemabakt and laboratory sample of *M. anisoplia* in wireworms control varied at 60-80 % levels. On pine strawberry against strawberry blossom weevil the highest BE, comparable with efficiency of Fytoverm preparation, was observed for Batsikol. It was shown that a combination of bioinsecticides and biofungicides can be helpful in pine strawberry pests control. Thus, together with some repellents and agrotechnical methods the biological preparations can provide reliable protection of vegetable and berry crops, and potato against the main insect pests, at least in the conditions of the Leningrad Province.

Keywords: Leningrad Province, vegetable and baccate crops, potato, insect pests, microbiological preparations, agrotechnical methods, biological efficiency.

The use of microbiological preparations for plant protection from pests is constantly expanding in the world practice. According to experts, the share of biopesticides on the market will reach 20 % in 2020 amounting to $8 billion [1]. The scale of the use of the *Bacillus thuringiensis* Berliner (Bt)-based products ranks first in the world. In 2009, they were used in the area of 50 million ha, and the proportion of the United States was 33 million ha (2). These agents are

effective against the pests that belong to various classes, including phytopathogenic nematodes [3]. The low toxicity to target objects and persistence of bacteria in the environment, as well as the possibility of including bacterial genes responsible for the synthesis of toxic metabolite proteins in the plant genome, contribute to the promotion of transgenic crops in agriculture [4, 5].

Entomopathogenic products based on the fungi *Metarhizium anisopliae* Metchn. and *Beauveria bassiana* Balsamo are important in the click beetle larvae control [6, 7]. Although the biological effectiveness (BE) of the samples with only *B. bassiana* was low in field trials [8], when used together with the biochemical agent created on the basis of soil actinomycete *Saccharopolyspora spinosa*, an increase in BE was achieved [9]. An area associated with entomopathogenic nematodes (EPN) is developing rapidly. Five commercial companies in the United States and five in European countries produce EPN-based products that are effective against a wide range of pests [10].

In Leningrad Province, agricultural joint-stock companies mainly use chemical plant protection products (PPP) [11]. The microbiological method is used in limited areas due to the higher cost of biologics, lesser biological efficacy compared to the chemical method, and the difficulty of complex plant biological protection. A short list of microbiological preparations that are authorized for the use in the territory of the Russian Federation for pest control is presented in the State Catalog of pesticides and agrochemicals. In 2014, the biological method amounted to only 1.9 % of the total pest control events [12].

The search for effective microbial plant protection products is one of the main directions [13-15]. The emergence of new biopreparations based on *B. thuringiensis* (Batsikol) and entomopathogenic nematodes (Nemabakt, Entonem-F) made it possible to develop integrated biological protection of cabbage from cruciferous flea beetles (genus *Phyllotreta*), cabbage fly (*Delia brassicae* Bouche and *Delia floralis* Fallen), diamondback moth (*Plutella xylostella* L.), latge (*Pieris brassicae* L.) and small (*Pieris rapae* L.) whites, back in 2001. However, the profitability of biological control was low (52 %) due to the cost of Nemabakt used against cabbage fly [16). In 2005, high biological efficacy of Nemabakt against cabbage flies was found when the seedlings were sprayed in trays prior to planting in open ground [17]. The possibility of combined treatment with bio-fungicides and bio-insecticides was also demonstrated [18].

Further scientific research was aimed at increasing the number of products (repellents and biochemical insecticides) against major pests of vegetable crops and potatoes which can be used in organic agriculture [19]. It was necessary to select boipreparations and develop the application of control techniques against click beetle larvae, the wireworms that cause significant harm to potato fields in the North-West of Russia and other countries [20]. Some authors have noted the possibility to use mustard crops to control wireworm as the plants contain glucosinates and isothiocyanates that are toxic to wireworms. At this, the maximum BE was reached with the embedment of 550 cwt/ha of mustard plant mass into the soil [21].

The greatest losses in berry crops yield, red raspberry and strawberry (80 %), occur at cultivation according to the organic technology [22]. The integrated protection of berry fields in the northern European countries focuses on the use of attracting traps, pyrethroid preparations, the use of entomopathogenic fungi and predatory insects. Bt-based preparations (such as Turex) are used for the strawberry tortrix *Acleris comariana* Lienig and Zeller control only [23, 24]. To protect plants from strawberry blossom weevil, the products based on azadirachtin of an insecticidal plant (NeemAzal-T/S) and on biochemical preparations of

Spinosad and Novodor based on *B. thuringiensis* ssp. *tenebrionis* are suitable [25]. At this, the preventive chemical treatment against the weevil is noted to be ineffective [26]. Two-time treatment with pyrethroid preparations prior to the opening of 50 % of strawberry buds prevents the increase of blossom damage by blossom weevil [27]. The list of biological preparations allowed for berry crops is very limited in number, and the products for strawberry are not registered in the Russian Federation.

Therefore, it was decided to evaluate the efficacy of microbiological agents in the insect and mite control in strawberries, black currants, and raspberries [28-31]. A significant contribution to the development of methods for pest monitoring and control in fruit crops is made by Finnish scientists who investigate damage thresholds. So, for strawberry weevil, the numbers threshold at which pest control is necessary in strawberry is defined as getting 4-5 weevils in a bowl with the shake-off with 100 plants [32].

The purpose of this study was to identify environmentally safe methods and means of protection of vegetable crops, berry fields and potatoes which make it possible to replace chemical PPP, obtain products without residual pesticides, and improve the biocenotic regulation of harmful species abundance. This required to clarify the norms, terms, frequency, and rate of treatment with biological preparations, and to expand the list of biofungicides that can be combined in tank mixtures with bioinsecticides in vegetable crops and strawberry.

Technique. In 2005-2014, at experimental plots of the St. Petersburg State Agrarian University (SPbSAU), All-Russian Research Institute of Agricultural Microbiology (ARRIAM), All-Russian Institute of Plant Protection (VIZR), in horticultural farms and private farms (St. Petersburg and Leningrad Province) the efficacy of microbiological preparations bitoxybacillin (BTB) at 1-3 % concentration, Batsikol (3-5 %), Lepidocide (1 %), experimental sample of the fungus *Metarhizium anisopliae*-based biological preparation (conidia titer of $2{,}3\times10^{10}$ per 1 g), and Nemabakt (application rate of 0.5 million larvae per 1 m^2) were compared to one another, to the control (no treatment against pests) and to the standard for which a chemical (Arrivo) and biochemical (Fitoverm, Spintor, Vertimek) insecticides were used. BTB and Lepidocide were manufactured by LLC PO Sibbiofarm (Berdsk, Novosibirsk Region), Nemabakt and fungus *Metarhizium anisopliae*-based biological preparation were produced by VIZR, Fytoverm was manufactures by LLC Farmbiometod (Moscow); experimental sample of Batsikol was produced in ARRIAM. Treatment was performed using the Solo hand sprayer (Solo Kleinmotoren GmbH, Germany) at a rate of working liquid of 400-500 l/ha. The options of experiments (preparations and concentration) are presented in the tables and graphs.

Route surveys (monitoring of pests and entomophages and establishing the timing of the protective measures to start) were carried out in joint-stock agricultural companies of the Leningrad Province (Prinevskoe, Shushary, Detskoselskii, Taitsy). For vegetable crops (cabbage varieties of Kraut Krayzer in 2011, Valentina in 2012, SB-3 and Prestige in 2013 and 2014; carrot variety Berlikum royal; swede variety Novgorod; rape variety Lira), the plot sizes were 10-25 m^2. Registration was performed in cabbage, swede, and rape in 25-30 plants (5-6 samples of 5 plants per sample), in carrots in 5-10 plants; to determine the proportion of plants infested 100 plants were studied. All pest phases of insect development (imago, larvae) were taken into account.

The efficacy of biological preparations in potato variety Nevskii was estimated at a garden plot located in the southern region of Gatchina (Leningrad Province). Treatment against Colorado potato beetle (*Leptinotarsa decemlineata*

Say) was performed during the hatching of the age I larvae at the end of June (2001). Potato was treated against wireworms with Nematobakt during budding and early flowering, shedding the ridges with an entomopathogenic nematode larvae suspension. Three application techniques were tested for the experimental sample of *M. anisopliae*, i.e. dipping tubers in the suspension of fungus conidia (titer of 4.6×10^7/ml of working fluid), wetting of ridge surfaces (conidia titer of 1.7×10^7/ml of working fluid) and wetting of bottom grooves (conidia titer of 1.7×107/ml of working fluid). The number of Colorado potato beetle larvae was estimated on 10 potato plants in each variant, the number of wireworms was registered by soil excavation (sample size of 0.5×0.5 m and 0.5 m×1.0 m at a depth of 0.3 m). In some cases (low numbers of wireworms) continuous excavation was performed (area of 1 m×1 m). Potato tuber damage with the larvae of click beetles was estimated in 100 tubers.

Except for the effect of microbial PPP, the effect of autumn embedding mustard plants into the soil on the abundance of wireworms and of the combination of this agrotechnical method with biological preparations (*M. anisopliae* and nemabakt) was studied in potato. Mustard was seeded in late July, the plants were dug in the soil in the first ten days of September.

Pest control in strawberries was performed in Taitsy farm in industrial crop variety of Tsarskoselskaya; in garden plots located in the vicinity of Pushkin in the varieties of Polka, Surprise for Olympics, Tsarskoselskaya; in the southern part of the Gatchina Region of Leningrad Province in the Zinga-Zanga variety. Red raspberry (Novosti Kuzmina variety) was the study object at the same plot, as black currant was in the Educational and Experimental Garden of SPbSAU (Plotnokistnaya, Vologda, Vigorous and Memory of Alexander Mamkin varieties). Pest abundance in strawberry was estimated at the plots of 4-25 m^2; all the plants in the small size plots and 20-25 plants in the large ones were examined. Specifically damaged by strawberry blossom weevil buds and undamaged fruits elements were counted on each plant. The absolute numbers of insects and mites in the sample of 10-30 leaves in any experiment variant were counted in red raspberry and black currant.

In the presence of pests on plants prior to treatment, in both experimental and control variants, the following formula was used (1):

$$BE = \frac{O_i \times K - O \times K_i}{O_i \times K} \times 100\%, \quad (1)$$

where *BE* is biological efficacy, %; O_i, O are pest density at the experimental plot (initial and at the date of registration), ind./m^2; K_i, K are pest density at the control plot (initial and at the date of registration), respectively (ind./m^2, ind./plant). The efficacy of preparations was also calculated based on the reduction of potato tuber damage by wireworm and of strawberry buds by strawberry blossom weevil versus control using the formula (2), since at the time of treatment it was zero at all plots:

$$E = \frac{K - O}{K} \times 100\%, \quad (2)$$

where E is reduction of tuber and bud damage, %; O, K are tuber and bud damage at the experimental and control plots at the date of registration, %.

Mean values, standard error of the mean or percent were calculated. Significance of inter-variant differences was estimated using the Student *t*-test.

Results. A sufficient efficacy (90-100 %) against large white in Leningrad region was demonstrated by Lepidocide at a concentration of 1 %. The biological effectiveness of BTB against diamondback moth was somewhat lower than that of Lepidocide which was inferior to Fytoverm (Table 1).

1. Biological efficacy (%) of microbial preparations against the three insect species in vegetable crops depending on the post-treatment period (Leningrad Province, Educational and Experimental Garden of SPbSAU, 2010-2011)

Crop	Pest	Preparation, concentration	Period after treatment		
			1 week	2 weeks	3 weeks
Cabbage	Diamondback moth (*Plutella xylostella* L.)	Bitoxybacillin, 1 %	64.8(1)	75.6(1)	54.3(1)
		Lepidocide, 1 %	67.7(1)	88.4(1)	75.8(1)
		Fytoverm, 0.2 %	71.2 (1)	91.8 (1)	100 (1)
	Cruciferous flea beetles (genus *Phyllotreta*)	Bitoxybacillin, 1 %	23.4 (1)	0 (1)	0 (1)
		Bitoxybacillin, 3 %	52*-70*(2)	52*-68*(2)	–
		Batsikol, 5 %	39-80*(2)	69*-72*(2)	0 (1)
		Fytoverm, 0.8 %	82.3*	72.5*	–
Swede		Batsikol, 5 %	70.8*(1) (registration in 10 days)		57.7*(1)
		Fytoverm, 0.8 %	80,4*(1) (registration in 10 days)		63.8*(1)
		Arrivo, 0.2 %	100*(1) (registration in 10 days)		88.1*(1)
Rape		Batsikol, 5 %	77.6*(1)	28.4*(1)	66.2*(1)
		Fytoverm, 0.8 %	85.6*(1)	40.7*(1)	48.7*(1)
		Arrivo, 0.2 %	100*(1)	78.2*(1)	55.1*(1)
Carrot	Carrot psyllid (*Trioza apicalis* Först.)	Bitoxybacillin, 3 %	27_2,35_2,36_1*(3)	33_2*-82*(3)	41_2*-56_2*(2)
		Batsikol, 5 %	16.8_1(1)	47.2_1*(1)	–
		Fytoverm, 0.4 %	27_1-48_2(2)	36_2*-92_1*(2)	0_2(1)
		Vertimec, 0.4 %	79.2_2*	51.8_2*	66.8_2*
		Spintor, 0.4 %	84.4_2*	60.3_2*	41.1_2
		Arrivo, 0.2 %	55.7_2*	39.0_2*	41.1_2*

N o t e. Asterisks denote statistically proven values (probability of differences from control of more than 99 %); in brackets: the number of independent replications. Similar indices mark the tests performed in comparative experiments at the same time under similar conditions. Intervals are given for timely (years) or spatially much separated replicates. The dashes mean that calculation has not been performed.

BTB demonstrated significant efficacy against crucifer flea beetles in cabbage only with the working concentration of 3 %, BE was slightly higher in Batsikol (5 %) and Fytoverm (0.8 %). In swede, differences in Batsikol and Fitoverm BE (the latter was superior) proved to be more significant, like the superiority of the chemical reference of Arrivo (0.2 %). In the first 2 weeks post-treatment, similar results in terms of cruciferous flea beetles were demonstrated in rape, but after 3 weeks a significantly better effect was observed when applying Batsikol. BE of BTB varied against carrot psyllid reaching 82.1 % at 2 weeks post-treatment. At this, Batsikol efficacy was significantly lower. In general, except for the first week after treatment, the efficacy of BTB against carrot psyllid proved to be comparable with the Arrivo (chemical reference) (0.2 %) and the better biochemical preparations. It should be noted that a Batsikol analogue, Batsiturin, has been approved in Belarus against carrot psyllid; this agent is produced based on *B. thuringiensis* var. *darmstadiensis* (Bt H_{10}). A single application of two samples of BTB (12 kg/ha) and Batsikol (20 l/ha) in 2011 against crucifer flea beetles in the Kraut Krayzer cabbage variety resulted in a significant reduction of pest population 1 week after reatment which was comparable to that of the reference Ffytoverm preparation (3.3 l/ha) (Fig. 1, A). However, the number of crucifer flea beetles began to increase later in all variants. With double treatments with Batsikol (total of 40 l/ha) at an interval of 10 days (2014), the growth of pest numbers in the two studied varieties of cabbage (SAT-3 and Prestige) was prevented (see Fig. 1, B).

At farming conditions, both microbiological preparations (BTB, Lipidocide) and biochemical Fytoverm approved for cabbage can be used to control the leaf-eating lepidopteran pests. We have found that whitefly caterpillars, survived the treatment with biological preparations, were colonized with the entomophages *Apanteles glomeratus* L., and in late August they were eaten by predatory bugs (*Ricromerus bidens* L.).

In potato, BE of 5 % Batsikol (20 l/ha, 2011) against Colorado potato beetle larvae of age I was 100 % which was comparable to the reference of Arrivo (0.4 %, 1.6 l/ha). Similar results were obtained in 2006 when potato was treated with BTB.

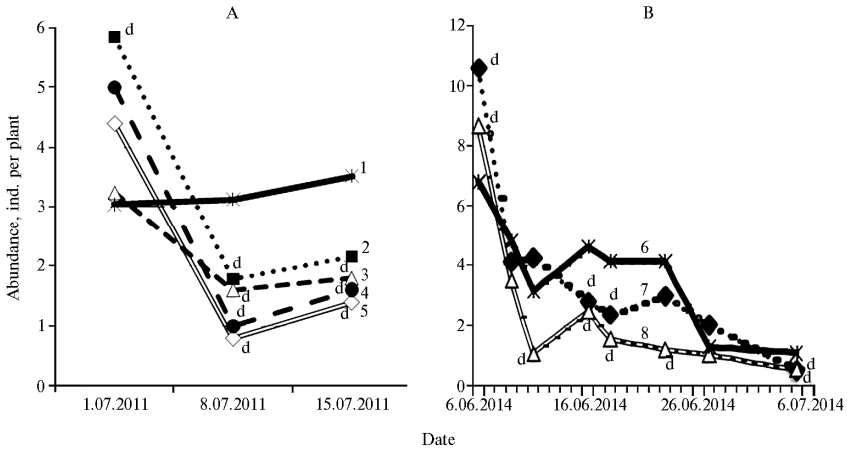

Fig. 1. Population dynamics (June to July) **of cruciferous flea beetle (*Phyllotreta undulata* Kutschera) at a single treatment of cabbage variety of Kraut Krayzer with Fytoverm or microbiological preparations** (A) **and at double treatment of cabbage varieties of SB-3 and Prestige with Batsikol** (B)**:** 1 — control (no treatment), 2 and 3 — Bitoxybacillin of various manufacturers, 4 — Batsikol, 5 — Fytoverm, 6 — control (no treatment, Prestige variety), 7 — Batsikol (SB-3 variety), 8 — Batsikol (Prestige variety). Doses and manufacturers are specified in the section «Technique»; letter d denotes the values significantly different from control at the date of registration ($p < 0.05$ according to Student *t*-test) (Educational and Experimental Garden of SPbSAU, Leningrad Province).

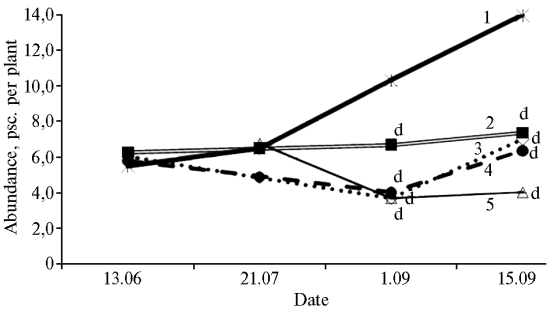

Fig. 2. Population dynamics (June to September) **of wireworms at Nevskii potato variety treatment with Nemabakt and at various modes of the *Metarhizium anisopliae* experimental sample application:** 1 — control (no treatment), 2 and 3 — *M. anisopliae* along bottom grooves and over the entire soil surface, 4 — treatment of tubers with *M. anisopliae*, 5 — Nemabakt. Doses and manufacturers are specified in the section «Technique»; letter d denotes the values significantly different from control at the date of registration ($p < 0.05$ according to Student *t*-test) (Educational and Experimental Garden of SPbSAU, Leningrad Province, 2012).

Wireworm control is most difficult. In 2012, we compared the efficacy of Nemabakt and three modes of treatments with the experimental sample of *M. anisopliae* (Fig. 2). BE of the *M. anisopliae* experimental sample when applied over the entire surface of the soil prior to potato planting was the greatest (54.2-67.5 %) and slightly inferior to that of Nemabakt. Nemabakt BE was 65.8-72.4 % which was consistent with the data on EPN reported later [33].

The prospects of *M. anisopliae* (Fig. 3, A) and Nemabakt (see Fig. 3, B) application in combination with embedding green mustard plants (*Sinapis alba* L.) variety Rhapsody into the soil was demonstrated. Such a method was more effective compared to the application of only biological products or embedding mustard into the soil (digging) [34].

In 2013, we continued the evaluation of various technologies for Nemabakt application against wireworms (see Table 2), but significant differences were not found.

BTB and Batsikol were effective against raspberry mite in raspberry. BTB was comparable to Fytoverm in its BE against spider mite in the same crop (Table 3). The death of strawberry transparent and spider mites due to BTB has been proven in strawberry in the open ground. However, better results were obtained with a combination of spraying with biological products and predatory mite *Amblyseius* colonization (see Table. 3).

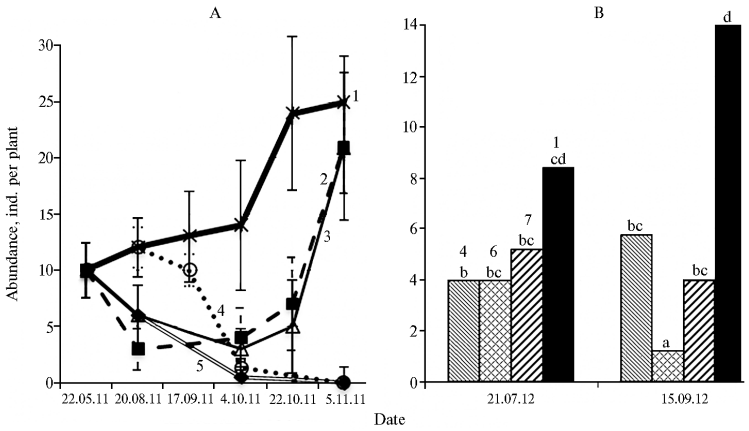

Fig. 3. Population dynamics (May to November) **of wireworms in the Nevskii potato variety at treatment with *Metarhizium anisopliae*** (A) **and Nemabakt** (B) **in combination with embedding mustard *Sinapis alba* L. plants:** 1 — control (no treatment), 2 — repellent Dachnik, 3 — *Metarhizium anisopliae*, 4 — mustard, 5 — mustard + *Metarhizium anisopliae*, 6 — mustard + Nemabakt, 7 — Nemabakt. Doses and manufacturers are specified in section «Technique». Confidence intervals of 0.95; similar letters mark the values not significantly different ($p > 0.05$ according to Student *t*-test) (garden plot, Gatchina Region, Leningrad Province).

2. Efficacy of various treatment with Nematobakt against wireworms in the Nevskii potato variety (garden plot, Gatchina Region, Leningrad Province, 2013)

Parameter	A	B	A + B	Control (without treatment)
Number of wireworms prior to planting ±SE, ind./m^2	3.5±0.96^b	3.0±0.65^b	3.5±0.63^b	3.0±1.29^{ab}
Number of wireworms at harvesting ±SE, ind./m^2	1.8±0.55^{ab}	1.3±0.36^a	1.1±0.32^a	4.3±1.58^{ab}
Biological efficacy, %	64.4	71.2	78.6	0
Damaged tubers ±SE, %	5.0±1.54^c	8.0±1.57^d	4.8±1.06^c	16.0±3.67^e
Damaged tubers ±SE, %	69±26.4	50±15.1	70±22.5	0

N o t e. A is application along bottom grooves prior to planting, B is spraying during budding; SE means standard error of the mean or percentage. Similar letters mark the values not significantly different ($p > 0.05$ according to Student *t*-test)

With high density of strawberry blossom weevil, Batsikol demonstrated a highly significant ($p < 0.001$) efficacy. Its BE with a double treatment was not inferior to Actellic. With low initial density of this pest, BTB at a concentration of 2.5-3 % demonstrated not a bad efficacy 3 weeks after treatment. This variant was comparable to Fytoverm and Actellic, and slightly inferior to Spintor and Vertimek (see Table 3).

3. Biological efficacy (%) **of microbiological and chemical preparations against pest insects in berry crops depending on the post-treatment period** (Leningrad Province, 2009-2013)

Pest	Preparation, concentration	Period after treatment		
		1 week	2 weeks	3 weeks
B l a c k c u r r a n t (educational and experimental garden of SPbSAU)				
Eriophyidae mites	Bitoxybacillin, 2 %	9_1-54_2^+(2)	16_2-30_1^+(2)	0_2-17_1^+(2)
	Fytoverm, 0.4 %	93_1*-100_2*(2)	71_2*-76_1*(2)	60.8_2*(1)
	Spark, 0.1 %	100_2*(1)	54.6_2*(1)	32.1_2(1)
R e d r a s p b e r r y (Gatchina Region)				
Raspberry mite (*Eriophyes gracillis* Nal.)	Bitoxybacillin, 3 %	47.9*(1)	59.6*(1)	58.8*(1)
	Batsikol, 3 %	96.3*(1)	–	89.8 (1)
Spider mite (*Tetranychus urticae* Koch)	Bitoxybacillin, 3 %	54*-68* (2)	94.6*-95.1*(2)	81*-89*(2)
	Fytoverm, 0.4 %	96.8*(1)	95.8*(1)	91.8*(1)
S t r a w b e r r y (Taitsy)				
Strawberry mite (*Tarsonemus pallidus* Banks)	Bitoxybacillin, 2-2.5%	25-46$^+$ (2) 99.2^{++}*	70$^+$-86* (2)	63^{+}*-73 (2)
Strawberry blossom weevil (*Anthonomus rubi* Hbst.)$^{+++}$	Bitoxybacillin, 2.5-3 %	30_4-38_5 (2)	32.5_4 (1)	40_4*-54_5*(2)

Continued Table 3

Batsikol, 5 %, 2 times	63.7_3*(1)	29.2_3*(1)	22.2_3*(1)
		47.0_3*(1)	39.7_3*(1)
Fytoverm, 0.4 %	40.3_4*	29.3_4	43.5_4*
Actellic, 0.1 %	56_5*-73_3*(2)	51.0_3*(1)	50_3*-51_5*(2)
Spintor, 0.4 %	58.2_5(1)	–	73.0_5*(1)
Vertimec, 0.4 %	50.8_5*(1)	–	59.7_5*(1)
Sochva, 1 %	62.0_4*(1)	42.6_4*(1)	47.2_4*(1)

N o t e. Asterisks denote statistically proven values (probability of differences from control of more than 99 %); in brackets: the number of independent replications; «+» — in combination with *Amblyseius*, «++» — in combination with *Amblyseius* in greenhouse, «+++» — estimation based on the damage to fruit elements. Similar indices mark the tests performed in comparative experiments at the same time under similar conditions. Intervals are given for timely (years) or spatially much separated replicates. The dashes mean that calculation has not been performed.

4. Efficacy of Batsikol and Fytoverm against strawberry blossom weevil (*Anthonomus rubi* Hbst.) in various strawberry varieties (*Fragaria ananassa*) (garden plot, St. Petersburg—Pushkin, 2013)

Variety	Average bud number per plant ±SE		Undamaged buds ±SE,%	BE, %
	damaged	total		
Control (no treatment)				
Polka	9.7 ± 0.50^{f}	27.7 ± 0.92^{jk}	65.1 ± 2.14^{d}	
Surprise for Olympics	12.5 ± 0.87^{g}	41.1 ± 2.24^{h}	69.7 ± 1.65^{cd}	
Tsarskoselskaya	10.8 ± 1.18^{fg}	40.6 ± 1.46^{h}	73.2 ± 1.59^{c}	
Batsikol (25 l/ha)				
Polka	4.2 ± 0.50^{e}	24.2 ± 1.52^{k}	82.5 ± 1.82^{b}	50.0
Surprise for Olympics	4.4 ± 0.47^{e}	33.1 ± 1.23^{i}	86.6 ± 1.40^{ab}	55.7
Tsarskoselskaya	3.5 ± 0.49^{e}	31.0 ± 1.09^{i}	88.7 ± 1.52^{a}	57.7
Fytoverm (3.3 l/ha)				
Polka	4.6 ± 0.80^{e}	24.4 ± 2.00^{k}	80.9 ± 2.13^{b}	45.4
Surprise for Olympics	3.8 ± 0.50^{e}	32.1 ± 2.10^{ij}	88.3 ± 1.38^{a}	61.3
Tsarskoselskaya	3.8 ± 0.38^{e}	31.4 ± 1.57^{i}	88.0 ± 1.37^{a}	55.0

N o t e. BE is biological efficacy, SE is a standard error of mean or percentage. Similar letters mark the values not significantly different within columns ($p > 0.05$ according to Student *t*-test).

Low efficacy of BTB against strawberry blossom weevil in the initial period after treatment in case the organic method of strawberry growing was used may be probably compensated by the additional application of the Sochva repellent (produced by pyrolysis of wood), and preparation Dachnik (produced of fir conifer) that have shown good results in this pest control in the strawberry in Taitsy farm [35].

Protection of strawberry from strawberry blossom weevil proved to be effective with triple treatments with Batsikol (Table 4).

Our experiments performed in the private garden demonstrated about the same biological efficiency (55-60 %) of Fytoverm and Batsikol, although in the Polka variety it was somewhat lower (45-50 %). Protection measures performed during budding made it possible to preserve significantly the crop in the Polka variety, a weakly stable and, therefore, more damaged by weevil.

Thus, our studies have shown the possibility of effective use of microbiological plant protection against the main pest insects and mites in vegetables, berries and potatoes under the conditions of Leningrad Province. By selecting different techniques, methods, timing, number of treatments, the efficacy of biological preparations comparable to chemical treatments can be achieved. A possibility of combined use of differently targeted biological preparations (biofungicides and bio-insecticides) in tank mixtures was found in garden strawberries. Biological preparations in combination with some repellents and agro technical measures can provide reliable protection of vegetable and fruit crops, and potatoes from pest species. In strawberry, additional monitoring is required to specify the timing and intervals between treatments. In most experiments carried out in recent years, the cost biological preparations was recouped better than in the beginning of the first decade this century. This is due to a rapid increase in prices of agricultural products compared to the cost of biological

preparations. Some tested microbiological preparations, primarily Batsikol, should be included in the plan of state registration trials for cabbage, potatoes, and garden strawberries.

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