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Address: build. 16/1, office 36, pr. Polesskii, Moscow, 125367 Russia
Tel: + 7 (916) 027-09-12
E-mail: felami@mail.ru, elein-k@yandex.ru **Internet:** http://www.agrobiology.ru

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CONTENTS

AGRICULTURAL BIOLOGY,
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CELLULAR AND MOLECULAR MECHANISMS CONTROLLING AUTOPHAGY: A PERSPECTIVE TO IMPROVE PLANT STRESS RESISTANCE AND CROP PRODUCTIVITY

(review)

C.K. RABADANOVA[1], E.V. TYUTEREVA[1], V.S. MACKIEVIC[2], V.V. DEMIDCHIK[2], O.V. VOITSEKHOVSKAJA[1]

[1]*Komarov Botanical Institute RAS,* Federal Agency for Scientific Organizations, 2, ul. Professora Popova, St. Petersburg, 197376 Russia, e-mail CRabadanova@binran.ru (✉ corresponding author), ETutereva@binran.ru, ovoitse@binran.ru;
[2]*Belarusian State University,* 4, pr. Nezavisimosti, Minsk, 220030 Republic of Belarus, e-mail v.mackievic@gmail.com, dzemidchyk@bsu.by
ORCID:
Rabadanova C.K. orcid.org/0000-0001-6342-8117
Tyutereva E.V. orcid.org/0000-0002-6727-6656
Mackievic V.S. orcid.org/0000-0002-5116-0157
Demidchik V.V. orcid.org/0000-0003-3765-8386
Voitsekhovskaja O.V. orcid.org/0000-0003-0966-1270
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Abstract

Under stress conditions, crops cannot reach the maximal level of productivity. Moreover, stress very often leads to plant death. Various stress factors limit the development and success of agricultural praxis. Under stress conditions, plants generate multicomponent metabolic, physiological and genetic responses which help them to adapt to suboptimal environment. At the level of cells, recent research has demonstrated that part of cellular content can be 'eaten' by the cell upon stress, producing energy and metabolites for survival. This process is known as autophagy (J.H. Hurley et al., 2017). Apart from this, some cells can die in the course of so-called programmed cell death (PCD), to provide better conditions for survival of other cells under stress (W.G. van Doorn et al., 2011). Both these processes are highly conservative in the evolution of eukaryotic organisms; they are very important for plant stress response and survival in suboptimal environment. Both autophagy and PCD are being intensively studied in yeast and animals since 1960ies. In plants, studies of autophagy and PCD began rather recently, and it should be kept in mind that these processes in plants bear several important features, which distinguish them from similar processes in heterotrophic eukaryotes. These features are related to the peculiar structures of plant cells. Nowadays, the problem of crop resistance to drought, salinity and extreme temperatures has become especially acute in a number of regions. Therefore, research on stress-induced autophagy is of special interest, as this process is most probably a universal component of the stress response to the abovementioned factors (V. Demidchik et al., 2017; M.E. Pérez-Pérez et al., 2017). Unraveling the mechanisms regulating the stress-induced autophagy and PCD may provide a key to genetic and chemical control of plants stress resistance, life cycle and productivity. Constitutive (i.e. not induced by stress) autophagy is an important mechanism of renewal of defect cell components; in plants, enhancement of autophagic flux by overexpression of the genes encoding autophagy-related proteins leads to an increase in stress resistance and to delayed senescence. In course of plant development, many types of plant cells undergo autophagy followed by PCD at the terminal stage of differentiation. In particular, autophagy and PCD are indispensable for seed germination, formation of vascular system and development of generative organs. Autophagy also participates in the regulation of leaf and petal senescence. So-called 'nocturnal' autophagy takes part in the degradation of transient leaf starch and sustains the assimilate transport to economically important plant organs such as fruit, tubers and storage roots. Thus, autophagy as a process directly affecting stress resistance, senescence and translocation of water and assimilates, represents a potentially very important target for regulation of plant functions, which thus far has not been used for generation of new crop varieties or in other applications in agriculture. The review discusses the structural types of autophagy (S. Reumann et al., 2010), molecular pathways of autophagy regulation (F. Reggiori et al., 2013) and cellular mechanisms of assembly of autophagic machinery, fo-

cusing on their potential use in agricultural technologies (Y.-Y. Chang et al., 2009; S. Han et al., 2015), first of all, to counterpart the deleterious effects of abiotic stress factors.

Keywords: autophagy, potassium, programmed cell death, senescence, stress, assimilate transport, crop yield

Plants overcome adverse environmental effects (drought, salinization, drastic changes in temperature, etc.) without having any possibility to physically avoid them. Annually, a significant part of the crop yield is lost in the world due to adverse environmental factors. There is an acute need to create technologies providing an increase in the resistance of plants, first of all, important agricultural crops, to abiotic and biotic stresses. One of the targets of directed selection for stress-resistant agricultural crop plants may be the autophagy process providing the survival of adverse environmental conditions by the plant at a cellular level.

Autophagy is an intracellular process, resulting in the removal of damaged sub-cellular structures, renovation of organelles and recycling of macromolecules [1-3]. During autophagy, cellular components are subjected to degradation in acidic lytic compartments, and the released low-molecular-weight compounds and energy are used for building new structures. Autophagy is inherent to all types of eukaryotic cells and is an ancient, evolutionary highly conservative catabolic program; however, its mechanisms in animal, yeast and plant cells are different [3]. Thereby, it should be noted that research on autophagy in plants significantly lags behind the studies of this process in animals and yeasts.

The processes of programmed cell death (PCD) in plants have also been studied to a significantly less extent than those in animals. There is still no significantly clear morphological classification of PCD in plant cells. In contrast to animals, it is uncommon to refer to apoptosis in plants because the features of cellular organization of plants exclude the manifestation of a number of morphological features characteristic for this type of PCD [4], although there are mentions of an apoptosis-like pathway and the formation of apoptosis-like bodies in plant cells [5]. According to one classification, there are two main types of cell death in plants, vacuolar and necrotic [4]. It is known that PCD in plants occurs at the renovation of root cap cells, elimination of cells in the endosperm aleuronic layer at the completion of seed sprouting, providing the growth of pollen tube to the embryo sac, the formation of xylem vessels and phloem sieve tubes [6-8]. At the same time, both programs, autophagy and PCD, are an important part of the response to stress.

In the present review, the authors will discuss how autophagy occurs in plants, what its main functions are in the plant organism in the absence of stress, and also assess the role of autophagy in the stress response: its cytoprotective function and participation in the starting stages of development of vacuolar programmed cell death. In connection with the identified role of cytoplasmic potassium as one of the crucial regulators of plant response to stress, including the triggering of the autophagy and PCD programs, the components of regulation of the amount of cytoplasmic potassium have been reviewed for the determination of potential targets for increasing plant stress resistance.

Role of autophagy in physiological processes. In plant cells, as well as in animal and fungal cells, damaged (used and oxidized) proteins or those which are required to the cell no more are removed via autophagy. In contrast to the proteasome degradation system responsible for the removal of short-living proteins, the autophagy process enables the cell to remove long-lived proteins [9]. Moreover, autophagy is involved in degradation of entire cellular organelles. It was initially found that autophagy is induced in response to stress factors, in which connection it was believed that its role consists predominantly in

adaptation to adverse conditions [10, 11]. However, as it was found later, autophagy (basal or constitutive) also occurs in the absence of stress effects and serves as one of the key factors of maintaining cell vitality [3, 7, 12, 13].

Constitutive autophagy is necessary for maintaining homeostasis at a cellular level because proteins in the cell are inevitably oxidized during metabolic reactions, and also by air oxygen. The plants mutant in autophagy genes and incapable of carrying out this process are susceptible to early aging even under beneficial conditions [7]. Moreover, basal autophagy provides for the replenishment of the pool of amino acids and other nutrients required by the cell as a building material for carrying out anabolic reactions.

It was shown that autophagy is involved in the plant development processes. Lytic cleavage of starch and reserve proteins contained in seeds during sprouting of the latter occurs at its involvement [14]. The formed low-molecular-weight compounds (sugars and amino acids) are transported to the cells of forming organs. At maturation of seeds, nutrients obtained as a result of autophagic degradation of proteins in aging leaves may be delivered thereto [7, 15]. However, no significant disorders in development were observed under normal conditions in most mutants in the *atg* gene incapable of carrying out autophagy. This allows making a conclusion that constitutive autophagy does not play a significant role in growth processes and plant development in the absence of stress. On the contrary, increased sensitivity of such mutant to carbon and nitrogen deficiency and also to other stress conditions was established [3, 16].

Nocturnal autophagy was found relatively recently. It was found that mutants of *Arabidopsis* and tobacco in the specific autophagy genes (autophagy-related genes, *ATG*) are incapable of recycling starch overnight, accumulated in leaves during daily photosynthesis [17]. Treatment with autophagy inhibitors has led to the same effect. As a result of thorough cytological studies in the mesophyllic cells of the wild-type plants, bodies were found that contain starch, which were subjected to degradation in vacuoles. These bodies were not present in the cells of the plants incapable of autophagy due to genetic defects in the *atg* genes or due to exposure to inhibitors. The authors have suggested that enzymes catalyzing starch breakage are partly localized in lysosomes and at the nocturnal breakup of leave starch the bodies are first gemmated from chloroplasts which are then subjected to degradation according to the autophagy mechanism [17].

However, the most important role in plants is played by the so-called stress-induced autophagy. The activation of this autophagy type is very often associated with the production of active oxygen forms [18, 19]. Activation of autophagy in root cells of higher plants occurred in response to salinization, hypoxia and reaeration, water deficiency, treatment with oxidizers, gene-toxic agents and ionization radiation [18]. The leading role of autophagy in the immune response of plants was established. It facilitates the development of hyper-sensitivity reaction in response to the attack of necrotrophs or non-virulent biotrophs, but thus limits its spontaneous uncontrolled expansion. Autophagy also enhances the resistance of plants to biotrophs and necrotrophs based on the salicylate and jasmonate signaling system, participates in virus-induced gene silencing processes [20].

In this review, the authors will concentrate on the role of autophagy in plant resistance to abiotic stresses, which currently cause maximum harm to the productivity of agricultural crops compared to other stress types.

Structural types of autophagy. It was originally believed that autophagy is a non-specific pathway of cell component degradation. It is the non-specific mass degradation of various cellular structures simultaneously according to the autophagy mechanism that is activated in plants at nitrogen and carbon deficiency [3]. However, currently it has been convincingly proven that

autophagy may be highly selective, and autophagy types have been described that are highly specific to certain organelles: mitochondria (mitophagy) [21], chloroplasts (chlorophagy) [22], peroxisomes (pexophagy) [13, 23], ribosomes [24]. The selectivity is achieved involving receptor proteins specific to the particular organelles [7, 25].

Depending on the cytological mechanism, two structural types of autophagy may be identified: micro- and macroautophagy. At microautophagy, the delivery of cytoplasmic components to acidic lytic compartments (vacuoles in plant cells) occurs due to membrane invagination [3, 26]. Such type is activated, for example, at seed sprouting [14, 27]. The cytological markers of autophagy are double-membrane organelles called autophagosomes. The formation of autophagosomes begins with the formation of a preautophagosomal structure (also called a phagophore assemble site, PAS) around sub-cellular particles. Further, the growth of this structure occurs, which leads to the formation of a closed double membrane around the components to be recycled, after which their delivery to the place of degradation occurs (to a central vacuole of plant and yeast cells or to lysosomes of animal cells) [7, 22, 26]. In plants, autophagosomes first merge with lysosomes containing acidic lytic enzymes, their internal compartment being acidified, and autolysosomes are formed. Then the outer membrane of the autolysosome merges with tonoplast, and the partially degraded content of the autolysosome surrounded by one membrane (autophagy body) enters the vacuole [7]. Often, exactly this type of macroautophagy is meant as "autophagy" (Fig. 1).

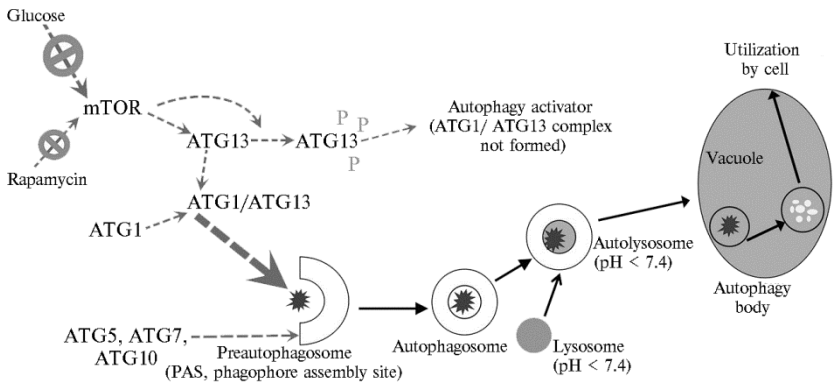

Fig. 1. Main organelles and proteins providing for inducing and progress of macroautophagy: mTOR — TOR-kinase; rapamycin, glucose — TOR-kinase inhibitors; ATG1, ATG5, ATG7, ATG10, ATG13 — component proteins of main autophagy complexes. Dashed arrows represent signal processes; black arrows represent the sequence of events at the level of sub-cellular structures.

Both structural types of autophagy were described in plants as well [10, 11, 14, 28]. In animal cells, apart from these, the third type of autophagy is known: chaperone-dependent autophagy. In its mechanism, chaperone proteins of the HSP family (heat shock proteins) are involved, which bind to the damaged proteins and deliver them to the lysosomal membrane [26]. In plants and yeasts, the Cvt pathway (cytoplasm-to-vacuole targeting) functions similarly; it is used for transporting the precursors of lytic enzymes to the vacuole [3]. Therefore, the Cvt mechanism is one of the selective types of autophagy, but it is more related to biosynthesis processes, not degradation processes [29]. Moreover, there are notes that autophagy is involved in the biosynthesis of the central vacuole. As a whole, there are more and more recent publications indicating that autophagic proteins and structural components, apart from carrying out the degradation of cellular components, may be involved in the circulation of cellular membranes,

including endo- and exocytosis [30, 31].

Molecular and genetic basis and mechanisms of autophagy development in plants. The genes encoding the protein components of the autophagy pathway (*ATG*) are highly conservative and are represented in all groups of eukaryotic organisms. Originally, the autophagy mechanism was discovered using the yeast model of *Saccharomyces cerevisiae*, and currently, about 40 *ATG*-genes are described for yeasts [25]. Most homologs of the *ATG*-genes were found in the plants as well [3]. Thereby, entire gene families in *A. thaliana* correspond to some single autophagic genes of *S. cerevisiae*. For example, homologs of *ATG12*, *ATG13*, *ATG8*, *ATG4* and *ATG18* are represented by several genes [32].

ATG proteins are classified in four groups involved at different stages of autophagy: ATG1-kinase complex (comprises ATG1, ATG13, ATG11, ATG17, ATG29, ATG31, ATG101); phosphatidylinositol-3-(PI_3)-kinase complex (VPS34, VPS15, ATG6, ATG14, ATG15, ATG38); ATG9-complex (ATG9, ATG2, ATG23, ATG27, ATG18); two Ubiquitin-like conjugation systems comprising complex 1 (ATG12, ATG5, ATG7, ATG10, ATG16) and complex 2 (ATG8, ATG4, ATG7, ATG3) [1, 3, 33]. Five main stages of autophagy may be identified: induction, formation of a preautophagosomal structure, maturation and expansion of an autophagosome, docking and merging with tonoplast, degradation of the autophagy body [2].

The key structure in the induction of autophagy is the kinase complex ATG1/ATG13 [34, 35]. The auxiliary proteins ATG17 and ATG11 participate in its formation. Their homologs have been identified in plants only recently [33, 36]. The formation of the preautophagosomal structure is initiated by binding of ATG17 to ATG29 and ATG31 [34, 37]. ATG1 binds to ATG17-ATG-29-ATG31 one of the first. Its binding to the three-component complex is mediated by the ATG13 protein, which has binding sites for both ATG1 and ATG17. These interactions facilitate an increase in the kinase activity of ATG1, providing for the addition of other proteins of the initiator complex. It has been recently discovered that ATG13 may facilitate the formation of dimers of the ATG1 protein, due to which the activation of this kinase according to the positive feedback principle is possible [34]. As a result of complex information interactions between ATG17 and ATG1, the initiator complex ATG1-ATG13-ATG17-ATG29-ATG31 (a scaffold of the newly formed autophagosome) emerges [3].

The next stage of autophagy is the growth or expansion of the autophagosome. For this purpose, the presence of phosphatidylinositol-3-phosphate (PI_3P) is necessary, which integrates into the membrane of the autophagosome. The amount of this phospholipid serves as another factor controlling the triggering of autophagy. Its content depends on the activity of the antagonist enzymes, phosphatidylinositol-3-kinases (PI_3K) and PI_3P-phosphatases. PI_3P is formed due to the activity of the PI_3-kinase complex 1 (PI_3K 1). It comprises the following proteins: VPS34 (vacuolar protein sorting-associated protein 34) that is related to class III phosphatidylinositol-3-kinases and plays a role of a catalytic sub-unit in the complex; VPS15 that serves as the activator sub-unit of the complex and anchors it in the autophagosomal membrane; ATG6 (homolog of mammal Beclin-1) [37]. The latter plays an important regulatory role: in animal cells, the binding of Bcl-2 and Bcl-1 serves as one of the key stages of autophagy initiation. However, no homolog of Bcl-2 was found in plants. In yeasts and mammals, another component of the PI_3K-complex is known — ATG14. There is still no data about the discovery of this protein in plant organisms in the literature and the GenBank database (NCBI) [33, 38]. Following from its important function, it is suggested that it must be present in plant cells [38].

Further, the assembly of conjugation complexes of Ubiquitin-like proteins begins. It is believed that the first event is the binding of ATG12 to ATG7. ATG7 has an E1-like activating ability and is required for the assembly of both complexes [39, 40]. Then ATG10, exhibiting E2-like conjugating activity, attaches to ATG7-ATG12. These enzymes perform reactions required for forming a bond between the ATG12 and ATG5 proteins [20, 40]. In order to bind the ATG12-ATG5 conjugate with phagophore, another protein is required, ATG16 [40, 41].

The main protein of the second complex is ATG8, an important regulator of autophagosome growth and formation. ATG8 is a small (14 kDa) ubiquitin-like protein. It is synthesized in a form of precursor and is subjected to significant post-translation modifications [42]. In the processing of ATG8, a redox-controlled enzyme, cysteine-dependent protease ATG4 is involved [43, 44]. Due to the cleavage of the amino acid sequence from the C-terminus of ATG8, its binding to the amino group of phosphatidylethanolamine (PE) becomes possible, which provides for the anchoring of the ATG8 protein in the autophagosomal membrane [45]. The E1-like enzyme ATG7 and E2-like enzyme ATG3 are responsible for the activation of ATG8 and the attachment of PE [41, 43]. Further, both complexes interact and the covalent binding of proteins of the second complex, ATG8 and ATG12 occurs via the protein of the first complex, ATG5, having E3-like ATG8-ligase activity. ATG12-ATG5 is involved in the transfer of ATG8 to the phagophore [46]. The lipids required for the further growth of the autophagosome are supplied from endoplasmic reticulum via the protein complex based on ATG9 [47]. The transfer of autophagosomes and autolysosomes in the cytosol is carried out with the mediation of cytoskeleton elements [48, 49]. Merging of autophagosomal membranes with lysosomes and with tonoplast occurs with the participation of the SNARE proteins [50].

Regulation of autophagy at the molecular level. At the present time, in plants two key regulators (inhibitors) of autophagy have been found that react to the concentration of nutrients: TOR-kinase [9] and the cytosolic isoform of the glyceraldehyde-3-phosphate dehydrogenase enzyme (GAPDH) [51, 52].

TOR-kinase (mTOR, the mammalian/mechanistic target of rapamycin) is a highly conservative serine-threonine protein kinase in eukaryotes, the most important activator of anabolism and the suppressor of catabolism in the cell [3]. TOR-kinase serves as a regulator for stress-induced autophagy, associated in the first place with an insufficient supply of carbon and nitrogen in the cell. In 2005, the dependence of the autophagy processes on the activity of TOR-kinase in the single-cell alga *Chlamydomonas reinhardtii* was confirmed [53]. It was proven in the paper by Liu *et al.* [54] that a decrease in TOR activity induces autophagy in a plant cell.

The blocker of TOR-kinase is rapamycin, an antibiotic of bacterial origin, synthesized by soil bacterium *Streptomyces hygroscopicus* [55]. It was reported earlier that despite the regulation of this process by Tor, the plants, in contrast to yeasts and animals, are not sensitive to rapamycin [54]. It was then established that rapamycin exerts an inhibitory action on the plant TOR-kinase; however, only in concentrations higher than the one in case of animal cells [9]. According to Xiong *et al.* [9], the concentrations, at which the rapamycin effect was manifested in plant cells, are 100-1000 nM, whereas in animal ones these are 10-50 nM. The presence of rapamycin in the said concentrations decreases TOR activity, which is morphologically manifested in slowing the root growth in *Arabidopsis thaliana* [9].

The key mediator in the induction of autophagy in response to stress is

the ATG13 protein [3, 35, 36, 56]. Under normal physiological conditions, the TOR-kinase phosphorylates ATG13. Such hyper-phosphorylated form of ATG13 has low affinity to ATG1, and the ATG1/ATG13 complex that initiates the formation of autophagosome is not formed. Binding of ATG1/ATG13 only becomes possible at a decrease in the activity of the TOR-kinase [3, 34, 56]. A deficiency of nutrients in the cell becomes a signal inhibiting the phosphorylation cascade of PI3K/TOR kinases, and results in a decrease in the activity of the TOR-kinase [54]. I.e. stress caused by carbon or nitrogen deficiency initiates autophagy (see Fig. 1).

Recently, another autophagy inhibitor was found in plants: glyceraldehyde-3-phosphate dehydrogenase enzyme (GAPDH) [51, 52]. The *Arabidopsis* forms deficient in the cytosolic isoform of this enzyme demonstrated enhancement of constitutive autophagy and also a high degree of oxidative stress and activation of PCD [52]. Production of ROS by cells in response to the pathogen attack was, on the contrary, decreased in such plants [52]. It was proved by the example of tobacco cells that GAPDH directly interacts with the component of the second system of ubiquitin-like conjugation, the ATG3 protein, suppressing its function; the inhibition is removed upon exposure to ROS [51]. Therefore, GAPDH, as well as the TOR-kinase, provide a direct relationship between the metabolic status of the cell and induction of autophagy, but this relationship is under redox control.

In yeasts and mammals, the important regulators of autophagy are kinases: AMPK (AMP-activated protein kinase) in mammals, SNF1 (sucrose non-fermenting 1) in yeasts [57]. They react to the alteration of energy charge, which is described as

$$([ATP] + {}^1/_2[ADP])/([ATP] + [ADP] + [AMP]),$$

and activate autophagy (directly or by inhibiting TOR-kinases). Several homologs of SNF1/AMPK are known in plants. For one of them (the KIN10 kinase in *Arabidopsis*), a role of autophagy activator has been recently shown under deficiency, hypoxia and water deficiency conditions [58].

Relationship of autophagy and programmed cell death. The role of autophagy in the development of PCD is ambiguous [15, 27, 59]. On the one hand, autophagy may serve as a method of avoiding cell death, and the cytoprotective function of autophagy is associated with this [60, 61]. On the other hand, activation of autophagy in some conditions precedes triggering of cell death programs, and in this case, autophagy is one of the starting stages of PCD [62]. Thus, in the process of vacuolar cell death, a decrease in the volume of the cytoplasm and an increase in the volume occupied by vacuoles are observed. These events are accompanied by the enhancement of autophagy and the rupture of tonoplast, accompanied by the release of hydrolases, which leads to the destruction of protoplast. Up to the moment of tonoplast rupture, the integrity of the plasmatic membrane, mitochondrial membranes and those of other organelles is maintained [4]. The entire process takes, as a rule, a long time: up to several days [63]. In contrast to vacuolar death, necrotic death develops much more rapidly and is characterized by the shrinkage of the protoplasm, early destruction of the plasmatic membrane and membrane organelles, the disruption of mitochondrial functioning and the accompanied accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cytoplasm [63, 64].

The cytoprotective role of autophagy is demonstrated by studies of knock-out mutants, in cells of which its development is impossible. The insertion mutants of *A. thaliana atg5-1* [46] and *atg7-1* [39] are characterized at a long photoperiod by normal growth and development. However, at a short photoperiod, the mutants of both lines grow slower, have less seed productivity and

are subject to premature aging compared to the wild type. Moreover, they have elevated sensitivity to stress, especially to the deficiency of microelements. As a whole, these plants exhibit lesser viability and have a significantly lower survivability rate compared to the wild type, beginning from 10-day growth in a medium with decreased nitrogen content. At conditioning in darkness, the survivability decreases in *atg5-1* on the 2nd day already and that in *atg7-1* on the 4th day, whereas in natural ecotypes it is correspondingly on the 6th and 8th day [39, 46]. The *atg13* mutation in *A. thaliana* is phenotypically characterized in several lines [35]. They are differently susceptible to early aging under short day conditions. In the nitrogen-deficient medium, the growth of such sprouts is slower compared to the wild type. Chlorophyll synthesis in the leaves is disrupted. They are also more sensitive to carbon deficiency in the medium. Darkening for 10 hours does not affect these lines so strongly as the plants with the disrupted formation of ubiquitin-like conjugation complexes. However, already after 13 hours of conditioning in darkness, a marked difference in stability is found between the *atg13* plants and wild types, especially in double mutants [35]. The *atg10* mutants [41] were hyper-sensitive to carbon and nitrogen deficiency, and also demonstrated spontaneous development of PCD.

In the cells of plant roots upon exposure to abiotic stresses leading to the development of PCD, the autophagy symptoms are often observed [18]. It can be hypothesized that it is originally activated as a cytoprotective mechanism. But after passing through the "point of no return" it becomes a necessary stage of PCD development.

Hypothesis of potassium regulation of autophagy and programmed cell death. From the mid-1980s, in plant biology, the concepts of control and coordination of physiological reactions at stress via cytoplasmic Ca^{2+} and reactive oxygen species (ROS) are actively developed [65, 66]. It is known that the generation of ROS plays a significant role in the regulation of cellular metabolism. ROS are inevitably formed at redox reactions in the cell both under normal conditions and upon exposure to stressors (exposure to pathogens, drought, salinization). The discovery of elevated synthesis of ROS at the early stages of stress response was the beginning of studies dedicated to functions of these molecules. One of such functions is the regulation of the activity of ion channels [67, 68].

The rapid release of K^+ is related to events accompanying reaction to stress in the plant cell. In the recent years, the theory about the participation of potassium in plant response to stress was developed [18, 68-70]. Potassium is the most abundant metal and cation in the plant cell. Its content by dry weight is 3-10%; therefore, the deficiency of this metal extremely adversely affects productivity. Adequate potassium supply is the basis of high yield and resistance of plants to stress effects. Being an irreplaceable macroelement, comprised in the vital NPK (Nitrogen-Phosphorus-Potassium) triplet, potassium plays key functions in plant life. In particular, it is responsible for the water balance and hydroskeleton of the cell, transpiration, closure of air pores and stretching growth. The trans-membrane streams of potassium form the diffusion membrane potential on the plasmatic membrane, tonoplast and endomembranes, which serves as a basis for a high difference of potentials on these membranes. Also, potassium plays a role of a non-specific activator of dozens of crucial anabolic enzymes of the cytoplasm [71]. Possibly, it stabilizes the low activity of proteases and nucleases, preventing unplanned triggering of autophagy and PCD [18, 69].

The potential-dependent potassium channels and also several nonselective cation channels (NSCC) are involved in potassium release at stress and during some development processes [68, 69]. It is important to note that the slow re-

lease of K^+ occurs under normal conditions as well [70]. Moreover, it is necessary for carrying out important physiological processes, for example, air pore regulation of transpiration [72-73]. The increase in potassium release at salt stress was shown long ago [74]. It was established that the release of K^+ is mostly mediated by depolarization-activated outwardly-rectifying K^+-channels [75]. The detailed mechanism of this process and its effect on further events in the cell as well are still to be discovered.

The outwardly-rectifying K^+-channels providing for Goldman rectification of the outgoing potassium current are activated at the depolarization of the plasmatic membrane and are related to the Shaker type. These are usually homo- or heterotetramers [70]. Each subunit comprises six trans-membrane domains, a pore domain, and a voltage sensor. At the assembly of a tetramer, the pore domains containing a specific amino acid sequence (GYGD) are combined in such a way that four potassium-binding sites appear inside the pore, i.e. a selective filter is formed [76]. In the plasmatic membrane of root cells of *A. thaliana*, two types of outwardly-rectifying Shaker channels are synthesized: SKOR (STELAR K^+-outward-rectifier) and GORK (guard cell outward-rectifying K^+-channel). Thereby, the SKOR-type channels are represented in parenchymal cells and mediate potassium current in the xylem vessels, whereas GORK are predominant in epidermal cells and are involved in potassium release from the root [70]. Both of these types are directly activated by ROS [77]. Channel opening is induced via the ROS-sensitive site in the molecular structure. In the case of SKOR, the role of the ROS sensor is played by the cysteine residue (Cys168) in the peptide sequence of the S3 domain in the contents of the potential-sensitive complex S1-S4 [77]. Due to the fact that structurally similar GORK and SKOR are significantly similar, it is suggested that in GORK the ROS-dependent activation is provided in the same way [18].

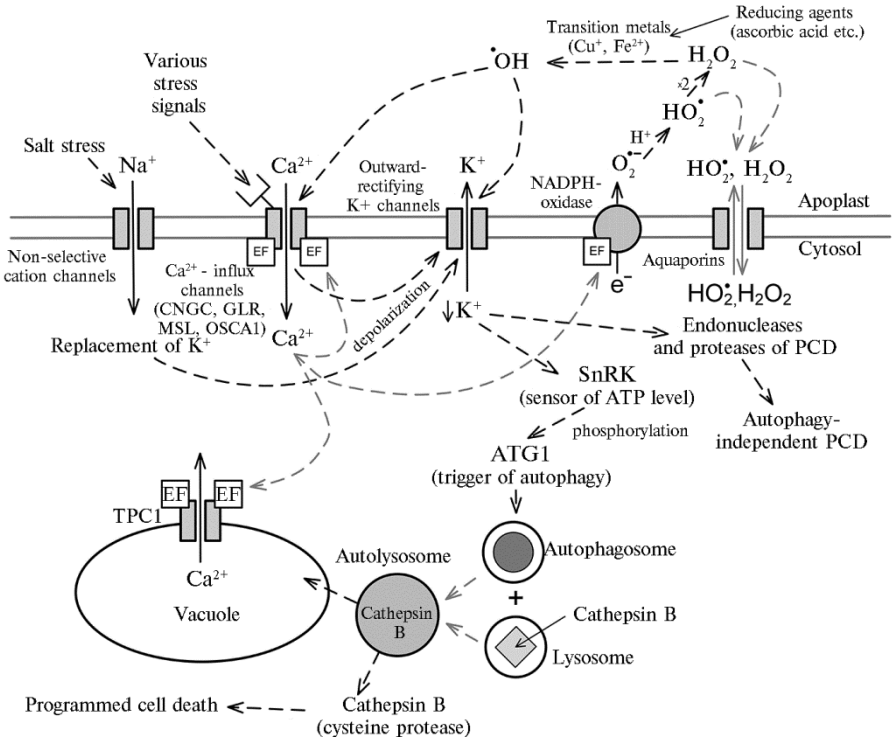

Fig. 2. Scheme of processes that are a basis of stress-induced autophagy and programmed cell death (PCD). The stress signals interact with specific receptors on the cell surface, which causes the depo-

larization of the plasmatic membrane, an increase in the cytoplasmic activity of calcium, an increase in the production of reactive oxygen species (ROS) due to the calcium-dependent activation of NADPH-oxidase. Depolarization also leads to the activation of the outward-rectifying potassium channels, which is further stimulated by ROS. A drastic drop in the concentration of cytoplasmic potassium leads to triggering of the autophagy and PCD reactions. ROS are also produced intracellularly and are transported into apoplast via aquaporins. The redox processes in the apoplast are controlled by the content of reduced transition metals and ascorbate (according to 18, with alterations). EF is EF-hand (protein domain); TPC1 is a two-port calcium channel. The grey arrows indicate secondary processes.

The activity of the GORK channel is stimulated by hydroxyl radicals ($^{\bullet}OH$), which are generated by the Ca^{2+}-dependent NADPH-oxidases at reaction almost to all types of stress, including salinization, drought, pathogen attack, etc. A decrease in the potassium concentration in the cytoplasm, in its turn, stimulates the activity of proteolytic enzymes, including caspase-like proteases, which play an important role in the PCD triggering mechanism in plants [45, 69, 70].

In a general form, the mechanism of developing a stress reaction involving the outgoing K^+ current in epidermal root cells may be represented as follows. At binding of the stress signal or pathogen elicitor with the plasmatic membrane receptor and also as a result of the supply of Na^+ via non-selective channels, the activation of Ca^{2+}-permeable cation channels occurs, which leads to an increase in the Ca^{2+} concentration in the cytoplasm. Calcium activates the NADPH-oxidase by binding to its cytoplasmic domain. Also, the Ca^{2+}-dependent activation of endonucleases and proteases may be observed [64]. NADPH-oxidase generates superoxide-anions ($O_2^{\bullet-}$), which react with protons, forming a hydroperoxide radical ($HO_2^{\bullet}$). Dismutation of $HO_2^{\bullet}$ gives hydrogen peroxide (H_2O_2), which becomes a source of oxygen for the synthesis of hydroxyl radicals ($^{\bullet}OH$) in Haber-Weiss reactions [78]. Peroxide and $^{\bullet}OH$ further activate the SKOR, GORK and Ca^{2+}-permeable channels, enhancing the supply of Ca^{2+} into the cytoplasm, and, in their turn, also stimulating NADPH-oxidase (Fig. 2). A positive feedback mechanism works, which may be stopped by systems for pumping Ca^{2+} away from the cytoplasm [79]. The membrane potential is restored by potassium release and due to an increase in the activity of the electrogenic H^+-ATPase pump. If membrane repolarization and restoration of the cellular potassium amount do not occur, the potassium-regulated plant proteases and endonucleases are activated, and the result is the initiation of the molecular PCD mechanism [70].

In the late 1990s, it was discovered that in mammal cells the decrease in the concentration of K^+ (together with the intake of Ca^{2+}) plays an important role in the apoptosis triggering mechanism [80, 81]. Potassium is a direct inhibitor of caspases, and its release from the cell activates these enzymes [82, 83]. Most probably, the similar mechanism exists in plant cells as well [18, 69]. According to the recent hypothesis, potassium in plants (depending on its content in cytoplasm) serves as a metabolism trigger, and the stress-induced release of K^+ may be a trigger of stopping the cell growth, inhibiting the biosynthesis, activation of catabolism and at strong stress at longer perspective, triggering the PCD [18]. An important step in developing this hypothesis is to test the potential stimulation of autophagy at the loss of K^+ by plant cells. It is still unknown whether autophagy is a K^+-dependent process in plants. It was shown that in *A. thaliana gork 1-1* mutants (in contrast to the natural ecotype Ws-0) at conditioning the roots in NaCl solution, the drastic accumulation of autophagosomes does not occur. The obtained data indicate that potassium loss plays a direct role in inducing autophagy [18].

Perspectives of studying autophagy. According to modern

concepts, autophagy plays a significant role in cell metabolism providing the renovation of cellular structures, the cleavage of damaged molecules and production therefrom of organic compounds required for the extraction and accumulation of energy. I.e. this effect is directed to cell survival. Autophagy is especially important at adaptation to various stress effects. It mainly defines the survival of plants under adverse and changing conditions. Despite the fact that autophagy is studied in sufficient detail, there are still many unresolved issues. In particular, it is not quite clear whether the rapid release of potassium ions from the cell always directly triggers the molecular autophagy mechanism (similarly to its induction of programmed cell death). The same concerns the relationship of autophagy with other intracellular processes. It was shown that the absence of autophagy adversely affects the endurance of plants to stress, that it influences biomass accumulation and seed productivity. However, the effect of autophagy is not so unambiguous because it is also involved in cell death processes. Nevertheless, these processes are an intrinsic part of organism development; they are necessary for the formation of many structures and passing of all life cycle stages.

In case the hypothesis that the regulatory cellular signal for triggering autophagy is a drastic decrease in the cytoplasmic potassium concentration is confirmed, its functions can appear to be even more significant, especially under conditions when the plant is exposed to adverse environmental factors. The directed manipulation with the degree of cellular component activity for maintaining the required potassium concentration in the cytoplasm at stress may be used at creation of stress-resistant plants, for decreasing their deaths and supporting growth processes under adverse environmental conditions.

Therefore, autophagy as a process directly relevant to the mechanisms of stress resistance, aging and to the transport of assimilates is an important potential target for regulation, which has not yet been used at creation of new cultivars and in practical applications in agriculture. Autophagy plays a double role: on the one hand, it is directed to the survival of the cell; on the other hand, it serves as part of the cell death process. In both cases, autophagy directly affects the development of plant organisms. In view of this, further studying of autophagy regulations, especially, more detailed disclosure of induction mechanisms of this process appears to be perspective.

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RESISTANCE OF GUAR *Cyamopsis tetragonoloba* (L.) Taub. TO HARMFUL ORGANISMS

(review)

E.E. RADCHENKO, D.V. SOKOLOVA

Federal Research Center the Vavilov All-Russian Institute of Plant Genetic Resources, Federal Agency for Scientific Organizations, 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail eugene_radchenko@rambler.ru (✉ corresponding author), dianasokol@bk.ru
ORCID:
Radchenko E.E. orcid.org/0000-0002-3019-0306 Sokolova D.V. orcid.org/0000-0002-9967-7454
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Abstract

Guar (clusterbean) *Cyamopsis tetragonoloba* (L.) Taub., a tropical annual legume crop of a multipurpose use, is promising for growing in the South Russia. The problem of resistance of guar to diseases and pests is discussed. The *Alternaria* leaf blight caused by *Alternaria cyamopsidis* Rangaswami & Rao and bacteria leaf blight caused by *Xanthomonas axonopodis* pv. *cyamopsidis* (Patel) Vauterin) are the most harmful diseases of guar. Seed infection promotes the extensive and fast spread of the disease. Anatomical and morphological characters are not having any relationship with *A. cyamopsidis* resistance in clusterbean plants. Sunshine, minimum temperature, cumulative rainfall and relative humidity in the evening were found significantly associated with *Alternaria* leaf blight severity (M.S. Saharan et al., 2004). The resistance of guar to bacteria leaf blight is oligogenically controlled (P.S.K. Anil et al., 2012). For *X. axonopodis* pv. *cyamopsidis* a differential interaction with plant host genotypes is characteristic. In the USA the two races of the pathogen (0 and 1) have been identified which differed not only by the virulence to guar varieties but serologically as well. A protocol of ELISA test for detecting virulent and avirulent strains of the bacteria is elaborated (G.K. Vijayanand et al., 1999). The pathogen isolates significantly differ in aggressivity when they are proliferated on resistant (HG 75) and sensitive (PNB) genotypes of guar. The analysis of the isolates with the use of molecular markers has revealed a significant polymorphism of the pathogen populations. The results obtained using two different approaches correspond to each other (B. Kaur et al., 2005). Plant infection with bacteria leaf blight and *Alternaria* leaf blight induces protective response (i.e. lignin and phenol compounds accumulation, increase of peroxidase activity). The induced resistance was observed when guar was inoculated with casual agents of charcoal rot *Macrophomina phaseolina* (Tassi) Goid., root rot *Rhizoctonia solani* J.G. Kühn, wilt *Fusarium solani* (Mart.) Sacc., and also with aphids infestation. The diversity of cultivated guar varieties for resistance to pathogens is not high. At the same time a differential interaction with plant host genotypes is revealed not only for bacteria leaf blight causal agent but also for *M. phaseolina* (S. Purkayastha et al., 2006). This means that varieties with different resistance genes should be grown for prevention of epiphytoties. The introgression of resistance genes from the wild species *C. senegalensis* Guill. & Perr. and *C. serrata* Schinz is considered as a promising approach for broadening genetic diversity (S. Kumar et al., 2017). However, interspecific crosses and phenotypic selection are the main breeding methods applied to date. In recent times the intercropping of guar with other crops (millet, okra, and castor) is also used for controlling populations of harmful organisms.

Keywords: guar, *Cyamopsis tetragonoloba*, bacterial blight, *Alternaria* leaf blight, root rot, insect pests, resistance

Guar, or clusterbean *Cyamopsis tetragonoloba* (L.) Taub. (family *Fabaceae* L., $2n = 14$), a new for Russia crop, presents great interest for selection and genetic research. The plant originates from India where its basic planted areas are concentrated; recently, it has also been cultivated in other countries of Asia, Africa, America (mainly in the USA) and in Australia. Guar is used for food (its seeds contain a significant amount of protein and fat oil) and for cattle forage;

however, the most demanded product is guar gum which is formed in the secondary endosperm of guar seeds. Natural gum is applied in the food-processing industry as a consistence stabilizer, increasing viscosity and enhancing the gelatinizing properties of the substance, as well as in cosmetology, paper, textile, coal and oil-drilling industries. The urgent necessity of guar gum import substitution, primarily, having an industrial purpose, as it is used at drilling of oil wells, has caused the actualization of the problem of the cultivation of this tropical crop in the climatic conditions of Russia.

Guar is not resistant to some fungal, bacterial, virus, nematode diseases and pests that periodically bring essential damage to the plant. In fact, the list of phytopathogens including obligate parasites and hemibiotrophs is rather extensive. The most harmful diseases of guar are *Alternaria* leaf blight caused by *Alternaria cyamopsidis* Rangaswami & Rao [1], and bacterial leaf blight caused by *Xanthomonas axonopodis* pv. *cyamopsidis* (Patel) Vauterin. So, yield loss in India owing to the distribution of *Alternaria* leaf blight on guar crops reaches 60% [2], and of bacterial leaf blight 68% [3]. Guar gall midges *Contarinia texana* (Felt) can destroy up to 30% of grain yield [4, 5], and is one of the most serious phytophages. Plant greenflies, thunder flies, frog-flies, white flies, Coleoptera pests [6-8] also bring a lot of harm. Thus, the necessity of the analysis of the Russian phytopathogenic landscape of all prospective regions for guar cultivation is obvious. The risk of damaging the new crop by pests-oligophages and widely specialised pathogens is especially great. The fungus *Fusarium solani* (Mart.) Sacc., causing wilt and root rot in India [9, 10], is propagated everywhere in Russia. Two diseases resulted in yield losses at experimental guar crops at the Ust-Labinsky District of the Krasnodar Territory in Russia *Alternaria* leaf blight and bacterial rot [11].

The purpose of the present review is to summarize available data about guar interaction with harmful organisms, the plant resistance to the most dangerous pathogens and pests and the possibility of guar selection for the development of immunity to them.

Resistance to bacteriosis. The bacterial leaf blight agent of guar *X. axonopodis* pv. *cyamopsidis* was first revealed in two states of India in 1952 [12], and soon in the USA [13], Brazil [14], and other countries. The infection contamination is retained in seeds, which promotes the extensive and fast spread of the disease [15]. Epiphytotic development of the disease (the affection of plants reaches 80%) is usually observed after a long period of showers [16, 17].

The studies investigating the factors of plant resistance to the pathogen are not numerous. It was found out that forms susceptible to the pest (first of all, the PNB variety) show a decrease in the activity of peroxidases and polyphenol oxidases. The absence of such a decrease or an increase in the activity of these ferments can be used as a marker in case of selection of plants resistant to the disease [18]. Contamination of the resistant sample HG 75 resulted in an essential increase in the levels of phenols and peroxidases in plants [19]. Susceptible (Pusa Nav Bahar), moderately resistant (HG 563, FS 277) and highly resistant (wild progenitor *Cyamopsis serrata* Schinz.) guar samples were investigated for the content of solvable and structural (cellulose, hemicellulose, lignin) carbohydrates after artificial inoculation of plants with *X. axonopodis* pv. *cyamopsidis*. The maximum decrease in the concentration of solvable sugars was noted in inoculated plants of the susceptible sample; the sample *C. serrata* had a minimum change in this parameter. The susceptible variety also showed the decrease in the level of structural carbohydrates; on the contrary, the resistant forms demonstrated the raised level of carbohydrates as well, and that indicates the formation of protective barriers [20].

The resistance of the samples HG 75 and RGC 137 to the pathogen is controlled by dominant genes, which interact in a non-allelic way [21]. In F_2 from the crossing of the samples HG 75 and HG 563 with sensitive testers, phenotypes segregated as 13 resistant to 3 susceptible. The authors believe that both HG 75 and HG 563 have two key genes; one of them controls resistance to the disease, and the second one inhibits its development [22]. Thus, with equal probability, it is possible to assume that each of these samples possesses both dominant and recessive genes of resistance to bacteriosis. Unfortunately, genetic control of guar resistance to bacteriosis and other harmful organisms is discussed only in two small articles. The starting point of the research was the work [23] that supposes a high productive transcriptome (RNA-seq) sequencing of two guar varieties (M-83 and RGC-1066), carried out recently. In this work, there are 62146 unique coding sequences, deciphered and annotated, 5773 microsattelite (SSR) markers and 3594 mononucleotide polymorphisms (SNP) which are identified.

X. axonopodis pv. *cyamopsidis* is characterized by differential interaction with host-plant genotypes. In the USA, two strains of the pathogen (0 and 1) were distinguished, which differ not only in virulence to guar varieties [24], but also serologically [25]. A protocol of Enzyme-Linked Immunosorbent Assays (ELISA) is elaborated for detecting virulent and avirulent strains of the bacterium [26]. *X. axonopodis* pv. *cyamopsidis*, collected in the north and the northwest of India, essentially differed in aggressiveness when they were proliferated on resistant (HG 75) and sensitive (PNB) genotypes of guar. The analysis of the isolates with the use of molecular markers based on the polymerase chain reaction (first of all, RAPD) has also revealed significant polymorphism of the pathogen population. The results obtained using two different approaches correspond to each other [27].

Resistance to *Alternaria* leaf blight. The causative agent of *Alternaria* leaf blight was first revealed in 1953 in India [28], then in the USA [29] and other countries where guar was cultivated. The extensive spread of the disease was promoted by the fact that the infection contamination retained in seeds [30]. It was found out that the development of the fungus mycelium is optimal at a temperature of 35 °C [31]. Other experiments demonstrated the most severe development of the disease in the case of the variety Pusa Navbahar (PNB) at 25-31 °C, 80% of relative air humidity and heavy rainfall [32]. Monitoring of *A. cyamopsidis* on moderately resistant (HG-75, HG-365), moderately susceptible (RGC-1000), susceptible (RGC-936, RGC-1002) and highly susceptible (FS-277) varieties of guar has shown some regularity. The disease affects susceptible samples more intensively, and the degree of leaves lesion development depends, first of all, on insolation, the minimum air temperature, precipitation and relative air humidity in the evening [2, 33].

Guar resistance to the disease is not related with its anatomic and morphological features. The anatomical characteristics of leaves of two moderately resistant (HG-75, HG-365), four susceptible (HG-258, HFG-119, RGC-936, RGC-1017) and highly susceptible (FS-277) to *Alternaria* leaf blight guar varieties were compared. All samples did not differ in the number and size of stomata on both surfaces of leaf plates. The upper surface of the leaves in the case of moderate stable forms had a bit larger number of hairs [23-25], in comparison with susceptible samples [13-17], but the pubescence of the bottom surface of the leaf plate of all varieties did not differ much. The amount of wax on leaves of the stable forms at all growth stages was a bit exceeded, in comparison with susceptible samples; however, distinctions were not statistically significant [34].

Guar inoculation with *A. cyamopsidis* resulted in the essential accumula-

tion of polyphenol oxidase and phenolic compounds [35, 36]. U.N. Joshi et al. [30, 36], investigating the biochemical composition of the guar samples, susceptible (IC 116835) and moderately resistant (IC 116903) to the *Alternaria* leaf blight causative agent, revealed the increase in the activity of enzymes, and the accumulation of phenols and lignin in response to infestation by the pathogen. The plants of the resistant variety RGC-986 showed the highest level of solvable protein and phenolic compounds in comparison with moderately resistant (RGC-1003) and susceptible (RGC-936) forms. Infestation by the pathogen resulted in the most essential decrease in the solvable protein level in the case of the susceptible sample and the increase in the content of phenols in the case of the variety RGC-986. The infected plants of the resistant sample demonstrated the greatest concentration of sugars [37, 38].

Resistance to other diseases. Data on guar resistance to causative agents of other diseases note mainly the presence of nonspecific reactions of plants in response to pathogen contamination. The induced resistance was observed in the case of inoculation of guar samples with different resistance to the causative agent of charcoal rot *Macrophomina phaseolina* (Tassi) Goid. The leaves and roots of resistant forms revealed the highest activity of peroxidase and some other emzymes and accumulation of phenolic compounds. The variety RGC 1031 [39, 40] appeared to be the steadiest. The marker SCAR-20 has been developed which allows identifying the sample RGC 1031 [41]. In the case of infestation with the guar wilt causative agent *F. solani*, the plants demonstrated the decrease in the protein level and the rise of activity of proteolytic enzymes [10].

Phytopathologic and DNA testing showed that isolates of *M. phaseolina*, collected on guar and other plants, differ in specificity to plant hosts and aggressiveness when they are proliferated on the susceptible variety FS 277 [42].

Addition of zinc ($ZnSO4 \cdot 7H2O$), copper ($CuSO4 \cdot 5H2O$) and manganese ($MnSO4 \cdot H2O$) fertilizers into the soil induced the formation of resistance to root rot (causative agents *Rhizoctonia* spp.) in the susceptible sample FS 277, what testified to the decrease in the lesion degree of the infected plants. Thus, the plants revealed the raised activity of oxidative enzymes and accumulation of phenolic compounds and structural carbohydrates [43-45]. System resistance of guar to *Rhizoctonia solani* J.G. Kьhn was also induced by processing of seeds of the variety Local with salicylic acid and/or *Pseudomonas fluorescence* Migula. The accumulation of PR-protein (chitinase, β-1,3-glucanase), phenolic compounds and lignin, as well as an increase in the activity of enzymes, were revealed in sample plants [46].

In India and Pakistan, papaya (PaLCuV) and tomato (ToLCV) leafroll viruses, a yellow tomato leafroll virus (TYLCV), and a recombinant guar leafroll virus (CyTLCuV) which are proliferated by whitefly *Bemisia tabaci* Genn., invoke deformation of leaf plates, contraction of interstices and stems of guar plants [47-51]. In India, tobacco streak virus (TSV) is revealed in guar, which invokes mosaic and necrosis of leaves and necrotic strips on stems [52]. Bean common mosaic virus (BCMV) is well spread in guar crops, which is transmitted not only by greenflies but also with inoculated seeds [53].

Resistance to insect pests. Articles connected with guar resistance to insect pests are not numerous. S.P. Singh et al. [54] investigated 40 guar samples and revealed 3 precocious forms (HG 365, HG 563, RGC 1066), poorly colonized by greenfly *Aphis craccivora* (Koch). The greatest number of pests is marked on late-ripening varieties. Guar colonization with *A. craccivora* resulted in accumulation in plants of phenolic compounds and ferments, and a decrease in the level of carbohydrates and amino acids. It is curious that the maximum gathering of natural gum was received from the plants with the raised

level of carbohydrates and phenols that caused the raised resistance to the phytophage [55].

In result of the research of 60 guar varieties, 5 samples (CH 14-2, HG 75, HG 94, HG 258, HG 365), characterized by resistance to whitefly *B. tabaci*, were revealed [56, 57]. Eight guar samples were estimated for resistance to leaf beetles (family *Chrysomelidae* Latreille), leafhoppers *Amrasca biguttula bigutella* (Ishida), *Empoasca* Walsh spp., greenflies *Aphis medicaginis* Koch and American clover miners *Liriomyza trifolii* (Burgess). High-yielding samples RGC-1031 and GAUG-13 were less invaded by the phytophages [58]. It was reported about complex resistance of the guar variety BR-99 to insect pests (leafhopper, whitefly) and root rot in Pakistan [59].

Breeding for resistance. The literature data note low genetic diversity of sources of guar resistance to pathogens. In the field and laboratory experiments, it was proved that the variety Brooks is resistant to *A. cyamopsidis* and bacteriosis in the USA [29]. Among the varieties resistant to bacteriosis and *Alternaria* leaf blight, one can mention the varieties Hall and Mills, and their derivative forms, the Kinman, Esser, and the high-yielding variety Lewis as well [60]. The variety Lewis is selected in F_8 from the cross of the line T64001-12-1-B-3-2-B-2 (Brooks × Mills) with the sample PI 338780-B from India [61]; the variety Santa Cruz [62] has an identical ancestry. Cultivation of genetically homogeneous varieties accelerated adaptable microevolution of the pathogen. There were already reports that the varieties Brooks, Hall and Mills began to be severely affected by the causative agent of bacteriosis [63]. The lineage of the guar varieties resistant to diseases from India is not discussed in the literature. Besides the above-mentioned varieties, resistance to *Alternaria* leaf blight was revealed in samples HFG-14, HFG-236, HFG-516, HFG-522, HFG-530, HFG-554 [64], CVS, RGC-619, RGC-677 and RGC-679 [65], HG-182 [66]. It is reported about resistance to diseases of samples RGC 986, RGC 1003, RGC 1002, RGC 1017, RGM 112 [67]. Resistance to the anthracnose causative agent *Colletotrichum capsici* f. sp. *cyamopsicola* (Desai & Prasad) is noted for the variety RGC 673 [68].

The overwhelming majority of selection and genetic works has been carried out in India until now, where the most extensive collection of guar is put together (about 5 thousand accessions). Intraspecific crosses and phenotypic selection are most often applied. Guar has highly variable morphological traits [69, 70], but the diversity of its cultivated varieties for the genes of resistance to phytopathogens is low [71].

The most widespread harmful organism, the causative agent *X. axonopodis* pv. *cyamopsidis*, is shown to differentially interact with host-plant genotypes. The problem of overcoming of plants resistance due to the spreading of new intraspecific forms of harmful organisms is relevant in the case of other economically important varieties. It means that for the prevention of epiphytotics and mass reproduction of pests, it is necessary to grow varieties with different resistance genes. The prospective approach for dilating of genetic diversity is introgression of resistance genes from the wild varieties *C. senegalensis* Guill. & Perr. and *C. serrata* Schinz. [71]. There are also some means of population control of the harmful organisms based on management of plants populations in space and time: incorporation, strain-change, mosaics of species. Incorporation of different varieties of plants has been popular in India recently.

Guar cultivation together with companion crops essentially reduced the number of harmful pests (leafhoppers, whiteflies, greenflies) on plants. Therefore, at the use of millet as intercropping, the colonization of guar by *A. craccivora* has appeared to be the lowest, and the yield the highest [72].

Companion guar crops, which were located either near to okra *Abelmoschus esculentus* (L.) Moench or as a shelterbelt along the edge and inside the field, caused a decrease in the number of sucking and gnawing depredators of okra, and also attracted entomophages [73]. Intercropping of castor-oil plant (*Ricinus communis* L.) and guar in the ratio 2:1 also significantly reduced the number of harmful pests on castor-oil plant and involved useful entomofauna [74].

Phytosanitary monitoring of guar crops in Russia. In 2017, the authors carried out guar phytosanitary investigations (nurseries, collection study, and ecological testing) at the VIR Kuban experimental station (Gulkevichsky District, Krasnodar Territory) and the analysis of the infected vegetative material. In the beginning of July, the phytosanitary monitoring (shoots) showed the obvious domination of representatives of the family *Aphididae* (aphids) of the order *Homoptera* (homopterous) on juvenile plants. In all nurseries, the episode of a population explosion of black bean aphid *Aphis fabae* Scopoli was observed: the number of pests on some plants exceeded 2 thousand individuals on a propagule. The colonies of peach aphid *Myzus persicae* (Sulzer) and pea aphid *Acyrthosiphon pisum* Harris were also revealed. In the case of strong proliferation, the death of plants was noted. Predators were not found in aphids' colonies; the individuals mummified by parasites were rare. Spreading of the virus infection contamination, which was proliferated by aphids (yellowing and leaves marbling), began. The pathogenic mycoflora was represented mainly by the fungus species *Alternaria* Nees invoking *Alternaria* leaf blight. The beginning of spreading of bacterial spot was revealed. In the end of August (blooming-fructification), it was revealed that after insecticidal treatment there were only individual colonies of aphids on guar. After their mass reproduction, severe focal virus lesion of plants was observed. The analysis of rhizospheric pathogenic mycoflora demonstrated the domination of the fungus species *Verticillium* Nees and *Fusarium* Link. As during the first estimation, two general propagated diseases were revealed, the *Alternaria* leaf blight and bacteriosis; however, epiphytotic development was characteristic only for the last one. Mass wilting and death of plants of some samples were noted [75]. In three independent experiments, the collection guar samples were estimated in bacteriosis epiphytotics. The highest resistance to disease was revealed in k-52569 (Pakistan), k-52575 (USA) and k-52580 (India). Some forms were selected only in one of the experiments, which is probably conditioned by the heterogeneity of the samples. Apparently, lines resistant to the disease can be selected from the majority of collection samples [75].

Thus, the most harmful guar disease, in Russia as well, is bacteriosis. Sources of resistance to the pathogen are revealed in India and on the American continent, but the term of their use is circumscribed owing to the specificity of parasite-host interrelation. *Alternaria* leaf blight and vascular root rot are among the potentially dangerous diseases. Among phytophages, greenflies are the most harmful, as they are vectors of the viral infection. Infection by pathogens and colonization by phytophages induces defense response in guar plants. Because of differential interaction of harmful organisms with genotypes of a host, cultivation of genetically homogeneous varieties leads to mass reproduction of pests and diseases epiphytotics. Therefore, it is necessary to involve in the selection as many varieties as possible. In cultivation, it is better to alternate in time varieties with different resistance genes, to use mosaics (cultivation of many varieties with unequal resistance genes in the pathogen geographic range) and mixed varieties (the approach which was well proved against harmful pests). In breeding, it is perspective to create multilinear varieties (mechanical admixtures of phenotypic similar lines with unequal resistance genes) and pyramiding (merging of various

resistance factors in one genotype). It should be noticed that genetic researches of plant resistance and intraspecific variability of pathogens are still insufficient. In Russia, they became more dynamic within the frame of the project of creation of guar varieties with complex resistance (Vavilov All-Russian Institute of Plant Genetic Resources). In this program, the search for molecular markers and genes-candidates of economically valuable traits is parallel to sequencing allelic gene variants encoding guar resistance to diseases and pests.

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Pho1a GENE FRAGMENT VARIABILITY IN TUBER-BEARING AND NONTUBER-BEARING POTATO SPECIES (*Solanum* subgenus *Potatoe*) AND *S. tuberosum* L. CULTIVARS

E.O. SHMELKOVA[1], M.A. SLUGINA[1], A.A. MELESHIN[2], E.Z. KOCHIEVA[1]

[1]*Research Center of Biotechnology RAS,* Federal Agency for Scientific Organizations, 33/2, Leninskii prospect, Moscow, 119071 Russia, e-mail shmelkoffa@gmail.com (✉ corresponding author), ekochieva@yandex.ru, mashinmail@mail.ru,
[2]*Lorkh All-Russian Research Institute of Potato Farming,* Federal Agency for Scientific Organizations, 23, ul. Lorkha, pos. Korenevo-1, Lyubertsy Region, Moscow Province, 140051 Russia, e-mail a-mela@mail.ru
ORCID
Shmelkova E.O. orcid.org/0000-0002-1046-7742
Slugina M.A. orcid.org/0000-0003-1281-3837
Meleshin A.A. orcid.org/0000-0002-6018-3676
Kochieva E.Z. orcid.org/0000-0002-6091-0765
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Abstract

Starch is the main metabolite in potato tubers. Therefore structure and functional analysis of starch metabolism genes are of fundamental and applied interest. The final starch amount in sink organs (fruits, seeds and tubers) depends not only on the amylose and amylopectin synthesis, but also on the catabolic enzymes activity. Proteins that participate in starch biosynthesis are rather well studied, while the starch degradation reactions are not fully understood. To date, more data on the crucial role of starch-phosphorylases in this process have been reporting. Starch phosphorylases are widespread among plant species, but the coding genes structure and genetic diversity remain unclear. In potato tubers starch is cleaved by L-form of starch phosphorylase encoded by the *Pho1a* (*STP23*) gene. In the current work *Pho1a* gene fragment (exon II—exon IV) variability was analyzed for the first time in 15 wild and 81 cultivated potato accessions. The chosen gene fragment corresponds to the regulatory part of the glycosyltransferase domain and comprises glucose-6-P binding site, pyridoxal phosphate cofactor binding site and active site (glucose binding). The nucleotide and amino acid polymorphism is determined. A total of 96 potato accessions were used for allelic diversity analysis: 15 wild species from *Potatoe* and *Estolonifera* subsections (where *S. etuberosum* is a nontuber-bearing species), 67 cultivated potato varieties and 14 breeding lines of *S. tuberosum.* Nuclear DNA was isolated from young leaves using potassium-acetate method with phenol-chloroform additional purification. Primer combination Pho2F (5′-CTGAACATGAAGCAAGCGTA-3′)—Pho4R (5′-GGCTATGGACTTAGGTACA-3′) was designed for chosen fragment amplification. The sequences of all varieties and breeding lines of *S. tuberosum* had a length of 670 bp. The length of the obtained *Pho1a* sequences in species ranged from 666 bp (*S. vernei*, *S. lignicaule*) up to 672 bp (*S. pinnatisectum*). Totally 59 SNPs were detected, 15 of them localized in exons. It allowed us to identify 11 allelic variants, moreover 9 of them were found in wild species. Cultivated potato *S. tuberosum* has two allelic variants. The *Pho1a_A2* allelic variant was observed in the majority of analyzed potato cultivars and all the breeding lines. Interestingly, the same variant was detected in some wild potato species, belonging not only to superseries Rotata, but also to Stellata that is considered to be more ancient. The *Pho1a_A10* allelic variant was found in 9 cultivars (Bintje, Red Scarlett, Ushkonir, Karasaiskii, Aurora, Aladin, Chernskii, Plamya, Udacha). The *Pho1a* gene fragment translation revealed that 3 out of 15 exonic SNPs led to amino acid substitutions. In potato cultivars with the *Pho1a_A10* allelic variant neutral M139I substitution was detected**.** The other neutral substitutions M139L and T157S were identified in *S. circaefolium* and *S. vernei,* correspondingly. The only radical substitution R212S was detected in nontuber-bearing *S. etuberosum*. The potential role of the found amino acid substitutions in the functional protein domain requires the further investigation. Further search for the allelic variants associated with starch content in tubers can be used in potato breeding programs.

Keywords*:* starch phosphorylase, *Pho1a*, nucleotide and amino acid variability, wild spe-

cies, potato cultivars, *Solanum* subgenus *Potatoe*

Starch can be considered the main source of carbon and energy in plant cells. The final starch amount in heterotrophic organs (fruits, seeds, and tubers) depends not only on the synthesis of starch components, the amylose and amylopectin, but also on the activity of catabolic enzymes. Starch biosynthesis enzymes are rather well studied, while the starch degradation reactions are not fully understood [1]. The catabolism in the leaves is described in more details; some data proves catabolism in the endosperm of cereals, and less information is obtained for potato tubers [2].

In general, the starch-destroying enzymes can be divided into two categories: hydrolytic (ES 3.2.1.1, ES 3.2.1.2 amylases, 4-α-glucanotransferase ES 2.4.1.25, maltase ES 3.2.1.20, isoamylase ES 3.2.1.68) and phosphorolytic (α-glycan phosphorylase ES 2.4.1.1, maltose phosphorylase ES 2.4.1.8, α-glucan-H_2O-dikinase ES 2.7.9.4, phosphoglucan-H_2O-dikinase ES 2.7.9.5) [3-5]. Their comparative activity may vary depending on the plant stage of development or environmental conditions. Which of enzymes groups has bigger importance is a rather controversial issue. It is supposed that the process itself has been initiated due to glycans phosphorylation that makes the starch grains surface more hydrophilic and, thus, more accessible to hydrolytic enzymes, creating selective protein-carbohydrate and protein-protein interactions additionally [5-8].

Starch phosphorylases, the plant analogs of α-glycan phosphorylases (ES 2.4.1.1), need more attention among other phosphorolytic enzymes [9]. The fundamental role of starch phosphorylase is in the catalysis of starch decomposition due to the replacement of carbon with phosphorus in the glycoside bond with the formation of glucose-1-monophosphate [10, 11]. However, in the case of phosphate lack, starch phosphorylases can carry out the reverse reaction of starch synthesis [12].

Starch phosphorylases are widespread among plant species [13]. The presence of many isoforms is a characteristic feature of starch phosphorylases, which differ in kinetic properties and localization in the cell [13]. Most higher plants with known genomes and transcriptomes have two types of starch phosphorylases, the plastidic (Pho1/L-form/L-SP) and cytosolic (Pho2/H-form/r H-SP) [14]. The plastid protein of about 105 kDa has low affinity to branched glycans, while the cytosolic form of about 90 kDa is characterized by high affinity to linear and branched glycans and even to heteroglycans [15-17]. For the first time, various forms of plant starch phosphorylases have been found in potato tubers (*Solanum tuberosum* L.) and pea seeds (*Pisum sativum* L.) [18, 19]. Structure and genes polymorphism of starch phosphorylases are widely studied among the representatives of the Monocotyledons class: barley (*Hordeum vulgare* L.) [20], rice (*Oryza* sp.) [21], corn (*Zea mays* L.) [6, 22, 23]. At the same time, very few similar data are obtained for the Dicotyledons: the full-size sequence of the yam gene [24] and the cDNA of four potato varieties [25] are known.

Starch is the main metabolite in potato tubers; therefore, the structural and functional analysis of the genes of starch phosphorylases and evaluation of their variability is of not only fundamental but also applied interest because it can shed light on the function of these proteins, as well as be used in the selection of new varieties with high starch content, resistant to cold-induced sweetening.

Starch decomposition in potato tubers is carried out due to the L-form of starch phosphorylase, which is encoded by the *Pho1a* gene (*STP23*). The full-size sequence of this gene of potato is unavailable now. The NCBI GenBank contains only the corresponding *S. tuberosum* mRNA (NM 001288286.1); however, it is known that the gene is located on chromosome 3 [26], has a length of

16.4 kbp and consists of 15 exons and 14 introns [27].

In the present paper, the allelic polymorphism of the fragment of the *Pho1a* gene from exon II to exon IV on the broad samples, including both wild species of potatoes and the varieties and lines of *S. tuberosum* was analyzed for the first time. As a result, we identified 11 allelic variants that can be used further in breeding programs.

The work objective of the present investigation was to assess the variability of the starch phosphorylase *Pho1a* gene in the area of exons II-IV in tuber-bearing and non-tuber-bearing potato species (*Solanum*, the *Potatoe* subgenus), as well as in domestic and foreign varieties and lines of cultivated species of *S. tuberosum*.

Techniques. To investigate allelic polymorphism, 96 samples of potato were selected, including 15 wild species of the *Potatoe* and *Estolonifera* subsections, one of which was non-tuber-bearing (*S. etuberosum*), 67 varieties and 14 lines of cultivated potato of *S. tuberosum*. The sequence of exons of rice *Oryza sativa* (AK063766.1), corn *Zea mays* (NM 001309854.1), barley *Hordeum vulgare* (JQ277327.1), and yam *Ipomoea batatas* (L.) Lam. (M64362.1) available in the GenBank NCBI database were taken for additional evaluation of polymorphism. Seeds of wild species were obtained from the collections of Vavilov Institute of Plant Industry (Saint Petersburg, Russia) and CGN (Centre for Genetic Resources, Wageningen, the Netherlands). Varieties and lines are provided by Lorch Potato Research Institute (the Moscow Province, Russia).

Nuclear DNA was isolated from young leaves with the potassium acetate method with additional purification with the phenol-chloroform mixture [28]. Primer combination Pho2F (5′-CTGAACATGAAGCAAGCGTA-3′)–Pho4R (5′-GGCTA-TGGACTTAGGTACA-3′) was designed for chosen fragment amplification. The reaction mixture for PCR contained 1× buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 M $MgCl_2$, 20 mM dNTPs), 10 μm of the respective primer, 0.25 U of Taq DNA polymerase (Dialat Ltd, Russia) and 100 ng of potato genomic DNA. The temperature-time profile of PCR was as follows: the first cycle — 5 min at 95 °C; 30 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C (35 cycles); the final elongation — 1 min at 72 °C. PCR was carried out with commercial reagents (Dialat Ltd, Russia) in an amplifier Bio-Rad C1000 (Bio-Rad Laboratories, Inc., USA).

PCR products were visualized by electrophoresis in 1% agarose gel LE 2 Agarose (Helicon, Russia) with 1× TBE buffer stained with ethidium bromide, and documented in a BioDocII system (Biometra GmbH, Germany). Amplification products were sequenced with the same primers in the Bioengineering CCU on the platform Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, USA).

For alignment and analysis of nucleotide and amino acid sequences polymorphism, MEGA 7.0 software was used (https://www.megasoftware.net/) [29]. The sequence of *S. tuberosum* mRNA from the GenBank NCBI database (NM 001288286.1) was the reference. The functional effect of amino acid substitutions was calculated with Provean program (http://provean.jcvi.org/index.php).

Results. To analyze the polymorphism of the *Pho1a gene*, a fragment from exons II to IV encoding the N-terminal regulatory part of the functional glycosyltransferase domain (ID 10136827) was selected, which includes glucose-6-phosphate binding site, pyrodoxal phosphate binding site and the active binding site for glucose [20]. The selection of sites for primers annealing was based on the possibility of the *Pho1a* amplification in phylogenetically distant species, as well as the ability to discriminate the target isoform of the gene from paralogs of *Pho1b* and *Pho2*. The gene fragment was amplified and sequenced with the

help of the developed Pho2f-Pho4R primer pair.

Sequences of all varieties and breeding lines of *S. tuberosum* had the same length, 670 bp. The length of the obtained *Pho1a* sequences in species ranged from 666 bp (*S. vernei*, *S. lignicaule*) up to 672 bp (*S. pinnatisectum*). The sequence lengths varied due to the presence of insertions and deletions localized in introns only.

In total, 59 point nucleotide substitutions (single nucleotide polymorphisms, SNPs) were detected among 96 potato samples under investigation; 58 of these SNPs were found in wild species sequences. The joint degree of polymorphism for the entire selection was 8.75%, and for a set of tuber-bearing and non-tuber-bearing potato varieties was 8.6%. Only two polymorphic sites (0.29%) were found in the analyzed varieties of the cultivated potato *S. tuberosum*, the breeding lines had no substitutions at all (Table 1). The great majority of substitutions were localized in the intron sequences. The variability of the studied exon sequences was 5.95% due to the presence of 15 SNPs, 14 of which were found in the species and only one replacement was found in the varieties of *S. tuberosum*.

1. The number and proportion of variable sites in the composition of the studied *Pho1a* sequences (II-IV exons) of wild species as well as varieties and lines of *Solanum tuberosum*

Analyzed sequence	Wild species and the Prior variety	Varieties	Breeding lines	All samples
Full-length gene	58 (8.60 %)	2 (0.29 %)	0	59 (8.75 %)
cDNA	14 (5.55 %)	1 (0.39 %)	0	15 (5.95 %)
Protein	3 (3.57%)	1 (1.19 %)	0	3 (3.57 %)

Based on the presence of the above-mentioned substitutions in the exon sequences, it is possible to distinguish 11 allelic variants (Table 2 submitted online on the journal website http://www.agrobiology.ru). Nine of them are typical for wild species, and seven are unique to the selection under investigation. The *Pho1a_A1* allelic variant was typical only for the non-tuber-bearing variety *S. etuberosum*; however, despite its evolutionary distance from tuber-bearing species [30], the differences were of only one SNP. The multiple SNPs that distinguish the species of the *Rotata* superseries from the evolutionary more ancient species of the *Stellata* superseries were not identified as well (see Table 2 on the journal website http://www.agrobiology.ru). Moreover, some *Stellata* species (*S. polyadenium*, *S. chacoense*, *S. lignicaule*) had the same allelic variant *Pho1a_A2* that the majority of the analyzed cultivars of *S. tuberosum*.

The analyzed sequences of the cultivated potato *S. tuberosum* were represented by two allelic variants. The *Pho1a_A10* allelic variant was found in nine varieties of Russian and foreign selection (Bintje, Red Scarlett, Ushkonir, Karasaiskii, Aurora, Aladdin/Aladin, Chernskii, Plamya, Udacha). Varieties differed from wild species with the presence of nucleotide substitution G150A (the index corresponds to the number in the sequence of cDNA).

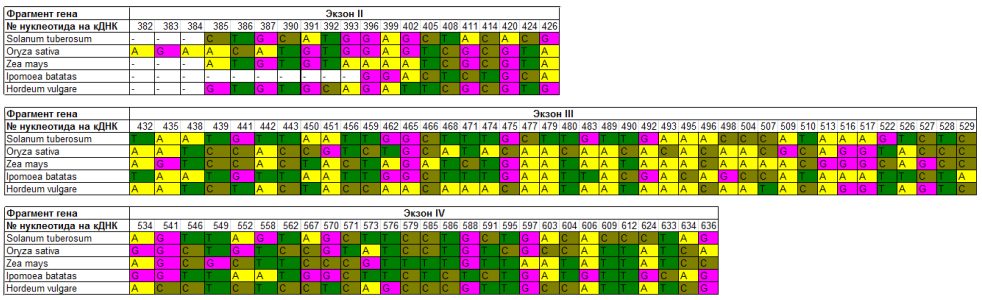
All 14 potato breeding lines were monomorphic in the *Pho1a* fragment and had one common *Pho1a_A2* allele with the majority of varieties, which was also found in a number of wild species (see Table 2). The same allelic variant of *Pho1a_A2* in wild species and most varieties can most likely be explained by the use of wild species samples in crossbreeding. For example, Charodei, Kholmogorskii, Sudarinya, Ocharovanie, and Sirenevyi tuman varieties – hybrids obtained with the use of *S. demissum*, *S. vernei*, *S. stoloniferum*.

The attempt to associate the presence of a certain allelic variant with the qualitative characteristics of tubers (starch content, cold resistance, cold-induced sweetening) was not successful.

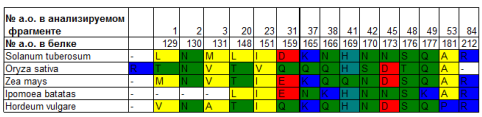
It is quite difficult to carry out a comparative analysis of the polymorphism of this part of the starch phosphorylase gene of potato and other plants since the *Pho1a* sequence is studied in a small number of monocotyledonous species [6, 20-23], and for dicotyledons, such information is extremely limited [24]. The polymorphism of the investigated plot of wild and cultivated tomato species was studied previously (*Solanum*, the *Lycopersicon* section). In total, 24 SNPs were detected in the composition of such sequences in 11 tomato species, 7 of them in the sequences of exons, 17 in the sequences of introns, the total polymorphism was 3.6% (M.A. Slugina, unpublished data). It is interesting that three species of wild potato (*S. limbaniense*, *S. bulbocastanum*, *S. pinnatisectum*) had indels common for all types of tomato in their introns sequences, which again reveal the phylogenetic relationships of tomato and potato.

Comparison of the *Pho1a* fragment (exons II-IV) in the studied potato samples with sequences in other species taken from the NCBI database showed a rather conservative structure of this gene area in the analyzed monocotyledonous and dicotyledonous plant species. At the same time, the homology of primary exon sequences was rather high, and that of intron sequences was very low. In the investigated area, the localization of introns coincided, but their lengths differed. The analysis revealed 87 SNPs (33.98%) (Fig., A, posted on the journal website http://www.agrobiology.ru). The greatest homology (89.45%) was in potato with yam. The most distant were the sequences of the *Pho1a* fragment of potato and corn (23.05%).

A



B



SNPs in exon sequences of the *Pho1a* gene fragment (exons II-IV) in the species *Solanum tuberosum* L. (NM 001288286.1), *Oryza sativa* L. (AK063766.1), *Zea mays* L. (NM 001309854.1), *Ipomoea batatas* (L.) Lam. (M64362.1), *Hordeum vulgare* L. (JQ277327.1) (A) **and substitutions of amino acids in the analyzed area of the protein** (B)**.** Gene fragment and nucleotide position on cDNA (A) and a.a.r. position in the analyzed fragment and in the protein (B) are indicated.

The Pho1 protein has a size of 966 a.a.r. and consists of four sections: N-terminal transit peptide, which delivers protein to plastids, a regulatory domain, central insertion L78 (distinguishes this isoform from Pho2), and a catalytic domain. The regulatory domain L78 and the catalytic domain form a functional glycosyltransferase domain, the function of which is to transfer sugar fragments from the activated donor molecule to the acceptor molecule, followed by the formation of a glycoside bond [20, 25]. When aligning the sequences of known starch phosphorylases of mono- and dicotyledonous plants, it is shown that the regulatory and catalytic domains are the most conservative [20].

Encoding sequences of the studied fragment (exon II—exon IV) of potato starch phosphorylase *Pho1a* gene were translated, which allowed estimating

their amino acid variability. The sequence available for analysis was 84 a.a.r. (positions from 129 to the 212 a.a.r.); it was a part of the regulatory domain and included glucose-6-phosphate and pyridoxal phosphate-binding sites, as well as the active binding site with glucose [20].

Only four of the 15 exon SNPs in the analyzed fragment led to the substitutions of amino acid residues. At the same time, A/T_{31} and G/T_{33} were parts of the same codon and led to the substitution of the same amino acid position: at position 139, methionine was replaced by isoleucine (M139I) or leucine (M139L). Substitution of M139I was typical for a group of varieties of Russian and foreign selection. Among them are Ushkonir, Karasaiskii, Aurora, Bintje, Chernskii, Red Scarlett, Aladin, Plamya, Udacha. Amino acid polymorphism was not found in other studied varieties and breeding lines. It is interesting that for the previously cloned and sequenced full-size cDNA of potato cultivars Diana, Theresa, Saturna, and Satina, amino acid polymorphism on the plot of the second and fourth exons has not been identified [25]. That is, the presence of variants of the sequence of starch phosphorylase is shown on a wider sample of varieties and breeding lines of *S. tuberosum* presented in this article.

Significant variability of the Pho1a fragment has been identified in the wild species. The substitution M139L typical for wild tuber-bearing *S. circaefolium* was detected. The amino acid substitution T157S unique for the selection was found in the *S. vernei* sample. However, according to the calculations in the Provean program [31], both substitutions are neutral and do not lead to a change in protein charge. R212S substitution in non-tuber-bearing *S. etuberosum*, in contrast to the above-mentioned, is the radical that may cause changes in the Pho1a protein structure and thus affect its functionality.

Alignment of the obtained amino acid sequences with translated sequences (exons II-IV) in rice, corn, barley, and yam revealed 14 amino acid substitutions, among which three were radical (see Fig. B, http://www.agrobiology.ru).

In general, the extremely low amino acid variability of the analyzed Pho1a regulatory domain in wild potato species and varieties is consistent with the data obtained for starch phosphorylases of other plant species [20], which indicates the important role of this domain for binding to substrates and cofactors, and thus explains the preservation of its conservatism during evolution. Amino acid substitutions identified in the regulatory domain can potentially affect the activity of the enzyme. In the future, it is necessary to continue the search for associations of detected substitutions with starch content in tubers, taking into account the prospects of their use in breeding programs.

Thus, the analysis of the area of *Pho1a* gene from exons II to IV in 15 wild species, 67 samples of cultivated potatoes and 14 breeding lines is carried out and description of its nucleotide and amino acid polymorphism is created on the basis of these data. Despite the conservatism of the studied exons, 59 nucleotide substitutions were described on this fragment, 15 of which were localized in exons, and three led to amino acid substitution. The unique replacement SNP636, leading to the substitution of arginine with serine R/S, found in non-tuber-bearing *S. etuberosum*, is radical and can potentially cause a change in protein conformation. In total, 11 allelic variants are determined. Further search for their associations with the starch content in potato tubers can be used in breeding programs. The influence of the identified amino acid substitutions in a functionally significant area and their potential impact on the activity of the starch phosphorylase enzyme requires further study.

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MANIFESTATION OF PRODUCTIVITY TRAITS IN *Triticum aestivum/T. timopheevii* INTROGRESSION LINES IN DIFFERENT ENVIRONMENTAL CONDITIONS

S.I. VAKULA[1], O.A. ORLOVSKAYA[1], L.V. KHOTYLEVA[1], I.N. LEONOVA[2]

[1]*Institute of Genetic and Cytology of National Academy of Science of Belarus,* 27, ul. Akademicheskaya, Minsk, 220072 Republic of Belarus, e-mail svettera@yandex.ru (✉ corresponding author), O.Orlovskata@igc.by, L.Khotyleva@igc.by;
[2]*Federal Research Center Institute of Cytology and Genetics SB RAS,* Federal Agency for Scientific Organizations, 10, prosp. Akademika Lavrent'eva, Novosibirsk, 630090 Russia, e-mail leonova@bionet.nsc.ru
ORCID:
Vakula S.I. orcid.org/0000-0002-2242-7107
Khotyleva L.V. orcid.org/0000-0003-0295-5022
Orlovskaya O.A. orcid.org/0000-0002-1187-1317
Leonova I.N. orcid.org/0000-0002-6516-0545
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Abstract

Common wheat lines containing introgression of alien genetic material are an important source and donors of pathogen resistance genes. However, for the effective involvement of lines in breeding, information is needed on their ecological plasticity and productivity in different environments. This paper is the first report on estimates of adaptive responses, stability and breeding value of common wheat lines with alien substitutions and translocations in geographically distant regions. The aim of our investigation was comparative analysis of manifestation of agronomic important traits of common wheat introgression lines containing alien genetic material, when grown in different eco-geographical zones, the Western Siberia (the Russian Federation) and Eastern Europe (the Republic of Belarus). Twenty one fungal disease-resistant *T. aestivum/T. timopheevii* introgression lines (BC_1F_{22-24}, $2n = 42$) from crossing of five common wheat varieties (Saratovskaya 29, Skala, Irtyshanka 10, Tcelinnaya 20 and Novosibirskaya 67) with tetraploid wheat *T. timopheevii* var. *viticulosum* were tested. Field evaluation of the lines and parental wheat cultivars was carried out in 2015 in the conditions of the West Siberian (Novosibirsk Region) and Eastern European (Minsk, Republic of Belarus) agro-climatic zones. The field experiment was arranged in two replicates on 1 m plots, 40-60 grains per row and 20 cm distance between rows, according to the systematic method. The evaluation of the tiller number, plant height, ear length, spikelet number, ear grain number, ear grain weight and 1000-grain weight were carried out according to the methodological recommendations of the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) (for 20-25 randomly selected plants of each line). Our results show conserved ranks of tiller number, ear length, number of spikelets per ear, and ear grain number in five groups of introgression hybrids in two agro-ecological regions, as influenced by growing conditions, while hierarchical structures for plant height, ear grain weight and 1000-grain weight dissociate due to effect of the genotype × environment interaction. In the West Siberian region, hybrid wheat lines, as a rule, overcome parental forms on productivity traits and are below them in height. In the Eastern European region, soft wheat varieties involved in crossings are inferior to the introgression lines created on their basis only on tiller number. The exception was cultivar Scala, which in the conditions of the Republic of Belarus was characterized as medium-sized with high spike-lengths and the number of spikelets per ear, but with low values of the ear grain number and ear grain weight. Approximation of the productivity of introgression lines and their parents by PCA also indicates a significant influence of environmental conditions. Observations corresponding to the Western Siberian region and Belarus constitute two relatively distant dispersion clouds, differing in the degree of overlapping of the areas corresponding to different cross combinations and their positions relative to the parental forms in the PCA space. According to the results of the research, introgression lines created on the base of varieties Sara-

tovskaya 29, Skala and Irtyshanka 10 are recommended as sources of resistance genes without reducing productivity of recipient varieties.

Keywords: *Triticum aestivum,* common wheat, tetraploid wheat *T. timopheevii,* introgression lines, productivity, eco-genetic experiment

Common wheat (*Triticum aestivum* L.) is one of the most important crops, which plays a key role in providing food for people all over the world [1]. To obtain high yields of common wheat, it is necessary to develop varieties which combine high yields with low fluctuations of economically valuable traits when cultivated in regions with different soil and climatic conditions [2-4]. The range of ecological flexibility determines the area of optimal agro-ecological zoning of the variety [5-7].

Increasing productivity and maintaining a stable crop yield in changing environmental conditions depend on various factors, the most important of which are the varieties resistance to biotic and abiotic stressors. Fungal diseases lead to significant losses in yield of winter and spring common wheat: during the period of epiphytotics, the productivity of susceptible varieties decreases by 50-70%. A number of papers associate high yields of modern varieties and breeding samples of common wheat, including lines obtained by the involvement of foreign genetic material, with resistance to fungal pathogens [8-10].

Wild and cultivated relatives of common wheat are promising sources for expanding the genetic diversity of modern varieties for resistance loci. Currently, over 50 genes of resistance to leaf and stem rust, as well as powdery mildew, have been introduced into the genome of common wheat from relatives and cereals from distant taxonomic groups [11]. However, in the case of introgression of alien resistance loci, it is necessary to pay attention to the degree of influence of inherited genetic material on other economically important traits. For many alien genes, determining resistance to leaf-stem infections, there has been a decrease in the productivity of the ear and other yield components during gene transfer to commercial wheat varieties [12, 13].

Information on the ecological flexibility and adaptability of lines in various environmental conditions is needed in order to use introgression lines as sources and donors of fungal pathogen resistance genes. A collection of introgressive lines of common wheat, obtained with the participation of the tetraploid species *T. timopheevii* var. *viticulosum*, was studied earlier. Long-term observations have shown that the lines are characterized by effective resistance to leaf rust and powdery mildew, some of them are resistant to stem rust, septoria, and dust brand; a number of lines have group resistance to disease [14]. Effective genes which determine the resistance of introgression lines to leaf rust [15, 16] were mapped using molecular genetic analysis,

This paper for the first time presents estimates of the adaptive responses, stability, and breeding value of common wheat lines with alien substitutions and translocations, obtained in an eco-genetic study.

The purpose of the study was a comparative analysis of manifestation of economically important traits in common wheat lines containing alien genetic material of *Triticum timopheevii* when grown in Western Siberia of Russia and in the Republic of Belarus, located in different geographical areas.

Technoques. A total of 21 introgression lines of *Triticum aestivum/T. timopheevii* ($BC_1F_{22\text{-}24}$, $2n = 42$), obtained by crossing five varieties of common wheat (Saratovskaya 29, Skala, Irtyshanka 10, Tselinnaya 20 and Novosibirskaya 67) with tetraploid wheat *T. timopheevii* var. *viticulosum* [14] were used. Field testing of the lines and their parental forms was carried out in 2015 under the conditions of the West Siberian (Novosibirsk Province) and East European (Minsk, Republic of Belarus) agroclimatic regions.

The field experiment was laid out in 2 replications on plots with a width of 1 m, 40-60 grains per row and distance between rows of 20 cm, distributed over the plot according to the systematic method. The evaluation of the tiller number, plant height, ear length, spikelet number, ear grain number, ear grain weight, and 1000-grain weight were carried out according to the methodological recommendations of Vavilov All-Russian Institute of Plant Genetic Resources (VIR) (for 20-25 randomly selected plants of each line) [17].

The obtained data were processed with STATISTICA 10.0 software (StatSoft, Inc., USA) and Microsoft Excel package. Descriptive statistics methods (the arithmetic mean M and the standard error of the mean ±SEM were calculated) and nonparametric correlation analysis (the Spearman correlation coefficient r) were used in the study. Radar charts were constructed according to the difference between the mean value of the introgression line trait and the mean value of each parental form trait in two tests. The commonality of each of the variables and its contribution to the main components of variability were evaluated using Principal Component Analysis (PCA).

Results. The climate of Western Siberia is continental and is characterized by long winters and short hot summers. The climate of Belarus is moderately continental and is characterized by frequent Atlantic cyclones, mild and wet winters, warm summers. The comparative analysis of meteorological conditions in 2015 (http://rp5.by) showed that the accumulation rate of the effective heat sum (EHS) in the West Siberian region was 11-15% higher than the values noted for the Eastern European experimental site. In the Novosibirsk Region, EHS for the May-July period exceeded 1600 °C required for the ripening of spring wheat [18], which contributed to the acceleration of growth and reproductive processes, reduction of the crop vegetation period. During the whole period of growth and development of wheat, there was an insufficient amount of precipitation (the lack of precipitation was more pronounced in the conditions of the Republic of Belarus).

The constructed radar charts reflect the difference between the mean value of the introgression line trait and the mean value of each of the parental forms traits during two tests and allow comparing the productivity of the studied wheat samples in the conditions of the West Siberian and East European regions. Plants of the *T. timopheevii* species were characterized by a maximum tiller number (on the average 2.7±0.1 stems in Novosibirsk, 4.7±0.2 stems in Minsk) and in terms of this trait were superior to the *T. aestivum* varieties involved in the crossing and introgressive lines derived from them (Fig. 1). Under the conditions of the West Siberian region, the lines created on the basis of the varieties Saratovskaya 29 and Novosibirskaya 67 were inferior to the parental variety *T. aestivum* in terms of tiller number. Regardless of the growing conditions, reliable transgressions of the trait (in relation to the variety) were shown in combinations based on the Skala and Tselinnaya 20 varieties.

Significant differences were found in the height of plants grown in different agro-climatic zones. Both hybrid forms and initial varieties formed a higher straw under the conditions of the continental climate of Siberia. At the same time, in the West-Siberian region, 75 and 57% of introgressive lines were inferior to the original variety and *T. timopheevii* in height. According to the results obtained in the conditions of the Belarusian experiment, only 48% of introgressive lines were below both parental forms.

T. timopheevii plants formed a short ear (5.3±0.1 and 5.6±0.1 cm, respectively, in the conditions of Belarus and Russia) with a small number of spikelets (10.8±0.7 pcs.) regardless of the conditions cultivation. Its parameters were superior to the ear of Irtyshanka 10 × *T. timopheevii* (see Fig. 1, G, 3.3)

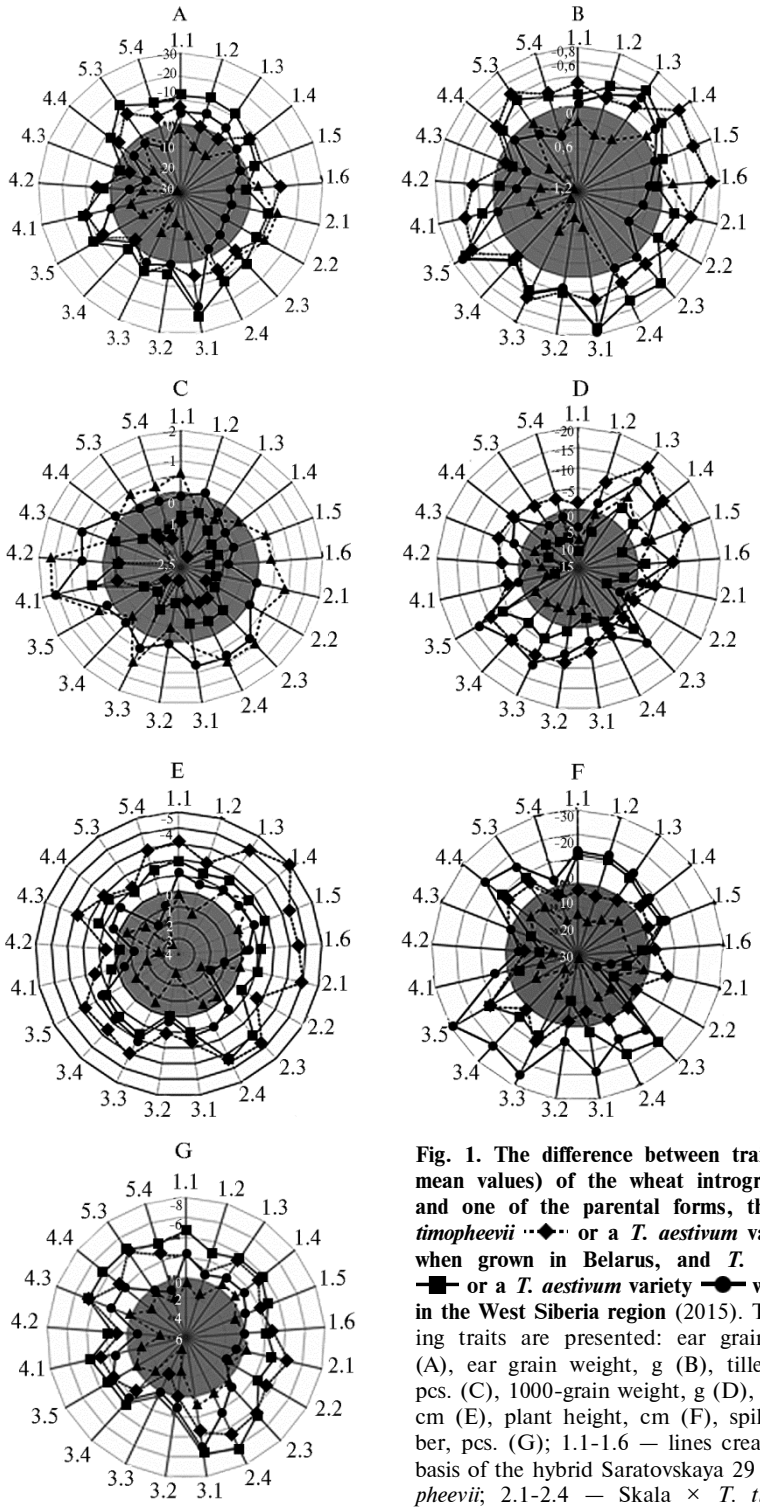

Fig. 1. The difference between traits (by the mean values) of the wheat introgression line and one of the parental forms, the *Triticum timopheevii* or a *T. aestivum* variety when grown in Belarus, and *T. timopheevii* or a *T. aestivum* variety when grown in the West Siberia region (2015). The following traits are presented: ear grains number (A), ear grain weight, g (B), tiller number, pcs. (C), 1000-grain weight, g (D), ear length, cm (E), plant height, cm (F), spikelet number, pcs. (G); 1.1-1.6 — lines created on the basis of the hybrid Saratovskaya 29 × *T. timopheevii*; 2.1-2.4 — Skala × *T. timopheevii*; 3.1-3.5 — Irtyshanka 10 × *T. timopheevii*; 4.1-4.4 — Tselinnaya 20 × *T. timopheevii*; 5.3-5.4 — Novosibirskaya 67 × *T. timopheevii*. The diagram dark sector means that the introgressive line is inferior to the parental form by the mean value of the analyzed trait, the bright sector means that the introgressive line is superior to the parental form by the average value of the analyzed trait.

and Tselinnaya 20 × *T. timopheevii* (see Fig. 1, G, 4.2). Under the conditions of

Belarus, an increase in the ear length compared to the variety was noted in four lines created on the basis of the Saratovskaya 29 variety (the average length of the ear in Saratovskaya 29 was 8.6±0.3 cm, in hybrids it could reach 10.3±0.1 cm). In the West Siberian region, more than half of the lines obtained on the basis of the Skala and Tselinnaya 20 varieties, and all the lines of the Saratovskaya 29 × *T. timopheevi* and Irtyshanka 10 × *T. timopheevi* combinations were significantly superior to the initial wheat variety in terms of the ear length. In the Eastern European region, more than 60% of hybrid lines were inferior to the parent variety in terms of the spikelet number. The trait transgression was noted for six lines (in combination with the Saratovskaya 29, Skala, Irtyshanka 10 and Tselinnaya 20). Introgression of the *T. timopheevii* genetic material into the Novosibirsk 67 variety resulted in a significant decrease in the ear length in hybrids regardless of growing conditions, while the number of spikelets almost did not decrease as compared with that in the cultural variety.

In general, according to two environmental tests, the average ear grain number and ear grain weight (see Fig. 1, A, B) varied from 11.6±0.8 pcs. and 0.4±0.04 g for line 4.3 (combination of Tselinnaya 20 × *T. timopheevii*) to 32.03±2.9 pcs. and 1.13±0.09 g for line 3.1 (Irtyshanka 10 × *T. timopheevii*). Under the conditions of the West Siberian region, *T. timopheevii* plants formed on average 15.6±0.5 ear grains with a weight of 0.61±0.03 g. When grown in Belarus, the seed number of the *T. timopheevii* ear reached 19.4±0.9 pcs., but the average grain weight decreased to 0.59±0.04 g. That is, some of the introgressive lines were inferior to the wild-growing ancestor in terms of the grains number (10 and 20% of samples, respectively, in the conditions of the West Siberian region and Belarus) and the ear grain weight (20 and 15% of samples). In the Eastern European region, seed numbers of the cultural variety were exceeded only by four lines created on the basis of the Skala variety and two lines based on the Saratovskaya 29 variety. In the West Siberian region, transgression by the grains number and grains weight was observed for the combinations of Irtyshanka 10, Saratovskaya 29 and Tselinnaya 20.

The growing conditions affected the 1000-grain weight in the *T. timopheevii* species. While in the West Siberian region, this indicator reached 37.9±0.8 g, exceeding the value of all parental varieties and being inferior to that only in eight introgressive lines (three lines of the combination Saratovskaya 29 × *T. timopheevii*; four lines of Irtyshanka 10 × *T. timopheevii*; one line of Scala × *T. timopheevii*), then in Belarus, the 1000-grain weight in the *T. timopheevii* was only 30.4±1.3 g, which is lower than in the majority of varieties and introgressive lines grown under the same conditions. In terms of the 1000-grain weight, 76.2% of the wheat introgressive lines in the West Siberian region and 9.5% of the lines in the conditions of Belarus were superior to the cultural varieties. It is possible that this is due to the difference in the size of the seeds that form the parental forms when growing conditions are changed.

Reliable correlations of the mean values of the four traits in two growing regions are shown for the five hybrid combinations (Fig. 2). Compared with the West Siberian region, there was an increase in the tiller number by 87.0%, in the number of spikelets by 17.0%, in the ear length by 15.0%, in the number of grains by 2.6% under the conditions of Belarus. Lines on the basis of the Saratovskaya 29 variety, which were undersized under the conditions of Belarus, were characterized by the maximum height of the stem in the West Siberian region. Compared with the Belarusian experiment, the lines created with the involvement of the Scala and Irtyshanka 10 varieties showed an increase in the ear grains weight and 1000-grain weight when grown in the West Siberian region. However, under the conditions of Novosibirsk, the seed number of the lines cre-

ated on the basis of the Saratovskaya 29, Tselinnaya 20 and Novosibirskaya 67 varieties decreased.

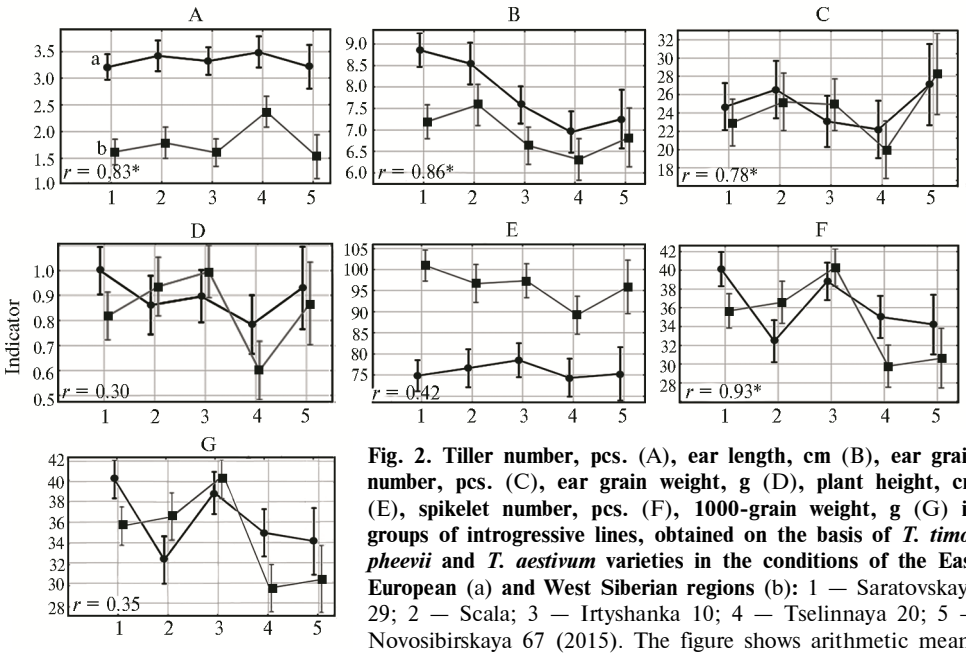

Fig. 2. Tiller number, pcs. (A)**, ear length, cm** (B)**, ear grain number, pcs.** (C)**, ear grain weight, g** (D)**, plant height, cm** (E)**, spikelet number, pcs.** (F)**, 1000-grain weight, g** (G) **in groups of introgressive lines, obtained on the basis of *T. timopheevii* and *T. aestivum* varieties in the conditions of the East European** (a) **and West Siberian regions** (b)**:** 1 — Saratovskaya 29; 2 — Scala; 3 — Irtyshanka 10; 4 — Tselinnaya 20; 5 — Novosibirskaya 67 (2015). The figure shows arithmetic means with confidence intervals (95%); *r* is the Spearman correlation coefficient between the mean trait values of the wheat introgression line grown in the West Siberian region and Belarus; the asterisk indicates correlation coefficients statistically significant at $p < 0.05$.

The Principal Component Analysis (PCA) estimates the commonality of each of the variables and its contribution to the main components of variability. The PCA is used to classify introgressive lines and parental forms according to a set of phenotypic traits and comparative analysis of productivity when grown in two agroclimatic regions (Fig. 3). The method reduced the number of variables describing wheat productivity to three main components (eigenvalues higher than unity). The first two PCA factors together provided 66.2% of the variance, the third factor explained additional 15.5%. For interpretation of the obtained solution, a two-factor PCA model which reproduces commonalities for six traits of productivity 0.60-0.89 (dispersion share for a given number of factors) was selected. Based on the factor loadings, the first main component explained the variability of the main ear traits (length, number of spikelets, number and weight of ear grains). The tiller number and the height of the plant correlated with the second selected component, which allowed interpreting this factor as the plant habit. The addition of the third main component to the analysis significantly increased the commonality of the solution only for the 1000-grain weight (commonality of the trait was 0.28 for the two main components, 0.85 for the three main components) and did not affect the sample classification diagram.

PCA evaluation of the productivity of samples of introgressive lines and their parental forms grown in Western Siberia and Eastern Europe indicated a significant effect of growing conditions. The results of observations, corresponding to the Novosibirsk Region and Belarus, form two dispersion clouds with the centers of gravity in the second and fourth quarters of the graph. Regional variants of the experiment could be divided by a diagonal line through the third and first quarters of the graph (see Fig. 3). There was an increase in the values of tiller number, plant height, size and yield of the main ear along this line. Projec-

tions of the individual introgressive lines onto the PCA plane were combined into planar clusters corresponding to the same crossing combination tested in each of the growing regions. The clusters position was correlated with the projections of regional variants of tested parental forms (*T. timopheevii*, Saratovskaya 29, Scala, Irtyshanka 10, Novosibirskaya 67, Tselinnaya 20).

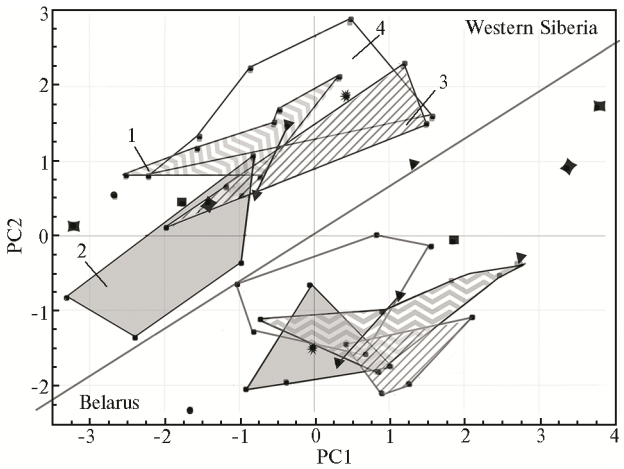

Fig. 3. The projection of observations of introgressive lines of common wheat Saratovskaya 29 × *T. timopheevii* (1), Tselinnaya 20 × *T. timopheevii* (2), Scala × *T. timopheevii* (3), Irtyshanka 10 × *T. timopheevii* (4), Novosibirskaya 67 × *T. timopheevii* (▼▼) and parental forms *T. timopheevii* (●), Saratovskaya 29 (■), Tselinnaya 20 (◆), Novosibirskaya 67 (▲), Scala (✷), Irtyshanka 10 (⚑) onto the two first PCA components (2015). The first main component: eigenvalue is 2.8, the variance explained is 39.8%; the second main component: eigenvalue is 1.9, the variance explained is 26.4%.

The total area of clusters overlapping and binding of the overlapping area to the analyzed samples for the conditions of Western Siberia and Belarus differed. The Belarusian experiment was characterized by greater compactness of the clusters, a wider range of spread and remoteness of the parental forms from the analyzed hybrid combinations. Under the conditions of the West Siberian region, the projections of parental wheat varieties were close to the region of variation in the group of introgressive lines based on the Tselinnaya 20 and Scala varieties. In the West Siberian experiment, the *T. timopheevii* fell into the range of variability of the hybrid combination based on the Saratovskaya 29 variety. The parent *T. timopheevii* showed lower values of the ear length, the number of spikelets and the number of seeds compared to the groups of lines created with the involvement of the Tselinnaya 20, Irtyshanka 10, Novosibirsk 67 and Scala varieties. The *T. timopheevii* species grown under the conditions of Belarus was characterized by a low value of the two main components (coordinates) compared to the varieties involved in crosses, which placed the clusters of introgressive lines of one crossing combination on the vector connecting the projections of the parental forms (see Fig. 3).

Factor analysis and its variants (principal component analysis, biplot analysis, etc.) are convenient for estimating components of a variety—year—growing conditions system, since they allow simultaneously estimating the variance and interrelation of a large number of traits, as well as reducing the number of experimental parameters to several factors explaining the observed variation of traits [19, 20]. Thus, Yu.F. Osipov et al. [21] identified five auto-compensatory systems based on factor analysis of 44 indicators of the winter wheat agrophytocenosis, which allowed determining the effect of photosynthetic activity, mineral nutrition and planting density on wheat productivity. In another paper, diversification of spring common wheat varieties according to the specificity of the genotype-environment response was carried out and the most productive and resistant varieties were identified for four sites of state variety testing in the Samara Region using the biplot analysis [22]. These approaches demonstrate a wide range of factor analysis capabilities for variety testing in specific soil and climatic conditions.

Currently, in addition to bioinformatics analysis methods, it has become possible to use molecular genetic methods that allow the dissection of economically important traits and identify minor loci (quantitative trait loci, QTLs) with various effects. As a result of an integrated approach, it is possible to identify genotypes containing combinations of QTLs in the genome, which make a positive contribution to the phenotypic manifestation of a number of traits under various environmental conditions [23-25].

Selection of the main factors determining the formation of the wheat yield in various ecological and geographical conditions allows creating varieties with high ecological flexibility, which has been convincingly demonstrated for various types of crops, including wheat [26, 27]. In this experiment, the principal component analysis used in the analysis of seven productivity traits allowed differentiating introgressive lines and parental forms of wheat grown in the conditions of the Republic of Belarus and in the West Siberian region, to distinguish two signs describing the main ear productivity and plant habit, and also to identify groups of introgressive lines that significantly change productivity depending on growing conditions (hybrids based on Irtyshanka 10 and Saratovskaya 29). It should be noted that in the case of parental forms and hybrids, in which productivity elements are less dependent on uncontrollable factors (as was observed, for example, in *T. timopheevii* parent plants), grain yield should be increased due to changes in the elements of crop cultivation technology.

The results obtained in the study show the prevailing influence of ecological and geographical conditions on the manifestation of economically important traits. At the same time, the most significant differences were noted for the tiller number, plant height, and ear length. Previously, using factor analysis of data on the assessment of lines in the conditions of the Novosibirsk Region, it was found that the genotype contribution to the phenotypic differences between lines and initial varieties is insignificant [28]. The identified fluctuations in the traits between groups of lines created on the basis of different common wheat varieties may be associated with a different response of the variety-founder to weather conditions, as shown by other researchers for both commercial varieties and introgressive lines [29-31].

Thus, introgression of the *Triticum timopheevii* genome in the *T. aestivum* varieties leads to a significant diversification of introgression lines according to a set of productivity traits. The degree of manifestation of the observed diversification depends on the growing conditions of the samples. In the West Siberian region, the hybrid lines of wheat, as a rule, are superior to the parental forms in terms of ear productivity and are inferior to them in terms of plant height. In the Eastern European region, the varieties involved in crossings are inferior to the introgressive lines created on their basis only in terms of tiller number. The exception is the Scala variety, which, under the conditions of the Republic of Belarus, is characterized as medium-grown with high values of ear length and the number of spikelets, but low values of the number and weight of ear grain. According to the study, introgression lines created with the involvement of the Saratovskaya 29, Scala and Irtyshanka 10 varieties are distinguished by higher adaptability to the stressful environmental conditions and can be recommended as sources of resistance genes that do not cause a decrease in the productivity of the recipient varieties.

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USE OF MORPHOPHYSIOLOGICAL MARKERS IN INTRASPECIFIC POLYMORPHISM ANALYSIS OF FLAX *Linum usitatissimum* L.

K.P. KOROLEV, N.A. BOME

Tyumen State University, 6, ul. Volodarskogo, Tyumen, 625003 Russia, e-mail bomena@mail.ru (✉ corresponding author), corolev.konstantin2016@yandex.ru
ORCID:
Korolev K.P. orcid.org/0000-0001-9595-3493 Bome N.A. orcid.org/0000-0002-8496-5365
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Abstract

In worsen environmental conditions sustainable agriculture and high quality food production rely on crop diversity and adaptiveness that requires the improved estimates of plant parameters. Flax which is among the recognized promising crops is presently rare in the Tyumen region. Our paper shows the most informative morphometric criteria of adaptiveness for several domestic and foreign flax varieties studied under local conditions. The criteria are for the first time improved by a rapid noninvasive method of chlorophyll measurement in leaves with a SPAD 502 optical counter. The purpose of this work was to estimate response of *Linum usitatissimum* L. genotypes to the environmental factors and to identify signs for use as indicators of adaptability. A total of 20 collection samples of flax of different ecological and geographical origin from Russia (6 samples), Belarus (7 samples), Czechia (2 samples), Ukraine (1 sample), France (1 sample), Canada (1 sample), Australia (1 sample), and Germany (1 sample) were used. Laboratory and field tests were conducted in 2016-2017. Seeds were germinated in Petri dishes on a filter paper moistened with distilled water at 20 °C, germination energy, laboratory germination rate, morphometric parameters and biomass of seedlings were determined. To test the plants at the initial stages of ontogenesis, 20 seeds were sown in 4-fold repeat in the vegetation pots. For field study (Biological station of the Tyumen state university «Biostation Kuchak», Tyumen Province, Nizhnetavdinsky District), 200 seeds of each sample were sown on 1 m^2 plots in 3-fold repetition. Chlorophyll content in leaves was determined using an optical counter SPAD 502 («Minolta Camera Co., Ltd», Japan), three times with a 5 day interval in the laboratory test and seven times (according to the phenological phases — full shoots, herringbone, rapid growth, budding, flowering, green ripeness, and early yellow ripeness) in the field test. As per results obtained the seeds of the studied flax samples had two types of color (brown and yellow), differed in length (3.0-5.4 mm), width (2.1-8.2 mm), weight of 1000 seeds (6.57-4.36 g), shape (elongated-elliptic, ovoid flattened), which indicates sample heterogeneity. Features of plant growth and development at the initial stages of ontogenesis in some cases were confirmed in the field. For example, the length of the shoot in plants of varieties Mayak, Rybin, and Iva in the laboratory test is the largest of the entire experimental group (13.1; 12.5; 12.1 cm, respectively), and in the field, these varieties are in the 'tall' group according to plant height (96.1-100.2 cm). The yield of the flax straw was more (166.6-171.7 g/m^2) in the varieties having shoots substantially predominating in the plant biomass (Grant, Mayak, Bertelsdorfer). Field germination rate (62.3-77.3 %) and plant survival during the growing season (70.6-85.8 %) we refer to the informative criteria that characterize a number of ontogenetic interrelated processes. Also, we propose SPAD 502 readings (SPAD index) to evaluate the genotype × environment interactions. Our results show positive correlations between SPAD index and parameters of plant assimilation surface (linear size, area and number of leaves, and plant height), as well as plant survival during the growing season. Significant differences are revealed between the studied samples in the average daily chlorophyll accumulation in the leaves before flowering and chlorophyll degradation at green and early yellow ripeness phases. Significant advantage in flax straw and seed yields have samples with a relatively rapid increase in SPAD index during the first half of the growing season with further uniform decrease towards maturity phase. Thus, Grant, Mayak (Belarus), Bertelsdorfer (Germany), Svalof (Czech Republic), Ottava 770 B See (Canada) are the varieties that combine high adaptive and productive properties.

Keywords: *Linum usitatissimum* L., flax, collection sample, chlorophyll counter SPAD 502, simulated and natural conditions

Stresses from environmental factors, known as stochastic events, represent significant deviations from normal conditions and can cause significant

damage to the plant population [1]. Sustainable crop production in the south of the Tyumen Province is possible with a combination of the following conditions: selection of plant species tolerant of limiting environmental factors, the creation of new highly adaptable varieties, their accelerated reproduction, and proper environmental placement [2]. Climate changes, manifested in an increase in average annual surface air temperature by 0.7 °C on all continents over the last quarter of the 20th century [3], indicate the need for making adjustments to the strategy for selecting species and varieties of cultivated plants.

The provision of high-quality, environmentally friendly food products to a certain extent is achieved by increasing the number of crops, developing fundamentally new ways to evaluate plant forms and quickly processing information about the biology of their development. Genetic diversity of plants increases the choice and provides protection against adverse conditions.

Flax is one of the crops that deserve attention and introduction into agricultural production. Currently, in the Tyumen Province, it is practically not grown; there is no testing of varieties in state strain-trial stations (SSTS). At the same time, flax growing was developed in Siberia in the 1970s-1980s, and the area under flax in the south of the Tyumen Province was more than 10000 hectares [4]. The introduction and selection of flax varieties, ecologically adapted to local soil and climatic conditions, is important for agricultural practice.

When phenotyping characteristics on seedlings in controlled conditions (modeled in the laboratory) and on adult plants in natural field conditions, it is possible to accelerate the identification of selectively valuable plant forms. Morphological characteristics of seedlings are considered as biometric indicators of the reaction of the forming plant organism to environmental factors. Thus, the ratio of the root and the shoot lengths in standard and stressful conditions can serve as a convenient indicator characterizing ontogenetic development. This indicator is identified among the informative ones during the assessment of the degree of adverse effects on triticale [5], maize [6], safflower [7], and pine [8].

The physiological features characterizing the genotype × environment interaction include the chlorophyll content in the leaves. Stationary methods for determining this indicator using spectrophotometers are laborious, time-consuming, and require removal of plants from agrocenoses. The ability to quickly and accurately determine the amount of chlorophyll using the SPAD-502 optical counter (Minolta Camera Co., Ltd., Japan) in the leaves of wheat [9, 10], barley [11, 12], oats [13], rice [14], potatoes [15], tomatoes [16], celery [17], sugar cane [18], papaya [19], bay [20] and woody [21] plants, eucalyptus [22], peanuts [23], soy [24, 25], arabidopsis [26], and ornamental crops [27] was shown. Differences between species and varieties were revealed according to the amount of chlorophyll when exposed to salinity [28], contrast temperature [29, 30], lack of moisture [31] and in vitro culture [32]. In Russia, studies made using this instrument are fragmented [33].

In this paper, for the first time in the conditions of the south of the Tyumen Province, the variability of morpho-physiological traits has been studied using the example of 20 samples of ordinary flax of different ecological and geographical origins and the most informative criteria determining the adaptive potential of the crop have been established. Rapid diagnosis of the accumulation and degradation of chlorophyll in the leaves at different stages of plant ontogenesis was carried out using a SPAD 502 optical counter.

The purpose of the research was to study the response of ordinary flax genotypes (*Linum usitatissimum* L.) to the effects of environmental factors and to identify population and individual characteristics for use as indicators of adaptive properties.

Techniques. The authors studied 20 collection samples of flax of various eco-geographical origins from Russia (6 samples), Belarus (7 samples), Czech Republic (2 samples), one sample per Ukraine, France, Canada, Australia, and Germany. Laboratory and field experiments were carried out in 2016-2017.

Seeds were germinated in Petri dishes on filter paper moistened with distilled water at 20 °C in a thermostat TS-1/80 SPU (Russia) in accordance with RF State Standard GOST R 52325-2005 [34]. The germinative energy, laboratory germination rate of seeds, morphometric parameters, and seedling biomass were determined.

To test the plants at the initial stages of ontogenesis, 20 seeds were sown in 4-fold repetition in the vegetation vessels. 280 g of soil with a humidity of 60% of the total water capacity was placed in the vessel. Plants were grown on specialized racks (illumination of 5000 lux, 16 h photoperiod). Seed germination, plant height, chlorophyll content in leaves, the weight of the aboveground and underground parts were assessed. To characterize the structure of biomass, the ratio for shoots and roots was calculated.

A field study of flax collection samples was performed at the experimental field of the biological station of the Tyumen State University Lake Kuchak (Nizhnetavdinsky District, Tyumen Province, 57°21′N, 66°04′E) on cultivated sod-podzolic soil of sandy loam granulometric composition. Field trials, observations and records of traits were carried out in accordance with the guidelines [35]. Sowing was carried out in the first decade of May in an ordinary way with 200 seeds per plot with an accounting area of 1 m^2, repeated 3 times. Placement of the plots was randomized. The predecessor is spring barley.

The chlorophyll content in the leaves was determined using an optical counter SPAD 502 (Minolta Camera Co., Ltd., Japan) at $\lambda = 650$ nm (maximum absorption of chlorophylls a and b) and $\lambda = 940$ nm (taking into account the thickness of the leaf). The instrument was calibrated before starting the measurement. The amount of chlorophyll in 20 leaves from the top of typical plants of each sample was estimated. The middle part of the leaf blade was placed on the bottom of the device, clamped for a few seconds until a numerical value appears. The device automatically remembers all readings and calculates average values (SPAD units). The authors conducted 3 measurements with an interval of 5 days in the laboratory experiment and 7 measurements in the field at different stages of phenological development: sprouting, herringbone, rapid growth, budding, flowering, green ripeness, early yellow ripeness.

The plant height was measured at each phenological phase. During the harvesting period, the number of surviving flax plants in each plot was calculated, and survival was calculated in relation to the number of shoots.

Statistical data processing was performed according to B.A. Dospekhov [36], G.F. Lakin [37], A. Field et al. [38] using the Microsoft Excel spreadsheet processor and the STATISTICA 6.0 software (StatSoft, Inc., USA). Mean values (M) and standard errors of the mean (±SEM) are presented. Differences between mean values of the variants were evaluated using Student's t-test and were considered to be statistically significant at $p < 0.05$ and $p < 0.01$.

Results. The heterogeneous flax material differed in linear dimensions (length, seed width) and 1000-seed weight. In accordance with the International Classification of the species *Linum usitatissimum* L. [39], the samples were divided into two groups: with medium (3 samples) and small (17 samples) seeds (see Table).

According to the color of flax seeds, there are two types, dark (brown) and light (yellow). M.N. Yaglo et al. [40] regarded this trait as an important marker for variety identification. The yellow color is determined by the domi-

nant *YSED1* gene, the dark yellow color by the recessive *ysed2* gene, and the light yellow-brown color by the *rs1* gene [41]. The seed color of the studied samples was brown (of varying intensity) in 19 samples and yellow in one (Ottava 770 B See).

Characterization of seeds of the studied ordinary flax (*Linum usitatissimum* L.) samples (*M*±SEM, laboratory test)

Sample, phenotype (origin)	Seed size, mm		Length/width ratio	1000-seed weight, g
	length	width		
Medium seeds (n = 3)				
Rucheek, o (Russia)	5.21±0.83	3.54±0.11	1.47	6.12±0.17
Fleez, o (Russia)	5.21±0.12	3.30±0.77	1.58	6.00±0.91
Turquoise, o (Russia)	5.05±0.77	4.31±1.22	1.17	6.57±0.25
Small seeds (n = 17)				
36.3.-4, l (Russia)	4.42±0.81	3.25±0.50	1.36	4.51±0.66
Velizhsky Ridge, l (Russia)	3.02±0.12	3.12±0.71	0.97	4.48±0.59
Pechersky Ridge, l (Russia)	4.13±0.99	3.54±0.66	1.17	4.36±0.33
Grant, l (Belarus)	5.01±0.89	3.10±0.63	1.61	5.25±0.34
Mayak, l (Belarus)	3.06±0.12*	5.23±0.32*	0.59	4.50±0.65
Mara, l (Belarus)	4.21±0.54	3.42±0.65	1.23	4.83±1.03
Rubin, l (Belarus)	3.15±0.78	2.11±1.23	1.49	4.66±0.34
Iva, l (Belarus)	4.25±0.75	3.32±0.90	1.28	4.74±0.43
Yarok, l (Belarus)	4.21±0.57	3.15±0.80	1.34	5.00±0.72
Vesta, l (Belarus)	3.35±0.28	3.42±0.64	0.98	5.39±0.94
Glinum, l (Ukraine)	4.45±0.45	2.25±1.12	1.98	4.89±0.77
Bertelsdorfer, l (Germany)	4.06±0.10	8.21±0.21**	0.49	5.25±0.95*
Currong, l (Australia)	4.05±0.45	3.05±0.32	1.33	4.37±0.76
Svalof, l (Czech Republic)	5.34±0.90*	3.15±0.32	1.70	4.59±0.91
Hermes, l (Czech Republic)	4.21±0.91	3.11±0.36	1.35	5.03±0.49
Ottava 770 B See, l (Canada)	5.36±0.21	3.48±0.79	1.54	5.50±0.20
Alizee, l (France)	4.54±0.54	3.27±0.25	1.38	5.61±0.63
Mean of all specimens	4.31±0.55	3.58±0.62	1.30	5.08±0.60

Note. The samples are grouped according to 1000-seed weight [39]; l — long-stalked flax (spinning flax), o — linseed flax (oil flax).

*, ** Differences with the average value of the samples are statistically significant, respectively, at $p < 0.05$ and $p < 0.01$.

The average seed length was 4.31±0.55 mm and varied in samples from 3.02±0.12 (Velizhsky Ridge) to 5.36±0.21 mm (Ottava 770 B See). In seed width, the minimum value (2.11±1.23 mm) was observed in the Rubin variety, the maximum value (8.21±0.21 mm) in the Bertelsdorfer variety, with an average value of 3.58±0.62 mm in samples (see Table). In the majority of the samples studied, the seed linear dimensions did not differ significantly from the average population values. Significant differences were found in the Mayak and Svalof samples in the seed length, in the Mayak and Bertelsdorfer samples in the seed width. The calculation of the index of the ratio of the seed length and width allowed estimating their differences in shape. The seeds of 16 samples, in which the length and width ratio was 1.17-1.98, had an elongated-elliptical shape. In four samples, the seed shape was ovate oblate (length and width ratio 0.49-0.98). By 1000-seed weight, a significant difference with the average population value was noted only in the Bertelsdorfer sample.

Testing of the physiological quality of seeds is carried out by germinative energy and laboratory germination rate in strictly regulated conditions. However, it is believed [42] that these tests are not designed to predict the exact number of seedlings in field conditions, since the impact of stress factors (low soil temperatures, lack of moisture, pathogens, etc.) reduces the rate of germination. In this regard, informative addition to the assessment of the biological properties of seeds was the study of the initial ontogenetic development of plants on the basis of the variability of their morphological features. The germinative energy and laboratory germination rates confirmed the high sowing qualities of the seeds studied. By germinative energy, none of the samples was significantly different from the average value (95.6±0.74%) throughout the experimental group. Laboratory germina-

tive energy of seeds of the Grant (99.8±0.66%) and Glynum (97.1±0.21%) varieties was significantly higher ($p < 0.05$), the Yarok (93.7±0.90%) and Fleez (94.6±0.99%) varieties were below average.

The variation of the characteristic values as influenced by environmental factors is called phenotypic adaptation and is determined by the reaction norm [43]. The ability of seeds to germinate and to form full-fledged seedlings reflects the adaptive properties of the crop in new environmental conditions. The average field germination of seeds in the studied flax samples was 25% lower than in the laboratory, which is typical of the soil and climatic conditions of the Tyumen Province and is consistent with data from other crops [44]. The samples response to cultivation in the field was ambiguous. Indices for 6 samples differed significantly ($p < 0.05$) from the average for the collection, of which Grant, Mayak, Velizhsky Ridge were characterized by high (77.3-76.5%), Hermes, Bertelsdorfer, Rucheek – by low (66.3-67.9%) seed germinative energy. The variability of samples in field germination was higher than in laboratory tests (*Cv* of 25.18 and 9.13%, respectively).

The identification of growth features in regulated conditions and the possibility of interpreting these data in relation to the field conditions are of particular interest. So, when grown in pots, the longest shoot length was observed in the Mayak (13.1±0.77 cm), Rubin (12.5±0.94 cm), and Iva (12.1±0.31 cm) varieties. According to the results of the field assessment, these varieties were classified as tall (100.0, 96.1, 100.2 cm, respectively). That is, their high growth potential was maintained throughout the entire period of plant development.

Analysis of the structure of raw biomass revealed the predominance of shoots, making in samples 66.7% on average. The high share of shoots (87.5-90.0%) in the formation of plant biomass at the initial stages of ontogenesis was found in samples Velizhsky Ridge, Grant, Iva, Vesta, Glinum, Bertelsdorfer, Ottava 770 B See. In the field trials, stock yield was relatively high for Grant, Glynum, Lighthouse plants (166.5-170.0 g/m^2), seed yield for Grant, Mayak, Bertelsdorfer plants (66.5-77.7 g/m^2).

The environmental plasticity of the tested flax samples was characterized by 80% plant survival during the growing season. The variation of the trait was 30.33% and was higher than for seed germinating energy, laboratory and field germination. Consequently, the survival of plants was largely determined by the indices of individual viability of the samples. Grant, Mayak, Rubin, Currong, Velizhsky Ridge showed the best viability, and Fleez, Mara, Hermes, and Rucheek had the least plasticity. Samples with high resistance to environmental factors on the biological properties of seeds, their ability to germinate, as well as on indicators of linear growth of roots and shoots in early ontogenesis in most cases, minimally deviated from the average values for the collection. Samples with reduced survival often deviated from the mean value in opposite directions, i.e. towards increased shoot length (Mara) and field seed germination (Hermes), and towards reduced laboratory and field germination rates, as well as shoot length (Fleez, Rucheek).

Thus, we used the widely-used tests which are based on the study of the characteristics of plants in laboratory and field tests allow one to reveal the peculiarities of intrapopulation variation at the phenotypic level. However, there are opportunities to improve this traditional methodology. For example, the tests using physiological markers, such as the chlorophyll content, are seemed important.

When analyzing the dynamics of chlorophyll accumulation in leaves in a laboratory test, we did not reveal significant differences between the samples in the first and second measurements with the SPAD 502, which is probably due to

the low intensity of linear growth at the initial stages of ontogenesis. The third measurement showed an increase in intrapopulation differences. In six varieties (Mayak, Glynum, Iva, Velizhsky Ridge, Currong, Hermes), the indicators were significantly ($p < 0.05$) lower than the average value, the others did not significantly differ. From the beginning to the end of the experiment, the indicator varied from 1.29 to 9.14 SPAD units. To assess the variability of the chlorophyll content in the leaves, to give a complete statistical description of the analyzed population in laboratory and field conditions, as well as to compare one distribution with another, span diagrams (box and whisker pots) developed by J. Tukey [38] were used (Fig. 1).

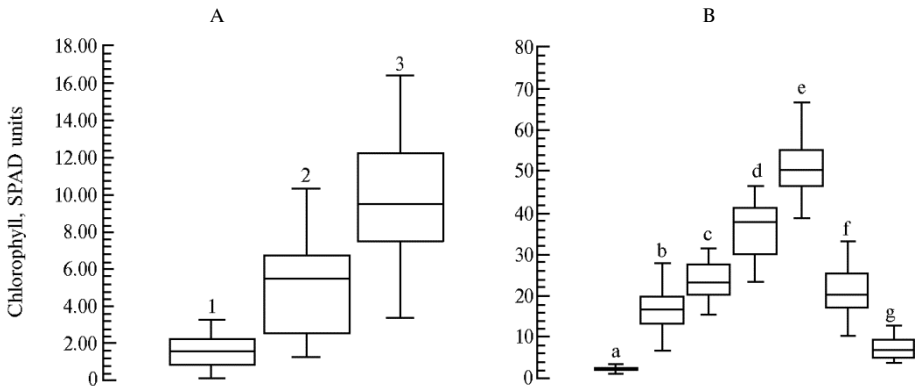

Fig. 1. Chlorophyll content in leaves of flax (*Linum usitatissimum* L.) collection samples with SPAD 502 (Minolta Camera Co., Ltd., Japan) in laboratory (A) **and field** (B) **trials:** 1, 2, 3 — counted with an interval of 5 days; a — full shoots, b — herringbone, c — rapid growth, d — budding, d — flowering, e — green ripeness, g — early yellow ripeness; line — arithmetic average; □ — standard error, ±; ⊥ — the trait minimum; ⊤ — the trait maximum ($n = 20$; field tests were performed at the biological station of Lake Kuchak, Tyumen State University, Nizhnetavdinsky Region, Tyumen Province, 2016-2017).

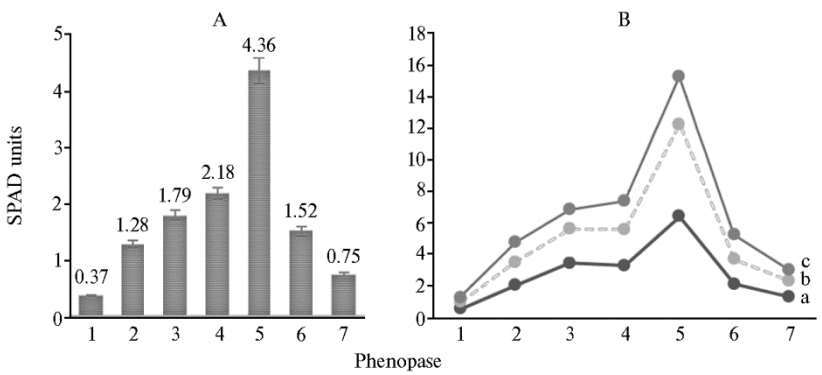

Fig. 2. Average daily accumulation and degradation of chlorophyll in flax (*Linum usitatissimum* L.) leaves during phenological phases for all samples on average ($n = 20$) (A) **and in the samples with significant differences** ($n = 3$) (B)**:** 1 — full shoots, 2 — herringbone, 3 — fast growth, 4 — budding, 5 — flowering, 6 — green ripeness, 7 — early yellow ripeness; a — Grant, b — Svalof, c — breeding sample 36.3.4 (field tests were carried out at Lake Kuchak biostation of Tyumen State University, Nizhnetavdinsky Region, Tyumen Province, 2016-2017).

Under natural conditions, the highest values (70.05 SPAD units) for chlorophyll were observed in flax leaves during flowering. During seed formation and ripening, the index dropped to 4.23-13.24 SPAD units. The nature of changes in the amount of chlorophyll can significantly affect seed yield and quality [45]. The minimum daily average accumulation of chlorophyll was recorded in the seedling phase (0.37 SPAD units). The further development of

plants was associated with an increase in the relative rate of chlorophyll accumulation during sprouting—herringbone period (up to 1.29 SPAD units), herringbone—rapid growth (up to 1.79 SPAD units), rapid growth—budding (up to 2.18 SPAD units), budding—flowering (up to 4.36 SPAD units). From flowering to green and early yellow ripeness, the indices sharply decreased and amounted to 1.52 and 0.75, respectively (Fig. 2).

Despite a common pattern of chlorophyll accumulation and degradation of, the studied samples differed significantly among themselves (see Fig. 2, B). Thus, the highest intensity of chlorophyll accumulation in the leaves was revealed in Grant, i.e. the pigment level during growing season increased daily by 2.71 SPAD units. Regarding other samples, the variety was characterized by a rapid increase in the chlorophyll content before flowering and pronounced degradation during seed formation. The variety showed a higher yield of stock (170.0 g/m^2) and seeds (68.3 g/m^2), with average population values of 147.1 and 59.85 g/m^2, respectively. During the growing season in the breeding sample 36.3.4, an increase in chlorophyll per day was slow (1.42 SPAD units), the lowest rate (1.53 SPAD units) as compared to other samples was during flowering phase, the stock yield was low (100.2 g/m^2). Destruction of chlorophyll in green ripeness (1.58 SPAD units) and early yellow ripeness (0.70 SPAD units) was relatively slow, and seed yield was 33.3 g/m^2. Svalof variety ranked an intermediate position between the described samples according to average daily chlorophyll accumulation in leaves (2.10 SPAD units during the growing season). It stood out by this indicator during plant flowering (2.43 SPAD units), seed formation and maturation (1.57-1.00 SPAD units), as well as by seed productivity (116.5 g/m^2). In general, for the studied samples, seed yield varied from 33.3 to 116.5 g/m^2, and stock from 100.2 to 171.7 g/m^2.

Correlation coefficients revealed interrelations of chlorophyll accumulation with other laboratory and field indicators. For plant height in the laboratory and field test the values were respectively $r = 0.65$ and $r = 0.89$; for the number of leaves $r = 0.36$ and $r = 0.25$; for leaf area $r = 0.35$ in the laboratory test (in the field correlation was weak). Positive correlations were found between the SPAD 502 measurements and plants survival rate in the budding phase ($r = 0.22$) and early yellow ripeness ($r = 0.24$).

The data we obtained are consistent with the concept that chlorophyll content in leaves is a significant parameter of plant physiological status [46, 47]. The chlorophyll content per unit of leaf area (chlorophyll density) [48] is an informative indicator of photosynthetic activity, growth and development of many crops. SPAD 502 readings are considered convenient criteria to estimate photosynthesis process with regard to changes caused by environmental factor, which allows selection of genotypes that adapt to stress [49-51].

According to the results of variance analysis, differences in factors were found in terms of the proportion of influence on the content of chlorophyll in the leaves in the total phenotypic variability. The accumulation of chlorophyll was primarily determined by the conditions of growing flax plants (46.2%), as well as the interaction of this factor with the genotype (34.4%). Genotypic differences were 16.6%. The simultaneous effect of other factors on the trait was insignificant.

So, by laboratory and field tests, population and individual traits crucial for flax plant adaptation to new agro-ecological conditions are identified. Testing of seeds shows that morphological traits of seedlings and young plants, the indices of root and shoot development, the structure of biomass can be informative criteria for biological state of varieties, along with traditional indicators of germinative energy and laboratory germination rate. The patterns of variability of

some traits (shoot length, plant biomass, chlorophyll content) detected in flax samples at the initial stages of ontogenesis are confirmed in field trials, which gives grounds for selecting genotypes with useful traits in simulated laboratory conditions. In the field, the field germination rate and plant survival during the growing season can be used to identify flax varieties resistant to hydrothermal stress, since these indicators sufficiently characterize a number of interrelated ontogenetic processes and reflect the plant response to environmental factors. Assessment of genotypes in the field trial is based on a number of traits: plant height, linear dimensions, leaf area and number. When selecting flax genotypes, it is convenient to use the SPAD 502 optical chlorophyll counter, which allows breeder significantly reduce the time of evaluation without losing objectivity. The relationships between the measured indicators (SPAD units), morphological features and plant survival at different stages of ontogenesis are revealed. By a comprehensive study, Grant, Mayak (Belarus), Bertelsdorfer (Germany), Svalof (Czech Republic), Ottava 770 B See (Canada) varieties combining high adaptive and productive properties are suggested as a starting material in breeding genetic programs.

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THE RESPONSE OF in vitro CULTURED CELLS OF *Linum grandiflorum* Desf. ON THE ACTION OF POLLUTANT AND HERBICIDE

E.A. GONCHARUK[1], T.N. NIKOLAEVA[1], L.V. NAZARENKO[2], E.A. KALASHNIKOVA[3], N.V. ZAGOSKINA[1]

[1]*Timiryazev Institute of Plant Physiology RAS,* Federal Agency for Scientific Organizations, 35, ul. Botanicheskaya, Moscow, 127276 Russia, e-mail goncharuk.ewgenia@yandex.ru (✉ corresponding author), niktat2011@mail.ru, nzagoskina@mail.ru;
[2]*Moscow City University,* 4, 2-i Selskokhozyaistvennyi proezd, Moscow, 129226 Russia, e-mail nlv.mgpu@mail.ru;
[3]*Timiryazev Russian State Agrarian University—Moscow Agrarian Academy,* 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail kalash0407@mail.ru (✉ corresponding author)
ORCID:
Goncharuk E.A. orcid.org/0000-0002-9280-9450
Nikolaeva T.N. orcid.org/0000-0003-1528-1020
Nazarenko L.V. orcid.org/0000-0002-2924-1612
Kalashnikova E.A. orcid.org/0000-0002-2655-1789
Zagoskina N.V. orcid.org/0000-0002-1457-9450
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Abstract

Scholarly papers on in vitro culture of large-flowered flax (*Linum grandiflorum* Desf.), an ornamental crop for urban gardening, are very few and mainly elaborate morphogenesis, embryo culture, and cell line resistance issues. Heavy metals and herbicides are typical urban pollutants which cause oxidative stress. Our study for the first time reveals differences in morphophysiological characteristics and biosynthesis of phenolic compounds between in vitro cultured flaxseed cells influenced by Cd and herbicide glyphosate. With the aim to specify the mechanisms involved in stress tolerance of *L. grandiflorum* we have estimated the accumulation of antioxidants of phenolic nature, including phenylpropanoids and flavonoid in the callus culture. When studying the effect of stress factors, cadmium (60 μM), glyphosate (10 μM) or cadmium and glyphosate simultaneously (in the same concentrations) were added to the nutrient medium. Control was the standard nutrient medium. Callus cultures were analyzed at the end of the passage (day 28 of culture). Phenolic compounds extracted with 96 % ethanol from a plant material frozen with liquid nitrogen at –196 °C. The content of the sum of soluble phenolic compounds was determined with a Folin-Denis reagent, flavonoids with a 1 % solution of $AlCl_3$ at 725 and 415 nm. The concentration of phenylpropanoids was determined by direct spectrophotometric analysis of the extracts at 330 nm. To study the composition of the callus flax cultures phenolic complex compounds, the method of a thin layer chromatography on cellulose plates, solvent BUW (n-butanol + acetic acid + water in the ratio 40:12:28) were used. Callus of *L. grandiflorum* had a loose consistency, yellowish-green color, low growth index (by the end of the passage — 150 %) and high water content (95-97 %). As it grows, the content of soluble phenolic compounds and phenylpropanoids (biogenetically early polyphenols) increased 2.7 times by the end of the passage. The content of flavonoids, one of the most common representatives of phenolic compounds, in the first half of the cultivation cycle increased 2.25 times, and by the end of the passage slightly decreased. The application of cadmium caused the formation of dark colored necrotic cells on the surface of callus. In contrast, the application of glyphosate did not give such reaction. The application of cadmium did not affect the biosynthetic capacity of callus cultures in terms of the total accumulation of phenolic compounds, phenylpropanoids and flavonoids, the quantity of which was almost equal to that of the control. Under the application of glyphosate, however, the level of these secondary metabolites in cultures decreased, especially flavonoids. Under the simultaneous use of cadmium and glyphosate the total content of phenolic compounds in calluses increased, but we observed the decrease in the amount of phenylpropanoids and especially flavonoids. The data obtained show the differences in the response of large-flowered flax cells to the effect of stress factors on their morphology and the level of biosynthesis of phenolic compounds, the substances with high biological and antioxidant activity.

Keywords: large-flowered flax, *Linum grandiflorum*, callus cultures, tolerance, cadmium, glyphosate, phenolic compounds, phenylpropanoids, flavonoids

One of the problems of recent times is a change in the environmental situation due to active industrial and agricultural human activities. Technogenic pollution of the environment occurs with various pollutants, including heavy metals [1]. The most common is cadmium, which is 2-20 times more toxic to plants, animals, and humans than other metals [2]. Cd has a negative effect on the processes of photosynthesis and respiration, water regime, mineral nutrition, the functioning of the antioxidant system. Cadmium damages the light-harvesting antenna complexes of photosystem I and photosystem II [3, 4], competes with ammonium ion, thereby affecting nitrogen metabolism [5], promotes the activation of free-radical processes [6], changes the accumulation of various antioxidants [7, 8]. All of this leads to a disruption of natural plant communities, and to significant crop losses in cultivated species due to changes in growth and development [1].

In modern agroecological conditions, there is a problem of weed grass infestation of field and decorative crops, for which herbicides that can suppress plant growth are widely used [9]. These include glyphosate N-(phosphonomethyl) glycine, a post-emergent non-selective herbicide of systemic action, which ranks first in the world in production among preparations of similar action [10]. Glyphosate is a unique inhibitor of the shikimate biosynthetic pathway key enzyme for aromatic compounds 5-enolpyruvilshikimate-3-phosphate synthase, as a result of which the synthesis of proteins and secondary metabolites of a phenolic nature is suppressed in plants and deregulation of energy metabolism is also observed [11].

Phenolic compounds are secondary metabolites synthesized in all cells and tissues of plants [12]. They vary widely in structure, chemical properties and biological activity. Their functional role is diverse and associated with the processes of photosynthesis, respiration, regulation of enzymatic activity, protection of cells from stress effects [13]. The antioxidant properties of these plant metabolites are due to the presence of hydroxyl groups in their structure, which easily interact with free radicals, thereby contributing to the inhibition of radical-chain oxidation under stressful conditions [14].

Flax (class *Dicotyledoneae*, fam. *Linaceae*) is one of the most important crops of complex use [15]. It is characterized by a diversity of species groups and varieties, which opens up wide possibilities for its use in the agricultural, textile, and pharmaceutical industries, as well as in decorative and landscape agriculture [16, 17]. In the latter case, the annual decorative look of flax *Linum grandiflorum* Desf. (large-flowered flax) is used successfully as it has a well-developed vegetative part and flowering continues until autumn [18]. Since this crop is planted in urban areas where the soil and the environment are contaminated with pollutants, and various herbicides are used to control weeds, it is advisable to study its resistance to their effects.

The method of culturing cells and plant tissues in vitro makes it possible to investigate metabolic processes, as well as the response of cells to the action of stress factors, at a simpler level of organization compared to an intact plant [19, 20]. In vitro culture of flax has a long history. At the same time, the preservation of biodiversity, the study of the structure of plant cells and their resistance to stress factors [21-23] were the main areas of research. But in general, there are very few works on *L. grandiflorum*, and they mainly deal with issues of morphogenesis, embryo culture production, and study of resistance of cell lines [24, 25].

The present study is the first to identify differences in the in vitro response of cultured flaxseed flax cells to stress factors such as a pollutant (cadmium) and

a herbicide (glyphosate). This is manifested both in morphophysiological characteristics and in the biosynthesis of phenolic compounds, the substances with high biological and antioxidant activity.

The aim of this work was to evaluate the growth activity of the large-flowered callus culture of flax and the accumulation of phenolic compounds in it, including phenylpropanoids and flavonoids, as well as the culture response to the action of pollutant (cadmium) and herbicide (glyphosate).

Techniques. Callus cultures of large-flowered flax were grown on a Murashige-Skoog medium with added 2% sucrose and 2 mg/l of 2,4-dichlorophenoxyacetic acid in a factor-static chamber (Timiryazev Institute of Plant Physiology RAS) at 25 °C, relative humidity 70% and a 16 h photoperiod (illumination intensity 5000 lx).

When studying the effect of stressors, cadmium $Cd(NO_3)_2$ (60 μM), glyphosate (Monsanto, Belgium) (10 μM) or cadmium and glyphosate were added to the main nutrient medium (at the same concentrations). The control was the usual nutrient medium. Callus cultures were analyzed at the end of the passage (day 28), fixing them with liquid nitrogen for subsequent biochemical studies. Growth rate of calluses and their morphophysiological characterization were accounted. The water content in callus tissues was determined by the standard method after drying to the constant weight in a thermostat at 70 °C [26].

Phenolic compounds were recovered with 96% ethanol from plant material frozen with liquid nitrogen at –196 °C. After 45 min, the homogenate was centrifuged (16000 rpm, 15 min) and the supernatant was used for spectrophotometric measurements. The content of the sum of soluble phenolic compounds was determined with Folin-Denis reagent, flavonoids with 1% $AlCl_3$ solution at $\lambda = 725$ nm and $\lambda = 415$ nm [27]. Rutin calibration curves were built. The amount of phenylpropanoids was estimated by direct spectrophotometry of extracts at $\lambda = 330$ nm, using caffeic acid to construct a calibration curve [28].

To study the composition of the phenol complex of callus flax cultures, thin-layer chromatography was used on plates with cellulose (Merck KGaA, Germany), solvent BV (n-butanol + acetic acid + water in the ratio 40:12:28). Preliminary identification of phenolic compounds was carried out on an ultrachemiscope DESAGA UVIS (DESAGA, Holland) using specific bright blue or blue fluorescence in UV light at $\lambda = 254$ nm and $\lambda = 366$ nm. To detect phenolic compounds, chromatograms were treated with a mixture of 1% solutions of $FeCl_3$ and $K_3Fe(CN)_6$ (1:1), and phenolcarboxylic acid reagent (diazotized p-nitroaniline) was used, followed by treatment with 20% Na_2CO_3 [27].

Experiments were performed in 5-fold biological and 3-fold analytical replicates. Correlation and factor analysis (ANOVA) was carried out in the SigmaPlot 12.3 software (https://systatsoftware.com). The tables show the arithmetic mean values of the obtained values (*M*) and their standard errors (±SEM). Superscripts denote the statistical significance of differences in average values for the Tukey test at $p < 0.05$.

Results. The callus culture of large-flowered flax grown on the main nutrient medium was mostly yellow, although some areas were light green (Fig. 1, A). This may indicate the initial stages of the formation of chloroplasts in it, since growth occurred under the influence of light. The fact that these organelles are formed in in vitro cultures has been reported in the literature [19]. It should also be noted that the flax calluses had a loose structure, low growth during the entire passage, and high water content (Table 1). Similar characteristics of callus culture of large-flowered flax were cited by other authors [24].

In vitro plant cells retain many properties of intact tissues, including the ability to synthesize phenolic compounds [19, 20]. As the large-flowered callus

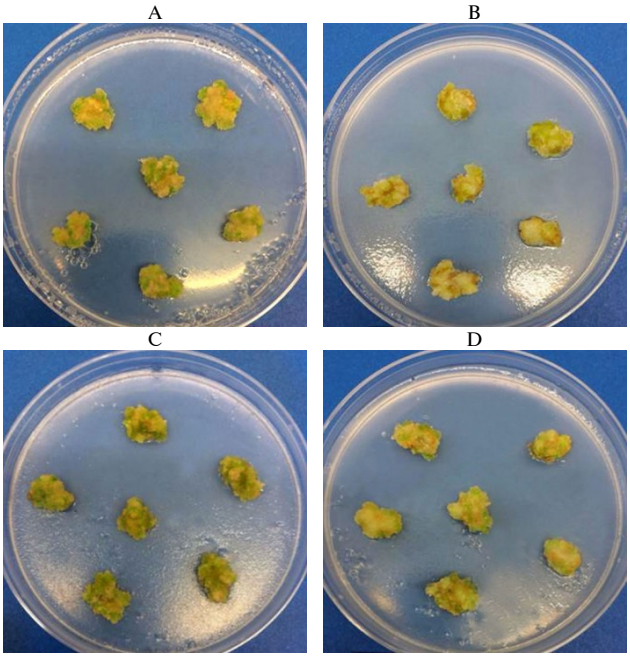

Fig. 1. The appearance of callus culture of large-flowered flax (*Linum grandiflorum* Desf.) grown on the main nutrient medium (A)**, as well as on a nutrient medium with cadmium** (60 μM) (B)**, glyphosate** (10 μM) (C)**, cadmium and glyphosate** (D) (day 28).

flax culture grew, the total content of phenolic compounds increased (see Table 1). The most significant changes occurred from day 6 to the day 14 of culture, when the number of phenolic compounds increased 2.4 times. The maximum total content was noted at the end of the passage, being 2.7 times more compared to the initial stages of growth (day 6). Consequently, the greatest intensity of biosynthesis of phenolic compounds was confined to the first half of the culture growth cycle, which once again confirms the high ability of young plant cells to form these metabolites [12].

Phenylpropanoids are biogenetically early representatives of phenolic compounds synthesized in plant tissues [12]. Their accumulation has been reported in various members of the *Linum* genus [29]. The tendency of accumulation of phenylpropanoids in callus of large-flowered flax was similar to that for the number of phenolic compounds (see Table 1). Based on this, it can be assumed that they are the main components of the phenolic complex of cultures and determine the nature of the accumulation of phenolic compounds.

1. Morphophysiological and biochemical characteristics of callus culture of large-flowered flax (*Linum grandiflorum* Desf.) on the main nutrient medium during passage (*M*±SEM)

Indicator	Culture age, days		
	6	14	28
Increase in the callus weight, %	115 ± 5^{A}	125 ± 6^{A}	150 ± 5^{B}
Water content, %	94.42 ± 0.48^{A}	97.27 ± 1.24^{A}	97.254 ± 1.01^{A}
Total content of phenolic compounds, mg eq. rutin/g dry weight	7.43 ± 0.31^{A}	18.07 ± 0.92^{B}	20.23 ± 0.74^{B}
The content of phenylpropanoids, mg eq. caffeic acid/g dry weight	10.58 ± 0.63^{A}	24.60 ± 0.65^{B}	28.97 ± 0.54^{B}
The content of flavonoids, mg eq. rutin/g dry weight	2.92 ± 0.74^{A}	6.56 ± 0.86^{B}	6.03 ± 0.71^{B}

N o t e. Superscripts (A, B) denote the reliability of differences in average values for the Tukey test at $p < 0.05$.

Flavonoids are among the most common representatives of phenolic metabolism in plant tissues [12]. Their accumulation in callus cultures increased in the first half of the cultivation cycle and by the 14th day was 2.25 times higher than the same indicator in a 6-day culture. By the end of the passage, the content of flavonoids was slightly reduced, but remained higher than at the beginning of the passage (almost 2 times). That is, at the final stages of culture growth, namely during the stationary phase, there was a tendency to a decrease in the content of these metabolites, which is also characteristic of plant tissues [13].

The impact of stress factors such as cadmium and glyphosate leads to an increase in the formation of reactive oxygen species in cells [2, 11]. Under these conditions, an important role belongs to phenolic compounds, the low-molecular-

weight components of the antioxidant defense system [14, 31]. They are able to "interrupt" chain oxidation reactions caused by stress factors, as well as form complexes with heavy metals, preventing their toxic effect [1]. In this regard, the next task was to study the effect of cadmium and glyphosate on the growth of large-flowered callus crops, as well as the accumulation of phenolic compounds in them, which was estimated at the end of the passage, that is, during their maximum accumulation.

2. Morphophysiological and biochemical characteristics of callus culture of large-flowered flax (*Linum grandiflorum* Desf.) on media with cadmium (60 μM) **and glyphosate** (10 μM) (*M*±SEM, days 28)

Indicator	Stressor		
	Cd	glyphosate	Cd + glyphosate
Increase in the callus weight, %	135±7^{A}	165±8^{B}	150±6^{B}
Water content, %	97.20±1.01^{A}	95.90±1.01^{A}	97.71±1.12^{A}
Total content of phenolic compounds, mg eq. rutin/g dry weight	19.45±0.36^{A}	15.93±0.72^{A}	25.23±0.94^{B}
The content of phenylpropanoids, mg eq. caffeic acid/g dry weight	30.22±0.74^{A}	27.68±0.63^{A}	15.46±0.34^{B}
The content of flavonoids, mg eq. rutin/g dry weight	6.38±0.71^{A}	3.48±0.29^{B}	2.77±0.14^{B}

N o t e. Superscripts (A, B) denote the reliability of differences in average values for the Tukey test at $p < 0.05$.

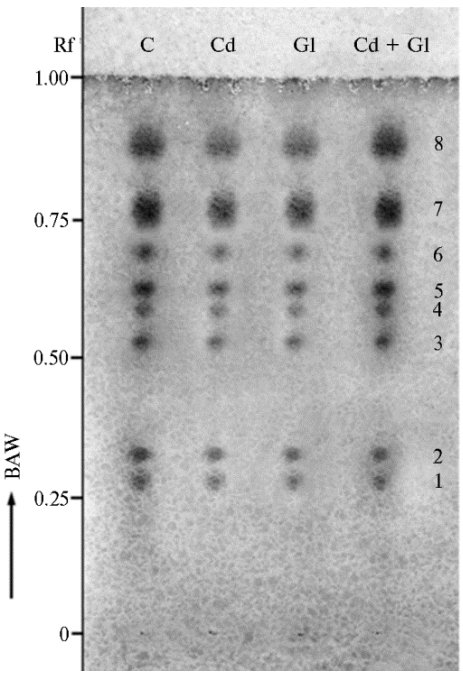

Fig. 2. Chromatographic separation of ethanol extracts from callus cultures of large-flowered flax (*Linum grandiflorum* Desf.) grown on the main nutrient medium (C) **and on media with cadmium** (Cd)**, glyphosate** (Gl) **and their combination** (Cd + Gl)**:** 1-8 – discovered substances of a phenolic nature. Thin layer chromatography on plates with cellulose, solvent composition (BAW) is n-butanol + acetic acid + water (40:12:28). The values of Rf (the ratio of the distance traveled by the substance to the distance traveled by the solvent) are indicated.

Callus culture grown on nutrient media with the addition of cadmium and glyphosate had a light-yellow color (see Fig. 1, B-D). There was a slight greening, but to a lesser extent than in the control version. In addition, small dark brown areas were formed on the surface, which could indicate cell necrosis, characteristic of other in vitro cultures in the presence of cadmium [30]. At the same time, the morphophysiological characteristics of calluses grown on the medium with glyphosate were better than in the control variant. They were denser and more compact, yellowish-green in color, with good growth (Table 2). The water content of the cultures in all the experimental variants had close values, approximately equal to those in the control.

The presence of cadmium in the medium had practically no effect on the biosynthetic ability of callus cultures (see Table 2). The total accumulation of phenolic compounds, the amount of phenylpropanoids and flavonoids were similar to those in the control (see Table 1). Most likely, *L. grandiflorum cells* are resistant to the studied metal concentration. This may be due to the fact that flax belongs to the group of accumulator plants, in which the toxic effect of the metal is expressed at higher concentrations of pollutant compared to other cultures, which are mainly exclusives that accumulate heavy metals in

Glyphosate serves as an inhibitor of one of the enzymes of phenolic metabolism responsible for the initial stages of the biosynthesis of these secondary metabolites [10]. When it entered the plants, in some cases a decrease in their accumulation was noted [31]. In the current experiment, a similar trend was observed, especially with respect to flavonoids (see Table 2). It can be assumed that glyphosate inhibited predominantly the flavonoid biosynthesis pathway for phenolic compounds and, to a much lesser extent, phenylpropanoid.

Accumulation of phenolic compounds in callus cultures was the most pronounced under the influence of combined cadmium and glyphosate. In this case, the total content of phenolic compounds significantly increased while reducing the amount of phenylpropanoids and flavonoids, which suggests the activation of the formation of other classes of phenolic compounds, in particular lignans, the compounds of a phenolic nature characteristic of flax plants [29].

To understand the peculiarities of the formation of phenolic compounds in plant cells, it is important to study not only their content, but also their composition [12].

3. Composition of the phenolic complex of ethanol extracts of callus cultures of large-flowered flax (*Linum grandiflorum* Desf.) in the control and all test variants (Murashige-Skoog medium)

Fraction No.	Rf	I	II
1	0.28	+	+
2	0.35	+	–
3	0.53	+	+
4	0.59	+	+
5	0.62	+	+
6	0.70	+	+
7	0.75	+	+
8	0.89	+	+

Note. Thin-layer chromatography on plates with cellulose was used for the separation, the solvent is n-butanol + acetic acid + water (40:12:28). Rf is the ratio of the distance traveled by the substance to the distance traveled by the solvent, I and II are the manifestation of a reagent for phenolic compounds and phenylpropanoids, respectively.

A thin-layer chromatography method revealed no differences in the composition of the complex of ethanol extracted phenolic compounds in the control and test variants (Fig. 2, Table 3). In all extracts, eight compounds of a phenolic nature were present, of which three dominated. Most phenolic compounds, as per their mobility, were conjugates of phenol carboxylic acids, i.e. p-hydroxybenzoic (compounds 1, 6, 8), p-coumaric (compounds 3, 7), ferulic (compound 4) and caffeic (compound 5) [27]. The presence of these substances in the phenol complex of flax plants was also reported by other authors [29, 30]. Since thin-layer chromatography allows only a preliminary estimate of the composition of the phenolic complex of plant cells and tissues and does not provide a complete picture, these studies will be further continued.

Thus, cultured in vitro cells of the large-flowered flax have the ability to form phenolic compounds, the biologically active substances with antioxidant activity, the greatest accumulation of which occurs at the end of the culture cycle. Their content increased rapidly in the first half of the cycle, reaching the highest values by the end of the passage, which points to the importance of the formation of phenolic compounds, including phenylpropanoids and flavonoids, in plant cells not only in vivo, but also in vitro. The heavy metal cadmium, when added to the culture medium, affected the morphophysiological characteristics of the callus culture; however, no changes in the accumulation of phenolic compounds, including phenylpropanoids and flavonoids, were observed. This once again confirms the significant resistance of flax cells to the action of heavy metals. The presence of glyphosate herbicide reduces the ability of the large-flowered flax culture to accumulate phenolic compounds, which is more pronounced for flavonoids. With the combined action of these two factors, the total content of phenolic compounds in cultures increases, and the amount of phenylpropanoids and especially flavonoids decreases, which may be due to the

formation of other representatives of phenolic compounds. Our findings confirm once again the species-specific response of plant cells to stress factors.

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PATTERN-TRIGGERED IMMUNITY (PTI) INDUCTION AND TRANSCRIPTIONAL REPROGRAMMING IN PERSISTANT ALLEXIVIRUS INFECTION

A.V. ARKHIPOV, V.K. VISHNICHENKO

All-Russian Research Institute of Agricultural Biotechnology, Federal Agency of Scientific Organizations, 42, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail iab@iab.ac.ru, batler51@yandex.ru, vish@iab.ac.ru (✉ corresponding author)
ORCID:
Arkhipov A.V. orcid.org/0000-0003-1195-6086 Vishnichenko V.K. orcid.org/0000-0002-6975-4885
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Abstract

In virus—plant interactions, one of the major mechanisms for plant antiviral immunity relies on RNA silencing, which is often suppressed by co-evolving viral suppressors, thus enhancing viral pathogenicity in susceptible hosts. However RNA silencing should not only be viewed as an antiviral mechanism that must be counteracted. In fact, many viruses encode weak or transiently active suppressors and probably do not use these viral proteins for control RNA silencing; for example, Shallot virus X (ShVX) do not code the active silencing suppressor and consequently use the another molecular mechanism to overcome the silencing immune barrier, establish the persistent infection and prevent catastrophic damage to its host. We hypothesized that this "non-suppressor" mechanism is the process of transcriptomic reprogramming (TRP) induced by the PTI (pattern-triggered immunity), the first layer of plant defence, which is triggered by specific recognition of conserved microbe- or pathogen-associated molecular patterns (MAMPs, or PAMPs, respectively) by pattern recognition receptors (PRRs) at the plasma membrane and the induction of defense signaling. Recently a role of PTI in antiviral defence has been demonstrated in *Arabidopsis* by showing that mutants in the PRR (PRRs, Pattern recognition receptors) coreceptor kinases exhibit increased susceptibility to different RNA viruses. Our preliminary results confirm this hypothesis and show that there is a negative correlation between the ShVX reproduction rates and the levels of RNA-dependent RNA-polymerase (RDR) and DCL proteins in roots and leaves of infected shallot plants. The task of this study is the experimental verification of our PTI-induced TRP hypothesis by quantitative real-time PCR (Comparative CT experiment, delta-delta CT algorithm; calibrator: healthy shallot seedlings; normalizer: 18S RNA; The 7500/7500 Fast Real-Time PCR Systems, Applied Biosystems, USA) to evaluate in vivo expression levels of transcripts coding PTI markers, factors of RNA-silencing, NB-LRR receptors and complex of TCTP-PIRL-GRF6-DBP1 proteins in the healthy and ShVX-infected shallot (*Allium cepa* L. var. *aggregatum* L.G. Don) plants. In this study for the first time we obtained the convincing data about PTI and TRP induction in ShVX-infected shallot plants. As result of TRP, repression of all factors of RNA-silencing, some NB-LRR receptors (e.c., *Tm2²*) and some proteins of TCTP-PIRL-GRF6-DBP1 complex take place in this virus—plant system. On the other hand, group of defense genes with high expression levels has been discovered in this system: *SOBIR* — log10RQ ~ 1.0; *ARM* (genes encoding armadillo protein family) — log10RQ ~ 2.0; Pathogenesis-related protein 1, *PR1* — log10RQ ~ 2.0; Pathogenesis-related protein 5, *PR5* — log10RQ ~ 4.0 (!); Pathogenesis-related protein 14 = nsLTP — log10RQ ~ 2.0. So, in leaves and roots of infected plants ShVX programs dynamical and coordinated process of TRP and downregulation of genes coding for core RNAi components and disease resistance proteins might be correlated with successful virus reproduction and persistent virus infection establishment. We are of opinion that plant viral-specific PRRs identification, plant viral PAMP-triggered PTI and PRR (Pattern recognition receptors)-mediated transcriptomic reprogramming mechanisms ascertainment are the main tasks of coming antiviral plant immunity research period. Cloning of plant PRRs involved in plant virus PAMPs recognition, and the inter-species transfer of plant virus-sensing PRRs are the promising future technologies for broad spectrum antiviral resistant plants creation (D. Bao et al., 2017).

Keywords: allexiviruses, Shallot virus X, *Allium cepa* L. var. *aggregatum* L.G. Don, persistant infection, RNA-silencing, plant innate immune system, Pattern-triggered immunity (PTI), transcriptomic reprogramming.

Plants possess complex protective antiviral system with the key role of RNA silencing (the molecular mechanism induced by viral double-stranded RNA) controlling virus replication and manifestation of the symptoms [1, 2]. In this context the activity of viral suppressor proteins which prevent viral RNA fragmentation or blocking of the viral RNA translation during silencing has been highlighted as an indispensable condition for successful reproduction of phytoviruses [3, 4]. It is known, however, that many phytoviruses encode inactive, very weak or transiently active suppressors [5]. In particular, as we have shown earlier [6], Shallot virus X (ShVX), the prototype of the genus *Allexivirus*, can successfully multiply and establish persistent symptomless infection in the absence of an active suppressor protein; therefore, it overcomes the immune barrier of silencing using some others mechanism(s). We suggest that such a mechanism could be transcriptomic reprogramming (TRP) [7, 8] caused by PTI (Pattern-triggered immunity), the first "defense line" of the innate immune system of plants [9]. As a result of TRP, expression of a number of target genes involved in the reproduction of phytoviruses can be selectively changed, in particular, expression of the RNA silencing key factors, i.e. DCL proteins, Argonaute proteins (Ago), and cellular RNA-dependent RNA polymerases (RDR), can be suppressed to a critical level. The results we obtained earlier testify in favor of this assumption: DCL and RDR genes' transcription have been repressed in roots and leaves of ShVX infected plants [10].

Experimental data obtained in the past 2-3 years in several foreign laboratories indicate that phytoviruses, like bacterial pathogens, induce a process similar to classical PTI [11-13], and in this context the double-stranded replicative forms of viral RNA act as virus-specific PAMPs (pathogen-associated molecular patterns) [12, 13]. Thus, at least two antiviral mechanisms triggered by double-stranded RNAs, i.e. RNA silencing and PTI, function in a plant cell. In Russia, the studies of the molecular mechanisms of antiviral phytoimmunity have not yet received proper development.

In is commonly deemed that the pattern recognition receptors (PRRs), RLKs (receptor-like kinases) or LRR-RKs (leucine-rich repeat receptor kinases), localized on plant cell plasma membrane, specifically recognize conservative PAMPs, such as bacterial flagellin, and thus initiate PTI [14-18]. During the interaction of molecular patterns with PRRs, immediate and intensive induction of transcriptomic reprogramming occurs. This particularly results in differential expression of a number of proteins which are the PTI markers [19]. Specific antiviral PRRs of a plant cell have not been identified to date, although their existence is quite convincingly proved by the recent study of the role of multifunctional co-chaperone Hop/Sti1 in the symptoms of potato virus Y infection [20].

This paper is the world's first attempt to obtain the experimentally evidence in witness of PTI induction in shallot plants under persistent viral infection and the participation of this mechanism in transcriptomic reprogramming. The objective was to experimentally test the authors' hypothesis [10] of the transcriptomic reprogramming key role as a mechanism of RNA silencing suppression, as well as the assumption that transcriptomic reprogramming is associated with PTI and, as a result, the expression of many genes involved in reproduction of phytoviruses (e.c. TCTP-complex) can be selectively changed [21]. In particular, our findings show that, due to the TRP, expression of all silencing factors and some R-genes, e.c. the *Tm2²* gene homologs, is suppressed.

Techniques. Shallot plants (*Allium cepa* L. var. *aggregatum* L.G. Don) were

grown from bulbs under ShVX persistent infection. In each experiment, 5-6 bulbs of one shoot cluster were planted, and in 3 days and 2 weeks the bulk samples of roots, seedlings and leaves were collected.

Total RNA isolation and ShVX detection by PCR method were described earlier [10].

Nucleotide sequences encoding homologs of selected target genes of *A. cepa* L. transcriptome (the species which is the closest to shallot plants) were searched using tblastn, tblastx software and the TSA database (Transcriptome Shotgun Assembly, https://www.ncbi.nlm.nih.gov/gen-bank/tsa/). The primers specific for the shallot homologs of the examined target genes were designed as described earlier [10, 23]; a set of primers was generated with Primer3 v.4.1.0 program (http://primer3.ut.ee/).

Transcripts, encoding target proteins, were detected by real time PCR using the Comparative CT experiment (delta-delta CT algorithm; virus-free shallot seedlings as a calibrator; 18S RNA as a normalizer) which seems to be optimal for dynamic process of transcriptomic reprogramming [22]. An amplifier was 7500/7500 Fast Real-Time PCR Systems or QuantStudio (Applied Biosystems, USA), a set of reagents was SYBR® Green Reagents (OAO Syntol, Moscow), 3-fold biological and 4-fold technical repetitions were used.

Target gene expression levels were calculated, statistical processing made and histogram constructed with embedded software Design & Analysis Software v.1.4.3 (Applied Biosystems, USA). The figures show the mean logarithmic RQ values and their deviations (RQ_{min} and RQ_{max}).

Results. Table 1 shows the PTI markers, and Table 2 shows the corresponding primers. In the first series of experiments, the profiles of transcripts of 10 classical PTI markers (Fig. 1) were investigated.

1. Target genes which expression was investigated in shallot (*Allium cepa* L. var. *aggregatum*) plants persistently infected by shallot virus X

Gene	Protein	Plant species, ortholog ID
	PTI marker genes	
ARM	Armadillo repeat family proteins	*Arabidopsis thaliana* AT3G02840
RBOHD	Respiratory Burst Oxidase Homologue D	*Arabidopsis thaliana* AT5G47910
EDS5	Mate Efflux Family Protein (Enhanced Disease Susceptibility 5)	*Arabidopsis thaliana* AT4G39030
LOX3	Lipoxygenase 3	*Arabidopsis thaliana* AT1G17420
BRI1	Bri1-like 3 (Brassinosteroid insensitive)	*Arabidopsis thaliana* AT3G13380
SOBIR	Leucine-Rich Repeat Protein Kinase Family Proteins (Suppressor Of Bir1-1/Evershed)	*Arabidopsis thaliana* AT2G31880
CRK4	Calcium-dependent protein kinase (CDPK) family proteins (Cysteine-rich receptor-like kinase 4)	*Arabidopsis thaliana* AT5G24430
SERK1	Somatic embryogenesis receptor-like kinase 1	*Arabidopsis thaliana* AT1G71830
PR1	Pathogenesis-related proteins group 1	*Arabidopsis thaliana* AT2G14610
PR5	Pathogenesis-related proteins group 5	*Arabidopsis thaliana* AT1G75040
NHL10	Late embryogenesis abundant (lea) hydroxyproline-rich glycoprotein family	*Arabidopsis thaliana* NP_181142.1
ACRE31	Avr9/cf-9 rapidly elicited protein 31	*Nicotiana tabacum* AAG43547.1
ACRE132	Avr9/cf-9 rapidly elicited protein 132	*Nicotiana tabacum* AF211532.1
	Other target genes	
DCL	Dicer-Like proteins	*Allium sativum* EPP005KGAA12S003959
RDR 6	RNA-dependent RNA polymerase 6	*Arabidopsis thaliana* NP_001327617.1
AGO	Argonaute family proteins	*Triticum aestivum* AGB34311.1
Tm2²	ToMV resistance protein	*Solanum lycopersicum* AAQ10736.1
PR6	Pathogenesis-related proteins group 6	*Arabidopsis thaliana* NP_199170.2
LTP	Lipid transfer proteins (Pathogenesis-related proteins group 14), (Antimicrobial protein Ace-amp1 precursor mRNA)	*Allium cepa* AF004946.1
WRKY	WRKY transcription factors family proteins	*Arabidopsis thaliana* AEC09374.1
TCTP	Translationally controlled tumor proteins	*Jatropha curcas* EF091818.1
PIRL	Plant intracellular Ras group-related LRR proteins	*Arabidopsis thaliana* NP_196204.1
DBP1	DNA-binding protein phosphatase 1	*Arabidopsis thaliana* NP_001324148.1
CBP60g	CBP60G (Calmodulin-binding protein 60 G)	*Arabidopsis thaliana* OAO89604.1
GRF6	G-box regulating factor 6	*Arabidopsis thaliana* NP_001190276
FRK1	FRK (Fertilization-related kinase 1)	*Arabidopsis thaliana* OAP09570.1

Note. The orthologs are indicated which allow us to construct corresponding primers (see Tables 2, 3).

2. Nucleotide sequences of primers used in determining the expression levels of Pattern-Triggered Immunity (PTI) markers in shallot (*Allium cepa* L. var. *aggregatum*) plants persistently infected by shallot virus X (qPCR)

Primer	Sequence	ID of the transcript *Allium cepa* L. in the database TSA (NCBI)
ARM 530-L	5′- ATGATGCGGGCCTAGTAGAC -3′	GETF01031504.1
ARM 766-R	5′- CTCCCTCGATCAGTCCACTC -3′	
RBOHD 2641-L	5′- GTTTGATCCTAGACGACGCG -3′	GBRN01002659.1
RBOHD 2868-R	5′- TCAACATACCCGACCCGAAA -3′	
EDS5 303-L	5′- TCGCTTGGTCTTGGCTTCTA -3′	GBRQ01023449.1
EDS5 549-R	5′- CGTCTGAGAATCCAACGACG -3′	
LOX3 1753-L	5′- ATGCCACTCGTACGCTTTTC -3′	GBRQ01024907.1
LOX3 1943-R	5′- GACGCCTGCATCATTAGAGC -3′	
BRI1 1541-L	5′- TGTTCCCGCTCAGCTGATTA -3′	GBRQ01031765.1
BRI1 1749-R	5′- TACTTTCGGTGGCAATGGGA -3′	
SOBIR 812-L	5′- CAAGTCATGCAAGCTTCCGT -3′	GBRQ01012958.1
SOBIR 1041-R	5′- CTGGAAAGATGATCGCGGTG -3′	
AtCRK4 1496-L	5′- CTTTCTTGACCTTGGCCTCG -3′	GBGJ01061169.1
AtCRK4 1731-R	5′- TCCCCAGCTAAGCACATCAA -3′	
SERK1 1291-L	5′- CTTCTTCAGCGGGAACATCG -3′	GBGJ01064935.1
SERK1 1478-R	5′- TCCACCTCCTCCATTTGTCC -3′	
PR1 443-L	5′- GTCAAGATCGGTTGCGCTAG -3′	GBJZ01171295.1
PR1 660-R	5′- CCAAGCAAACTCTCATCGCA -3′	
PR5 616-L	5′- ACTGTCTACGGGCCCAAAAT -3′	GBGJ01079964.1
PR5 803-R	5′- ATATGCTGCCTCCGGAACTC -3′	
18s rRNA-L	5′- CATCAGCTCGCGTTGACTAC -3′	[22]
18s rRNA-R	5′- GATCCTTCCGCAGGTTCAC -3′	

3. Nucleotide sequences of primers used in determining the expression levels of RNA silencing factors, NB-LRR receptors and genes involved in viral reproduction in shallot (*Allium cepa* L. var. *aggregatum*) plants persistently infected by shallot virus X (qPCR)

Primer	Sequence	ID of the transcript *Allium cepa* L. in the database TSA (NCBI)
Ago-L-672	5′-AACTCCCAAGAAGCTTTGCG-3′	GBGJ01050630.1
Ago-R-851	5′-CCCTCCTTGAGCAGTTCTGA-3′	
$Tm2^2$-L-3013	5′- TCGTGGGCTCTTTCACTGAT-3′	GBRN01023560
$Tm2^2$-R-3257	5′- CACCCGCTTCATTGGTGTAG-3′	
FRK1-L-421	5′-AGTCACGCTCAATGGCAATG-3′	GAAO01012059.1
FRK1-R-635	5′-CTGCCGCAACATCATAGCAT-3′	
NHL10-L-327	5′-TGCTCCTCACATCGTTCACA-3′	GBRQ01023138.1
NHL10-R-572	5′-AGCTCACCACCTTCACTCTC-3′	
ACRE31-L-473	5′-GCAGTTCTTCGAAAGCAGGA-3′	GBRO01073928.1
ACRE31-R-699	5′-ATTGAGCACATCCTCCCCTT-3′	
ACRE132-L-272	5′-GCCATGCCTCAACCTGATTT-3′	GBRQ01011764.1
ACRE132-R-451	5′-CCTTCTTGATCGGGAAAGCG-3′	
PR6-L-100	5′-ATGAGGGGTACATGGCAGAC-3′	GBRQ01165078.1
PR6-R-274	5′-AAGCATCGGAAGCGAAGAAG-3′	
LTP pcr-59- L	GCA-GTC-CGT-ATG-CAA-AT	AF004946.1
LTP pcr-274-R	TAG-GGT-TTC-GTC-TCA-GAC-CG	
WRKY-L-1367	5′-ACGTGGAAAGGGCATCAAAC-3′	GBRO01047677.1
WRKY-R-1550	5′-GCGACCGGTCTTTGAACATT-3′	
TCTP-L-172	5′-AGGGCAAGTGGGTAGTTCAA-3′	JR844934.1
TCTP-R-382	5′-TCCAATTTCGAAGGCAGAAGT-3′	
PIRL-L-84	5′-ATCATGGATCCAAGCCCCAA-3′	GAAN01019083
PIRL-R-377	5′-CTTTGCGAGGTCAACAGCTT-3′	
DBP-L-392	5′-AGGGTCGTTGTGCTCTTGTA-3′	GBRO01024689.1
DBP-R-623	5′-CAACGGTCAGCTCAACGTAG-3′	
CBP-L-429	5′- GAAGCAGAGGGAAAGCAACC-3′	GBRO01059419.1
CBP-R-673	5′- AAGCCAACACCATCATGCAG-3′	
GRF6-L-607	5′-TCCAGTCTTGAATTCGGCCA-3′	GAAN01023832.1
GRF6-R-829	5′-TTCGATCGAGCAGAAGGAGG-3′	
18S rRNA-L	CATCAGCTCGCGTTGACTAC	[22]
18S rRNA-R	GATCCTTCCGCAGGTTCAC	

Our results show (see Fig. 1) that, to some extent, viral infection affects expression of all the examined genes; however, a group of genes with very high expression both in leaves and in roots of the infected plants is revealed, viz.

SOBIR with log10RQ ~ 1.0, ARM with log10RQ ~ 2.0, PR1 with log10RQ ~ 2.0, and PR5 with log10RQ ~ 4.0 (!). Therefore, it can be concluded that ShVX during the initiation of infection interacts with the factors involved in PTI and TRP is triggered by the PTI induction.

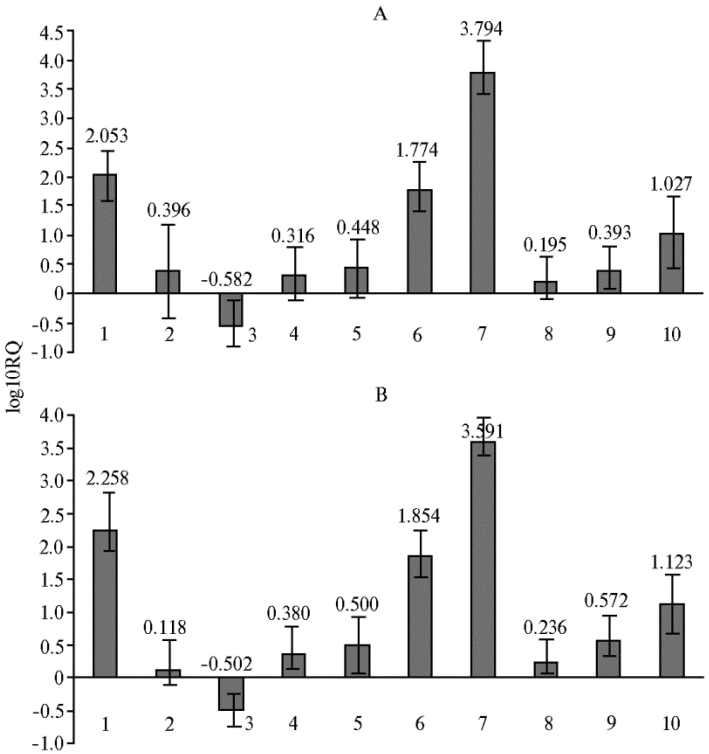

Fig. 1. Transcripts of the Pattern-Triggered Immunity (PTI) marker genes in leaves (A) **and roots** (B) **of shallot (*Allium cepa* L. var. *aggregatum*) plants persistently infected by shallot virus X 2 weeks after the bulb planting:** RQ — Relative Quantification (changes in mRNA expression level compared to the internal control RNA). Symbols of the PTI marker genes are given in Table 1.

At the initial stage of the infection in seedlings (Fig. 2, A), the expression of all target genes (Table 3) is activated (except for *AGO* and *PIRL*), including four additional PTI markers and nsLTP (= PR14, Pathogenesis-Related Proteins Group 14). After 2 weeks of infection, the opposite pattern is noted (see Fig. 2, B). The expression of most target genes, including all RNA silencing factors, three PTI markers, NB-LRR receptors and CBP60g proteins that control the synthesis of salicylic acid, is suppressed in the leaves (Argonaute proteins more than others). However, within the same time frames, the expression of ACRE 132 and LTP (PTI markers) in the leaves show a noticeable tendency to growth, while TCTP expression remaines quite high and stable.

In the roots (Fig. 3, A, B), inhibition of the target genes expression occurs at the initial stage of infection, and after 2 weeks most of the target genes are suppressed, but LTP expression is slightly reduced.

These results lead to the conclusion that in persistent infection ShVX induces the dynamic TRP in roots and leaves, a selective change in the expression of many target genes, including the genes encoding PTI marker proteins, RNA silencing factors, NB-LRR receptors, lipid transfer proteins, as well as the proteins involved in viral replication (TCTP complex). Ultimately, in the examined pathosystem, expression of all silencing factors and some NB-LRR receptors is

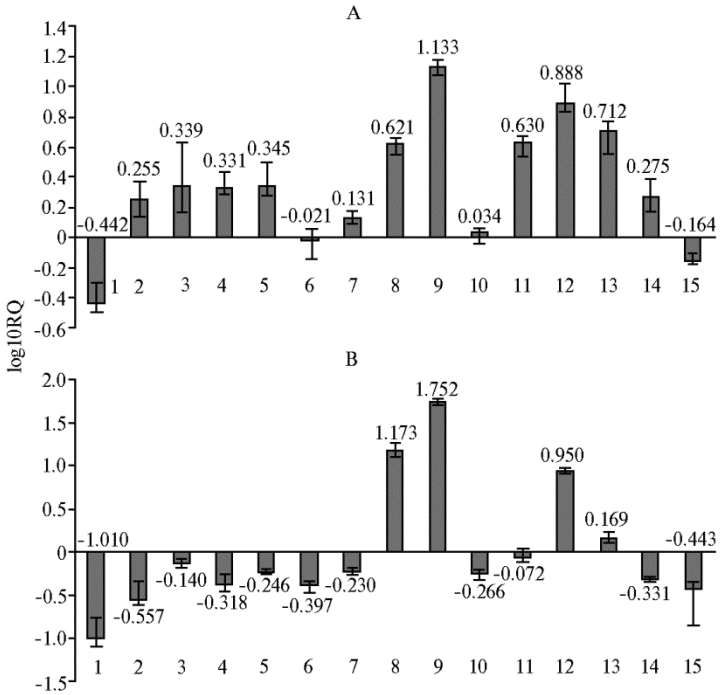

Fig. 2. Target gene transcripts in the above-ground organs of shallot (*Allium cepa* L. var. *aggregatum*) plants at different stages of shallot virus X infection: A — seedlings (3 days after the bulbs were planted), B — leaves (2 weeks after the bulbs were planted); RQ — Relative Quantification (changes in mRNA expression level compared to the internal control RNA).

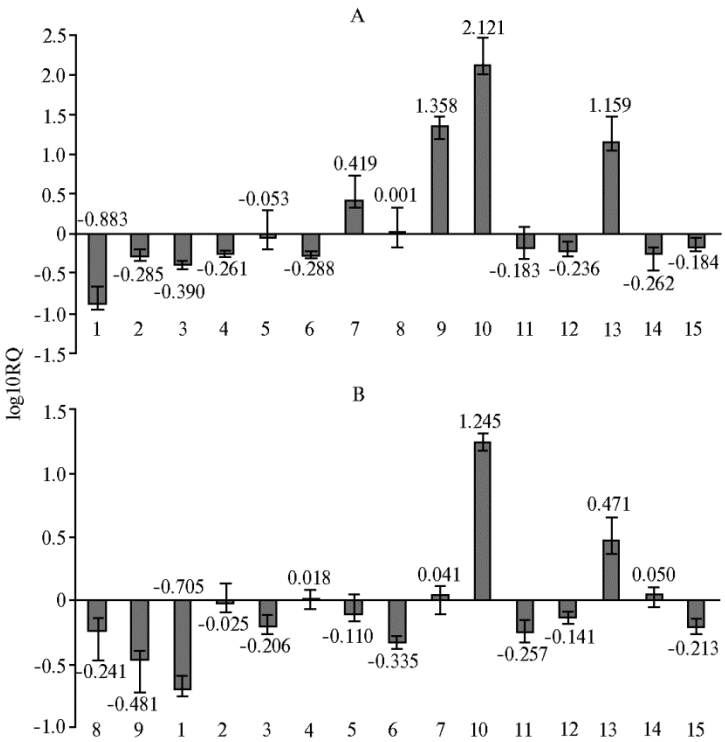

Fig. 3. Target gene transcripts in the roots of shallot (*Allium cepa* L. var. *aggregatum*) plants at different stages of shallot virus X infection: A — 3 days after the bulbs were planted, B — 2 weeks after bulbs were planted; RQ — Relative Quantification (changes in mRNA expression level compared to the internal control RNA).

suppressed, which confirms our initial hypothesis. High induction of the transcription factor WRKY (Group III) and the genes of PR proteins, e.c. PR1, PR5, and PR14 (nsLTP), should be noted as a characteristic feature of reprogramming.

We believe that the induction of PTI, ETI (Effector-triggered immunity) [9] and the resulting transcriptomic reprogramming are the mechanisms, common to all RNA-containing viruses. Due to receptor and signaling functions inherent to innate immune system factors, numerous and diverse immune responses are activated in plants, including RNA silencing. Their goal is to control the reproduction of the virus, but not completely eliminate it. For their part, viruses have developed mechanisms to counteract the host plant immune responses, in particular through suppressor proteins. Many phytoviruses encode very weak, transiently active or inactive suppressors. Thus, from the point of view of the virus, RNA silencing is not a perfect weapon of a cell: it can be ignored or weakened by the suppressor proteins or transcriptomic reprogramming. The virus's choice of the way to suppress the antiviral immunity depends on how the host plant resolves the dilemma of the concept of "growth-defense tradeoffs" [24].

The TRP process described in this paper has organ specific and a pronounced biphasic character. At early stages of the infection, the expression of most target genes is activated, and at later stages it is suppressed. Analysis of the experimental facts also leads to the conclusion that in seedlings, leaves, and roots of the infected plants the transcriptomic reprogramming is likely induced by PTI, but not silencing involving a paralog of Ago-protein family. This paralog is not identified in this work, but its expression, as follows from the data (see Figs. 2, 3), is intensely suppressed at all stages of the infection. However, it can be assumed that at some stage of infection, transcriptomic reprogramming may also be due to the activity of a specific complex of silencing factors (for example, Ago2 + DCL4 + RDR1), the expression of which was probably not suppressed under the conditions of this experiment. As a result of this activity, endogenous small interfering RNAs of an extensive class of virus-activated cellular siRNA (virus-activated siRNAs, vasiRNAs) are generated [25] the targets of which are different genes of a host plant. It is also possible that in persistent ShVX infection the induction of transcriptomic reprogramming may be due to RNA silencing involving small interfering RNA the source of which is the viral genome [26]. Moreover, a yet unknown mechanism of the studied pathosystem may change expression of certain types of microRNAs that control the TRP process [27].

In our opinion, identification of plant antiviral PRR involved in PTI and elucidation of the molecular mechanisms of interaction of these receptors with double-stranded replicative viral RNA will bring to better understanding of TRP induction and its role in antiviral phytoimmunity. The fundamental features of the PTI process induced by viral PAMPs, suggest that the cloning and interspecific gene transfer of plant antiviral PRRs may be promising for creating cultivars with high long-term resistance to a wide range of pathogenic viruses [28].

Thus, our experiments, for the first time in the world, shown that persistent infection of shallot virus X induces in the roots and leaves of shallot plants a dynamic process of transcriptomic reprogramming, resulting in a change in the expression of a wide range of target genes, including those encoding PTI marker proteins, RNA silencing factors, NB-LRR receptors, PR proteins, as well as the proteins involved in virus replication.

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EFFECT OF *Bacillus subtilis* BASED MICRROBIALS ON PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF SUGAR BEET (*Beta vulgaris* L.) PLANTS INFECTED WITH *Alternaria alternata*

O.V. LASTOCHKINA[1, 2], L.I. PUSENKOVA[1], R.A. YULDASHEV[1, 2], E.Yu. ILYASOVA[1], S. ALINIAEIFARD[3]

[1]*Bashkir Research Institute of Agriculture, Subdivision of the Ufa Federal Research Centre RAS,* Federal Agency for Scientific Organizations, 19, ul. Riharda Zorge, Ufa, 450059 Russia, e-mail oksanaibg@gmail.com (✉ corresponding author);
[2]*Institute of Biochemistry and Genetics, Subdivision of the Ufa Federal Research Centre RAS,* Federal Agency for Scientific Organizations, 71, prosp. Oktyabrya, Ufa, 450054 Russia, e-mail yuldashevra@gmail.com;
[3]*University of Tehran, Aburaihan campus,* PC 3391653775 Pakdasht, Tehran, Iran, e-mail aliniaeifard@ut.ac.ir
ORCID:
Lastochkina O.V. orcid.org/0000-0003-3398-1493
Pusenkova L.I. orcid.org/0000-0001-6341-0486
Yuldashev R.A. orcid.org/0000-0001-9033-6867
Ilyasova E.Y. orcid.org/0000-0001-5737-5469
Aliniaeifard S. orcid.org/0000-0001-9572-2839
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Abstract

Phytopathogenic *Alternaria* fungi are economically important causative agents of sugar beet (*Beta vulgaris* L.) leaf diseases which significantly reduce root yield and quality. Promising agents for plant disease biocontrol are *Bacillus subtilis* based biologicals due to the ability to stimulate plant growth and immunity to many biotic stressors. Starting our experiments, we could not find publications on *B. subtilis* effects towards physiological parameters of sugar beet plants affected by *Alternaria*. This paper is the first to report that *B. subtilis*-based biologicals including novel Bashkirian isolate *B. subtilis* 10-4 prevent a decrease in leaf photosynthetic activity in sugar beet plants affected by *A. alternata*, activate hydrolytic enzyme inhibitors, suppress proline production, and increase sugar content in roots. Our objective was to estimate effects of Fitosporin-M, Vitaplan, and endophytic strain *B. subtilis* 10-4 on leaf photosynthetic pigments (chlorophyll a, b and carotenoids), leaf area index, activity of hydrolases (proteases and amylases) and their inhibitors, as well as proline and sugar levels in leaves, root level of sucrose, and productivity in healthy plants as compared to those artificially infected with *A. alternata*. Our results show that Vitaplan, Fitosporin-M and strain *B. subtilis* 10-4 when used twice increase the concentrations of photosynthetic pigments (chlorophyll a, b and carotenoids) 1.2-1.9-fold in healthy plants whereas a decrease in photosynthetic activity in *A. alternata*-infected plants is 1.2-1.5 times lower, the leaf area is 30 % higher and leaf weight increases 1.8-2.9 times compared to the untreated plants. *A. alternata* infection increased the activity of hydrolases (proteinase, amylase) and suppressed their inhibitors, which indicates the intensive development of the pathogen and a decrease in plant resistance to enzymes produced by pathogen during plant tissue colonization. On contrary, biologicals suppress hydrolases and increase activity of their inhibitors both in infected and healthy leaves, which points out to the induction of protective reactions against *A. alternata* in plants. Interestingly, *B. subtilis* 10-4 and Fitosporin-M ensure the maximum activation of protective proteins. Furthermore, biologicals decrease stress-induced accumulation of proline and sugar, the markers of plant resistance to extremal factors in plants, which is in line with protective effect as well. Also, proline and sugar levels slightly elevated in healthy plants treated with the biologicals, which accentuate the role of these substances in induced resistance to *A. alternata*. Ultimately, larger roots with higher sucrose content confirm the positive effect of the used biologicals among which Fitosporin-M and strain *B. subtilis* 10-4 provide the maximum effect.

Keywords: *Bacillus subtilis*, photosynthetic pigments, hydrolases, sugar, proline, sucrose, *Alternaria alternata*, resistance, productivity, *Beta vulgaris* L., sugar beet

Leaf diseases caused by pathogenic *Alternaria* fungi significantly reduce the productivity and quality of sugar beet plants (*Beta vulgaris* L.), an important sugar crops which serves as a source of raw materials for the sugar, food, confectionery, alcohol industries, bioethanol, fertilizers, animal feed manufacture [1]. Affected plants suffer from Alternaria spot which is characterized by spot for-

mation on the leaves surface [2]. Physiological functions are violated, anatomic-morphological indicators change, yield decreases, separate parts of the plant die, which leads to their complete destruction [3]. The premature loss of the assimilation area of the leaf apparatus, caused by Alternaria spot, leads to loss of plastic substances of the roots, spent on the formation of new leaves, the growth of the roots mass slows down, and the sucrose content reduces [2].

The advantages of the use of biological preparations for plant health improvement in comparison with chemical means of protection are the ecological safety and systemic immunomodulatory action [3, 4]. Promising agents for plant disease biocontrol are *Bacillus subtilis* based biologicals due to their antagonism to pathogens and positive effects on the productivity of crops [4-6]. Growth-stimulating and protective effects of these drugs are shown in many plant species [7-9] and in relation to various stress factors of biotic and abiotic nature [10-12]. It is considered that this action is due to the ability of *B. subtilis* to produce biologically active substances (insecticidal and antimicrobial components, phytohormones, siderophores, and chelators) [13-15], to reduce the content of ethylene in plants, to improve nitrogen fixation, absorption of macro- and microelements [16], to launch mechanisms of systemic plant resistance in response to stress [17] by activating salicylate- and jasmonate-dependent signaling pathways [18-20].

Hydrolytic enzymes (amylases, pectinases, and proteases) and their inhibitors [21-23] play an important role in the induction of plant resistance to pathogens. On the model potato and sugar beet plants, it is shown that the introduction of biologicals on the basis of *B. subtilis* promotes the activation of protease inhibitor synthesis and protects plants from the penetration and development of pathogenic microorganisms [6]. The development of protective reactions of plants to stresses of different nature can also be judged by the degree of accumulation of proline and sugars in them, which serve as markers of the resistance formation in extreme situations [24-26]. At the same time, despite the significant amount of experimental data, the sequence of protective mechanisms induced by *B. subtilis* is not completely clear. Starting the experiments, the authors could not find publications on *B. subtilis* effects towards the photosynthetic activity of sugar beet leaves as an integral characteristic of the physiological state of the whole plant and the nature of changes in the content of proline and sugars in the leaves in the conditions of infection with the pathogens of the Alternaria spots.

As a result of the research, the authors have revealed for the first time that the introduction of biological preparations based on *Bacillus subtilis* prevents the reduction of photosynthetic activity of the sugar beet leaf apparatus induced by the pathogen of the Alternaria spots, and initiates protective reactions, including the activation of inhibitors of hydrolytic enzymes, increasing the content of proline and sugars. It reduces the damaging effect of the pathogenic *A. alternata* fungus on sugar beet plants and promotes the formation of large root crops.

The work objective was to estimate the effects of Fitosporin-M, Vitaplan, and strain *Bacillus subtilis* 10-4 on the physiological and biochemical parameters and productivity of sugar beet infected with *Alternaria alternata*.

Techniques. The investigations were carried out on sugar beet (*Beta vulgaris* L.) plants, Kampai variety (OOO AgroSem-Invest, Krasnodar). In the experiments, the biological preparations Fitosporin-M (*B. subtilis* 26D, NVP Bashinkom, Ufa) (P, 30 g/10 l), Vitaplan (*B. subtilis* 2604D + *B. subtilis* 2605D, ZAO Agrobiotekhnologiya, Russia) (SP, 20 g/ha) and a new strain of *B. subtilis* 10-4 (Bashkir Research Institute of Agriculture; 10^5 CFU/ml) were used [8]. Plants were sprayed with suspensions of biological preparations 2 times, in the phase of 2-3 pairs and 4-6 pairs of real leaves, at a flow rate of 300 l/ha.

Field trials were carried out in the pre-Ural steppe zone of the Republic of Bashkortostan (OOO Chishmy Agroinvest) in 2013 on small plots (5 m^2). The soil is leached chernozem (pH 5.25), Hg 5.50 mg eq/100 g of soil, humus content 8.69%, potassium and phosphorus 29.0 and 23.0 mg/100 g, respectively. Sugar beet was planted according the terms generally accepted for the region; seedlings emerged on days 12-14. The shoots were artificially infected by applying 100 μl of the *A. alternata* fungus suspension (10^6 CFU/ml). The disease development was assessed visually during the growing season by a 5-point scale: 0 points no symptoms, 1 point lesion from 1 to 25% of the leaf area, 2 points lesion from 26 to 50%, 3 points lesion from 51 to 75%, and 4 points more than 75%.

During vegetation (phases of 4-6 pairs of leaves, closure of leaves in rows, technical maturity), control (untreated and healthy) and experimental (treated with biological preparations and infected by *A. alternata*) whole plants or detached roots and shoots were selected three times to assess physiological and biochemical parameters.

Chlorophyll a and b content was determined according to S.W. Jefferey et al. [27]. Leaf samples were weighed, ground, mashed in a mortar calcium carbonate and 90% acetone (at the rate of 0.05 g of leaves per 10 ml acetone); the obtained extract was filtered. The optical density of extracted pigments was measured at λ = 436 nm and λ = 680 nm (a UV-2401PC spectrophotometer, Shimadzu, Japan). The concentration of carotenoids in the total extract of the pigments was calculated by the P. Wettstein's formula [28].

The proline formation in leaves was evaluated by method of L.S. Bates et al. [29] modified by L.G. Kalinkina [30]. For this purpose, 2 g of the test material was taken and poured with 2.5 ml of boiling distilled water. The tubes were brought to a boil in a water bath and cooled. Then, tubes with 2 ml of the cold test, 2 ml of ninhydrin reagent, 2 ml of glacial acetic acid were placed in a water bath, boiled for 1 h and cooled. The color density of the proline complex with ninhydrin was determined (λ = 522 nm, a SF-26 spectrophotometer, LOMO, Russia). The proline content was determined by the calibration curve using standard solutions of chemically pure L-proline (Sigma Aldrich, USA).

The enzymatic activity of proteinases, amylases, and their inhibitors was determined by the spectrophotometric method [21, 31], accumulation of sugar in leaves – by the photometric method with the use of 2,4-dinitrophenol according to GOST R 51636-2000, the amount of sucrose in roots by the cold water digestion method with the use of the polarimeter P161-M (Russia) [32]. The leaf area was measured with a photoplanner, aerial parts of plants and roots were mass with the weighed [33].

All experiments were carried out in 3-4 biological and 4-5 analytical repeats. Statistical processing was performed with STATISTICA 6.0 software (StatSoft, Inc., USA.) The figures and tables show the mean values (*M*) and their standard deviations (±SD) at P = 0.95.

Results. Alternaria spot affects the leaf surface of plants, forming spots, and leads to a decrease in the photosynthetic surface of the leaves [2]. Photosynthesis is the main process in the formation of plant productivity; the total biological yield of crops depends on its intensity [34]. In turn, the content of the main photosynthetic pigments (chlorophylls a, b, and carotenoids) is one of the indirect indices of the photosynthetic activity and the most important biochemical indicator of the plant, which determines the intensity of photosynthesis [24, 34]. In the experiments, infection of sugar beet with *A. alternata* led to a decrease in the content of chlorophyll a (up to 1.5-fold) and b (up to 1.2-fold) in the leaves compared to healthy plants (Fig. 1), which indicates a violation of the photosynthesis process and a decrease in the photosynthetic activity of plants.

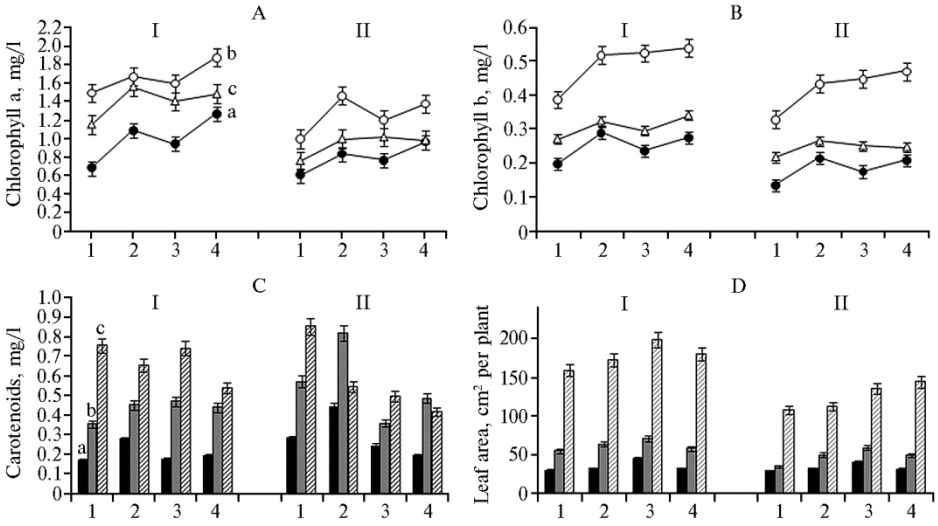

Fig. 1. The content of chlorophyll a (A)**, chlorophyll b** (B)**, carotenoids** (C) **and leaf area** (D) **in healthy** (I) **and infected with *Alternaria alternata*** (II) **sugar beet (*Beta vulgaris* L.) plants of variety Kampai treated with biological preparations:** 1 C — control, 2 — Fitosporin-M, 3 — Vitaplan, 4 — *Bacillus subtilis* 10-4; a — the first treatment, b — the second treatment, c — harvesting (OOO Chishmy Agroinvest, the Republic of Bashkortostan, 2013).

Inoculation with *B. subtilis* 10-4 and treatment with Fitosporin-M and Vitaplan biologicals restored the photosynthetic activity of plants. For example, 2-fold treatment with biological preparations prevented stress-induced reduction of chlorophyll a and b in all variants of the experiment (see Fig. 1, A, B). The content of carotenoids in the leaves increased when infecting with *A. alternata* (see Fig. 1, B). Treatment with Vitaplan and *B. subtilis* 10-4 contributed to the decline of their number, whereas after two treatments with Fitosporin-M, a significant accumulation of carotenoids more than the control values was observed. However, the content of carotenoids decreased to harvesting and was comparable with that in the variants in which Vitaplan and the strain 10-4 were used (see Fig. 1, B). It is necessary to note that in uninfected plants, although 2-fold treatment with biological preparations led to a slight increase in the number of carotenoids, this figure was lower than in the control variant (see Fig. 1, B).

The introduction of biological preparations under normal growing conditions stimulated the photosynthetic activity of plants, probably due to an increase in the content of physiologically active chlorophyll a. Indeed, the results obtained in assessing the leaf surface area (see Fig. 1, D) correlated with the effect of the studied compounds on the chlorophyll a and b content. Plants treated with bioactive preparations in all variants during the whole vegetation period were characterized by a much larger leaf area, both without and with *A. alternata* infection (see Fig. 1, D).

It is obvious that the revealed increase in the content of photosynthetic pigments in case of the use of biologicals in the conditions of infection with *A. alternata* (see Fig. 1, A, B, C), in addition to their direct role in the process of photosynthesis and increasing leaf size (see Fig. 1, D), may contribute to the development of protective reactions of plants [34]. In particular, carotenoids perform photoprotective and antioxidant functions [35-38] by preventing damages caused by the formation of singlet oxygen and triplet chlorophyll [37]. In addition, they can take the excitation energy of triplet chlorophyll, and then dissipate it as heat or extinguish singlet oxygen molecules [38, 39]. However, it is necessary to note that despite the obvious role of carotenoids in the antioxidant protection

of plants, data on changes of their content under the influence of stress are very contradictory [22, 24, 36]. Such effects can be explained, on the one hand, by the induction of carotenoids formation under the influence of the stress factor, on the other hand, by its enhanced degradation under severe stress [22].

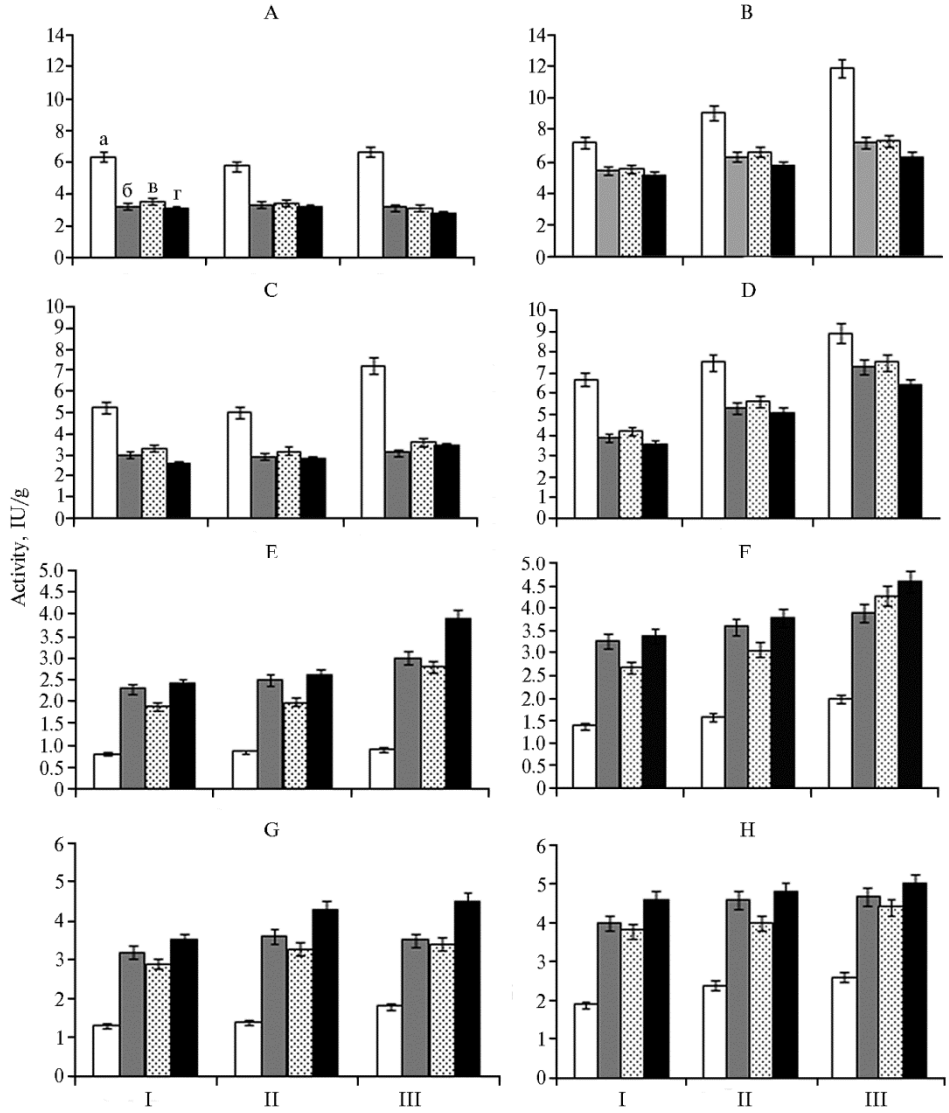

Fig. 2. Activity of proteinases (A, B)**, amylases** (C, D)**, proteinase inhibitors** (E, F) **and amylase inhibitors** (G, H) **in leaves of healthy** (A, C, E, G) **and infected with *Alternaria alternata*** (B, D, F, H) **sugar beet (*Beta vulgaris* L.) plants of variety Kampai treated with biological preparations:** a — control, b — Fitosporin-M, c — Vitaplan, d — *Bacillus subtilis* 10-4; I — the first treatment, II — the second treatment, III — harvesting (OOO Chishmy Agroinvest, the Republic of Bashkortostan, 2013).

Hydrolytic enzymes (proteases, amylases) and their inhibitors play an important role in the formation of plant protective reactions to pathogens [21]. In infection with *A. alternata*, the increase in the activity of proteinases and amylases (Fig. 2, B, D) in the leaves of sugar beet occurred which indicates the intensive development of the pathogen in plant tissues. Probably, this process was caused by changes in the metabolism of the host plant under the influence of the pathogen, as well as by the secretion of hydrolytic enzymes by the pathogen itself capable of macerating tissues and destroying the components of the

cell wall, which allows the pathogen to overcome the natural resistance of the host plant. Plants treated with the strain of *B. subtilis* 10-4, Fitosporin-M and Vitaplan were characterized by a decrease in the activity of hydrolases in the infected and healthy leaves (Fig. 2, A-D), and the greatest decrease in hydrolytic activity was observed in variants with the use of Fitosporin-M and the strain of *B. subtilis* 10-4.

A significant contribution to the regulation of the hydrolytic enzymes activity is made by protein inhibitors of plants that suppress the activity of their own and foreign enzymes, in particular, pathogenic fungi and bacteria [23, 40]. The activity of hydrolase inhibitors decreased in the leaves of sugar beet in response to infection with *A. alternata* (see Fig. 2, E, H), as a result of which, probably, the resistance of plants to the action of enzymes of the pathogen and its spread in tissues decreased. Treatment with the studied drugs, on the contrary, contributed to an increase in the activity of hydrolase inhibitors (see Fig. 2, E, H), indicating that the under their influence plants induct protective reactions against *A. alternata*. It is necessary to note that the maximum activation of protective proteins was caused by the use of the strain of *B. subtilis* 10-4 and Fitosporin-M.

Accumulation of sugar and proline in plant mass can be important biochemical markers of resistance formation [31]. Healthy plants of *B. vulgaris* gradually accumulated sugar in the leaves throughout the growing season (Fig. 3, A), which was quite typical and consistent with the available data in the literature [41]. The highest rate of sugar accumulation was observed in the initial stages of growth, when the plant formed leaves and roots vigorously, and slowed to the end of the formation of the third pair of real leaves. Apparently, it was due to the fact that in the phase of the closure of leaves in the rows, leaves growth slowed, the intense thickening and formation of root crops, accompanied by the continuation of sugars accumulation in them, was observed.

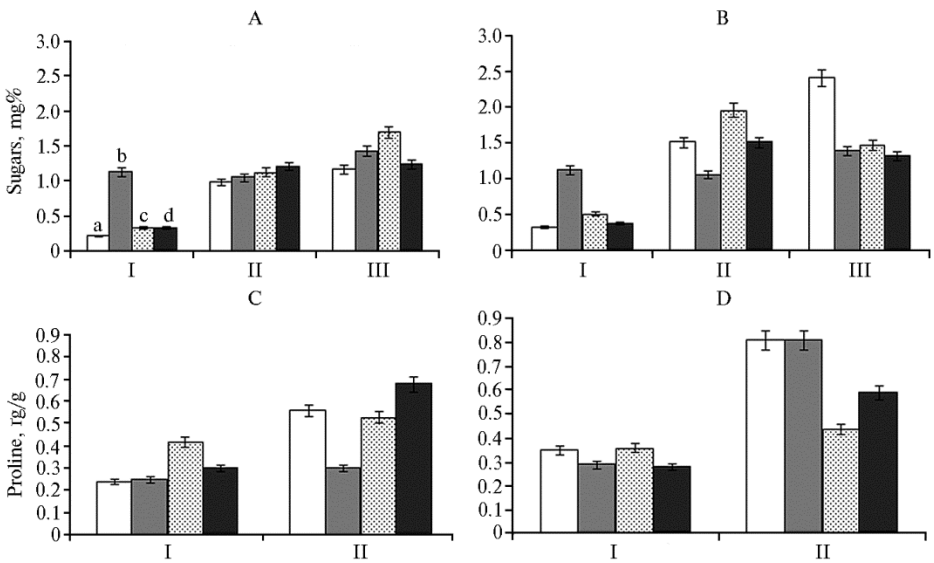

Fig. 3. The content of sugar (A, B) **and proline** (C, D) **in leaves of healthy** (A, C) **and infected with *Alternaria alternata*** (B, D) **sugar beet (*Beta vulgaris* L.) plants of variety Kampai treated with biological preparations:** a — control, b — Fitosporin-M, c — Vitaplan, d — the strain of *Bacillus subtilis* 10-4; I — the first treatment, II — the second treatment, III — harvesting (OOO "Chishmy Agroinvest", the Republic of Bashkortostan, 2013).

Infection with *A. alternata* led to a sharp increase in sugar content in the leaves compared to the control parameters of healthy plants, which apparently

performs a protective role and allows the plants to continue to grow under stress conditions (see Fig. 3, B). The properties of monosaccharides associated with an increase in the stability of biomembranes, anti-denaturation effects on proteins and antioxidant effect may contribute to this process [42]. In addition, accumulating carbohydrates help maintain the osmotic status of cells [33].

One of the most multifunctional plant stress metabolites is amino acid proline which plays the role of not only an osmolyte and antioxidant [24, 43] but also a low-molecular chaperone [40] involved in maintaining the native structure of enzymes [24]. Many investigations have reported an increase in the proline content in plants in response to the stress of different nature and its importance as a factor for plant survival in extreme situations [24-26]. However, we did not find any available data on changes of proline content in sugar beet plants in *A. alternata* infection and the use of *B. subtilis*-based preparations.

A. alternata infection led to a significant increase in the content of proline in sugar beet plants (see Fig. 3, D). At the same time, treatment with Fitosporin-M, Vitaplan and *B. subtilis* 10-4 contributed to the prevention of its accumulation, induced by stress (see Fig. 3, D). It is necessary to note that under the influence of biological preparations in healthy plants, a slight increase in the amount of proline, which further indicates the important role of this agent in the formation of induced resistance to the causative agent of Alternaria spots, was observed (see Fig. 3, B).

The combined indicator of the nature of physiological and biochemical processes for the entire period of vegetation can be the data on the external state of plants and the productivity of root crops. In the experiment, the artificial infection of plants with *A. alternata* led to a gradual increase in the affected leaf area. For example, to harvesting it reached 75% or more (4 points), while in plants treated with *B. subtilis* 10-4, Fitosporin-M, Vitaplan, less than 35% (2 points). The best effect was observed in use of *B. subtilis* 10-4 and Fitosporin-M. In these cases, the disease development did not exceed 25 and 30% respectively. At the same time, 2-fold treatment with biologicals led to a significant increase in the average weight of aerial parts of healthy plants 1.8-2.7-fold and roots crops 1.6-2.3-fold depending on the variant of the experiment (Table). Treatment with biological preparations preventes the stress-induced decline in the productivity of root crops in infection with *A. alternata* and contributes to stable growth of leaves and roots comparable to that in healthy plants.

Leaf and root weight in sugar beet (*Beta vulgaris* L.) plants of variety Kampai, healthy and infected with *Alternaria alternata*, as influenced by *Bacillus subtilis*-based microbial preparations

Variant	Aaerial part, g			Roots, g		
	I treatment	II treatment	harvesting	I treatment	II treatment	harvesting
Healthy plants						
Control	4.45±0.19	14.67±0.77	165.20±2.65	0.52±0.09	4.33±0.30	550.80±10.41
Fitosporin-M	6.11±0.30	27.96±0.82	304.80±2.32	0.92±0.19	10.42±0.48	971.00±11.89
Vitaplan	7.75±0.51	14.25±0.46	425.40±3.01	1.17±0.28	4.94±0.12	970.60±13.03
Bacillus subtilis 10-4	11.00±0.42	36.70±0.91	336.40±4.69	1.80±0.12	14.12±0.22	1142.40±12.62
Infected plants						
A. alternata	6.86±0.22	14.14±1.13	71.20±1.78	1.32±0.09	3.37±0.21	276.30±4.11
Fitosporin-M	8.84±0.14	15.61±0.91	182.00±1.99	1.81±0.12	7.11±0.29	463.00±5.33
Vitaplan	4.68±0.49	11.84±0.70	127.40±2.06	0.64±0.11	3.98±0.15	502.20±5.09
Bacillus subtilis 10-4	11.21±0.30	25.70±1.42	209.40±2.57	2.72±0.23	11.66±0.23	607.20±4.95

In addition to the positive impact on the intensity of growth processes and biomass accumulation, 2-fold application of biological preparations provided a higher content of sucrose in yield as compared to control of both healthy and infected plants. So, at harvesting in the control variant, the root crops contained 16.1% of sucrose, in tests from 17.9 to 19.0%. The maximum amount of sucrose

was in 2-fold treatment with Fitosporin-M and *B. subtilis* 10-4. In infection with *A. alternata*, the roots of all treated plants were characterized by an increased content of sucrose compared to untreated ones.

Thus, the preparations Fitosporin-M, Vitaplan, and *Bacillus subtilis* 10-4 contribute to increased synthesis of photosynthetic pigments (chlorophyll a, b, and carotenoids), increase activity of hydrolase inhibitors in leaves and reduce stress-induced accumulation of proline and sugars, providing a protective effect in infection of sugar beet plants with *Alternaria alternata*. When treated with *B. subtilis*-based microbial preparations, both healthy and infected plants show an increase in sucrose accumulation. The most effective variants were 2-fold use of Fitosporin-M and *B. subtilis* 10-4, in which the adverse impacts of *A. alternata* is smoothed to the maximum and root crops with the biggest weight and the highest sucrose content are obtained.

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REASONS OF CONTAMINATION OF PRODUCTION LOTS OF SUNFLOWER (*Helianthus annuus* L.) SEEDS BY MYCOTOXINS

A.A. BURKIN, M.I. USTYUZHANINA, E.V. ZOTOVA, G.P. KONONENKO

All-Russian Research Institute of Sanitary, Hygiene and Ecology — Branch of Federal Science Center Kovalenko All-Russian Research Institute of Experimental Veterinary RAS, Federal Agency of Scientific Organizations, 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail kononenkogp@mail.ru (✉ corresponding author), aaburkin@mail.ru, ustpuma@list.ru, ezotova63@gmail.com
ORCID:
Burkin A.A. orcid.org/0000-0002-5674-2818
Ustyuzhanina M.I. orcid.org/0000-0001-7405-7577
Zotova E.V. orcid.org/0000-0002-1479-8602
Kononenko G.P. orcid.org/0000-0002-9144-615X
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Abstract

In recent years, in our country there has been a clear trend towards sustainable growth in the production of sunflower oil. During long-term monitoring high risks of contamination with mycotoxins are established for the by-products of oil and oil-extracting industries, oil cakes and meals, which are traditionally in demand as valuable raw materials for mixed fodders (G.P. Kononenko et al., 2018). Among the possible reasons of contamination are the violations of the technology of production and storage of the final products, but the problem of the sanitary quality of oil seeds coming from the farms remains without attention. The purpose of our work was to survey the lots destined for the production of oil by comparing the contamination with mycotoxins of the main seeds and typical accompanying impurities. The average samples of sunflower oil seeds from the farms of the Belgorod, Voronezh, Kursk and Lipetsk regions of the 2016 year crop were fractionated into seeds and impurities (according to GOST 22391-2015) for separate study of their contamination. We also examined a batch of seeds stored after cleaning in two compartments, in one of which there was a shutdown of the ventilation system and, as a result, self-warming foci arose. In addition, sunflower growing plants that were collected in June-September 2016 and 2017 in the subsidiary farms of the Moscow, Tver, Voronezh and Rostov regions were analyzed for mycotoxins. Ground parts (n = 65) were cut at a height of 5 cm from the soil surface, dried in a shaded ventilated room and crushed whole. Another part of the plants (n = 29) was divided into leaves, stems and baskets before grinding. The mycotoxins group containing T-2 toxin (T-2), diacetoxycirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR) and ergot alkaloids (EA) were determined by the enzyme immunoassay. Our tests show that in seeds AOL is almost universally encountered, MPA and EMO are somewhat less frequent, the remaining mycotoxins are not detected. In contrast, in impurities, in addition to AOL, EMO and MPA, fusariotoxins (T-2, DAS, ZEN) are often enough, whereas CPA and CIT are more rare, and DON, STE, EA occur in a few samples. The ranges of AOL, EMO and MPA content are significantly wider and the average values for the samples are significantly higher than those found in the seeds. The newly discovered fact of multiple and intensive contamination of impurities with mycotoxins has a great practical importance, as it is an experimental justification for the need for thorough harvest cleaning for further processing. Additional experiments with vegetating sunflower plants show the maximum content of mycotoxins in leaves and pseudanthium (sunflower head) as compared to stems. A sharp increase in the mycotoxin accumulation both in seeds and in impurities occurs under self-warming conditions. The obtained results show that fungi of the genera *Alternaria* and *Penicillium* can cause damage to the harvested crop. We will elucidate their role in more detail in further studies.

Keywords: *Helianthus annuus*, sunflower, seeds, impurities, mycotoxins, *Fusarium*, *Alternaria*, *Aspergillus*, *Penicillium*, enzyme immunoassay

Sunflower (*Helianthus annuus* L.) is cultivated in many countries for oil

seeds widely used edible oil. At the same time, in different areas, based on the generalized data on the contamination of seeds and the products of their processing with mycotoxins, the real risks of the influence of these toxicants on a humans are confirmed [1-4]. Considering the frequent occurrence and high content of Alternaria toxins with genotoxic effect, seeds, and sunflower oil are classified as products that pose a serious threat to public health [5-7].

In Russia, where about 70% of the world's sunflower crops are located, sunflower oil production in recent years has shown a tendency to sustainable growth. For oil cakes and meals, the by-products of oil-pressing and oil-extracting industries, which are traditionally in demand as a valuable raw material for mixed fodders, a long-term monitoring has been established the significant mycotoxin contamination [4]. Among its possible causes, there are violations of technological schemes during seed processing, transportation and storage of final products at enterprises, but the problem of the sanitary quality of oil seeds, coming from agricultural producers, remains without attention.

In the present work, in mycotoxicological assessment of commercial batches of sunflower oil seeds, we determined for the first time the nature of the contamination and revealed an increased accumulation of mycotoxins in the impurities. A survey of vegetating sunflower plants before harvesting showed that the main contribution to the contamination is made by the fragments of baskets and leaves. The new data on a sharp increase in contamination of batches of seeds by mycotoxins that have undergone self-warming are of particular interest, since information about mycotoxin formation in such conditions is very limited.

The aim of the work was to compare the contamination by mycotoxins of seeds and impurities in the production batches, intended for the production of sunflower oil.

Techniques. The study was performed on samples from 19 batches, a bulk sample per each batch, of sunflower oil seeds (*Helianthus annuus* L.), produced at agricultural enterprises of Belgorod, Voronezh, Kursk and Lipetsk Regions in 2016. Before conducting the analysis, the samples from each batch were fractionated into main seeds and impurities, separating the small part (the entire passage through a sieve with holes of 3.0 mm in diameter) and organic impurities (husks, remains of leaves, stems, baskets) from the residue on the sieve [8].

While conducting the mycological analysis of the samples from the batch of seeds stored after harvesting in two compartments, in one of which the storage conditions were violated, superficially sterilized seeds and the grinding of unsterilized seeds were incubated on a nutrient medium followed by detachment and species identification of the fungi *Alternaria* and *Penicillium* by culture and morphological features [9, 10]. Toxin formation in typical strains was evaluated after culture on malt extract agar medium (MEA, Liofilchem, Italy) [11] in the dark at 25 °C for 7 days.

For the analysis of vegetative sunflower plants, they were selected in June-September 2016 and 2017 in the subsidiary farms of Moscow, Tver, Voronezh, and Rostov Regions. The ground parts (n = 65) were cut at a height of 5 cm from the soil surface, dried in a shaded ventilated room and crushed whole. Another part of the plants (n = *29*) before grinding was divided into leaves, stems, and baskets.

In the group of mycotoxins, determined by the enzyme immunoassay using commercial and certified research test systems [12, 13], there were T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR) and ergot alkaloids (EA).

The quantitative results were statistically processed by the single-factor

analysis of variance [14] using the R version 3.4.3 program (https://cran.r-project.org/bin/windows/base/old/3.4.3/) [15]. The differences between the mean s(M) were estimated by the significance level p = 0.05.

Results. The samples from the production batches of the sunflower seeds were fractionated into the main seeds and impurities to study separately the nature of their contamination (Table 1).

1. Occurrence (n^+) and the content of mycotoxins (μg/kg) **in seeds and impurities of batches of sunflower (*Helianthus annuus* L.) seeds** (Belgorod, Voronezh, Kursk and Lipetsk Regions, 2016)

Mycotoxin	Main seeds (n = 19)	Impurities	
		organic impurities (n = 19)	small part (n = 18)
T-2	–	15 (3-11-100)[a]	18 (2-12-100)[a]
DON	–	1 (1000)	2 (79, 1000)
DAS	–	14 (145-250-395)[a]	15 (130-305-935)[a]
ZEN	1 (15)	15 (25-34-50)[a]	16 (25-37-63)[a]
FUM	1 (79)	–	–
EA	–	1 (6)	4 (2-7-16)
AOL	15 (21-450-3080) [a]	19 (40-2390-7940)[b]	18 (44-1800-5620)[b]
AB_1	–	–	–
STE	–	2 (9, 12)	1 (20)
CPA	1 (200)	9 (77-145-250)[a]	7 (64-135-250)[a]
EMO	8 (12-42-125)[a]	17 (130-825-1620)[b]	17 (50-415-795)[c]
OA	–	–	1 (9)
CIT	–	12 (25-44-94)	6 (21-32-43)
MPA	6 (12-28-53)[a]	16 (16-245-2400)[b]	15 (27-260-1050)[b]
PR	–	4 (230-360-645)	2 (235, 300)

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, AB_1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid, PR — PR-toxin; n — the number of examined samples. The number of positive samples n^+ is indicated before the brackets, the minimum-average-maximum mycotoxin content in the positive samples is shown in brackets. A dash means that no positive samples were found. In one line, for values with different superscript indices (a, b, c), the differences are statistically significant at p = 0.05.

2. Occurrence (n^+) and the content of mycotoxins (μg/kg) **in leaves, baskets, stems and whole sunflower (*Helianthus annuus* L.) plants** (Moscow, Tver, Voronezh and Rostov Regions, 2016 and 2017)

Mycotoxin	Whole plants (n = 65)	Parts of plants (n = 29)		
		leaves	baskets	stems
T-2	14 (2-6-20)	23 (2-3-6)[a]	16 (2-15-145)[a]	1 (2)
ДАС	24 (97-165-265)	25 (130-265-645)[a]	9 (130-205-315)[a]	–
ДОН	–	2 (76, 100)	–	–
ЗЕН	–	4 (28-34-39)[a]	5 (26-29-33)[a]	–
ФУМ	–	–	–	–
ЭА	52 (2-14-60)	22 (2-14-100)	–	2 (2, 3)
АОЛ	42 (14-32-91)	23 (12-40-100)[a]	14 (15-110-775)[a]	3 (20-200-415)
AB_1	–	1 (2)	1 (4)	–
СТЕ	17 (12-14-25)	11 (10-16-27)	1 (25)	–
ЦПК	64 (89-235-500)	29 (130-415-980)[a]	20 (50-150-400)[b]	4 (115-130-140)
ЭМО	27 (19-37-10)	26 (25-47-100)[a]	17 (20-61-225)[a]	2 (26, 30)
ОА	7 (4-5-7)	3 (5-5-6)	1 (6)	–
ЦИТ	12 (32-42-50)	16 (29-44-63)	4 (21-42-60)	–
МФК	15 (15-52-225)	11 (13-21-35)[a]	11 (13-42-215)[a]	2 (13, 36)
PR	9 (150-180-265)	20 (130-300-500)	4 (135-195-260)	–

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, AB_1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid, PR — PR-toxin; n — the number of examined samples. The number of positive samples n^+ is indicated before the brackets, the minimum-average-maximum mycotoxin content in the positive samples is shown in brackets. A dash means that no positive samples were found. In one line, for values with different superscript indices (a, b), the differences are significant at p = 0.05.

Out of the 15 studied mycotoxins, AOL was almost universal, MPA and EMO were less frequent, and ZEN, FUM, and CPA were found in single samples. On the contrary, along with AOL, EMO and MPA, the impurities quite often contained fusariotoxins (T-2, DAS, ZEN), more rarely CPA and CIT, in

few cases DON, STE, EA and PR. There were no significant differences in the nature of the contamination of the organic impurities and the small part in most mycotoxins. The content ranges of AOL, EMO, and especially MPA in impurities were significantly wider and, by average values, significantly exceeded the indicators of seeds. The fact of multiple and intense contamination of impurities, described for the first time, is of practical importance, since it experimentally substantiates the need for thorough cleaning of sunflower seeds supplied for further processing.

It was of interest to identify which parts of the plants that get into the seeds during harvesting make a major contribution to contamination. To do this, we studied the component composition of mycotoxins in the ground parts, as well as leaves, baskets, and stems of a vegetative sunflower (Table 2). The contamination of plants was multiple; most often there were CPA, EA, AOL, EMO, followed by STE, CIT, and MPA, less often OA and PR, out of fusariotoxins, T-2 and DAS were detected with a frequency of 14/65 and 24/65. The accumulation of T-2, DAS and AOL could be caused by phytopathogenic fungi *Fusarium* and *Alternaria*, whereas almost constant detection of CPA, EA, and EMO in the samples seems to be associated with the members of genera *Aspergillus* and *Penicillium* [16-18]. They often accompany the pathogens of fungal diseases of sunflower in very small quantities [19], but their role in the production of toxins is yet to be assessed.

In leaves and baskets, the complex of the main contaminants as a whole corresponded to the established one for whole plants, and toxic metabolites were found extremely rarely in the stems (see Table 2). These results are consistent with the previously published ones for a smaller sample [20]. The heterogeneous nature of the distribution of mycotoxins among the plant organs is seen as a manifestation of the complex associative connections of these organisms with microscopic fungi, mainly endophytes [21, 22]. According to the obtained data, the content of all toxins, except for ZEN and MPA, was less in the baskets in comparison with the leaves. At the same time, a statistically significant decrease in the content of CPA was observed. The extensive information, which has appeared in recent years, on the composition and content of fungal metabolites in lichen thalli [23], and now in sunflower, has become an important contribution to understanding the biochemical mechanisms of regulation of coenotic interactions. The advanced comprehensive studies of endogenous fungi and the metabolic profile, formed in plants with the participation of the entire associated community of organisms, seem to be especially promising.

We had the opportunity to analyze the samples from a batch cleaned and splinted for storage between two premises in one of which technical problems led to the appearance of self-warming foci. In the main seeds under unfavorable condition, a much larger amount of AOL and MPA was detected (Table 3). According to the results of the mycological analysis in the seeds of these compartments, there were both similarities and differences. The number of affected representatives of the genus *Alternaria* was equally extensive (85-95%), and the frequency of *Penicillium* spp. in the affected part was significantly higher (248×10^3 CFU/g vs. 7×10^3 CFU/g); moreover, with a frequency of 10 %, they were accompanied by one of the species of *Aspergillus glaucus* Gr. From morphological features, the species composition of *Alternaria* and *Penicillium* fungi in both parts was fairly uniform. The typical strains of *A. tenuissima* (Nees & T. Nees: Fr.) Wiltshire and P. *stoloniferum* Thom in the laboratory express tests were highly active and formed AOL and MPA of more than 10000 ng/ml of the medium. The ability of *A. tenuissima* to produce AOL was described previously [24], and for the species *P. stoloniferum*, subjected to multiple taxonomical movements [25] and

currently recognized as synonymous with *P. brevicompactum*, the possibility of MPA biosynthesis was repeatedly confirmed by foreign and Russian researchers [26-28]. In addition, *A. pseudoglaucus* Blochwitz from *Aspergillus glaucus* Gr. also belong to MPA producers [26]. Accounting a general trend of increased AOL and MPA amounts in sunflower seeds with changing external conditions, there were no effects of growth inhibition and suppression of specific toxins biosynthesis between these species. The reports on the mutual influence of toxigenic fungi of different genera coexisting on the same biological substrate are still few. Nevertheless, it was shown that the rate of colonization of wheat caryopsides with the fungus *A. tenuissima* and the amount of formed AOL significantly increased after the preliminary treatment of fusariotoxins DON or ZEN [29]. Recently, the relationship between aggressive trichothecene-producing species *Fusarium* and *Alternaria* fungi in oats has been characterized as symbiotic [30].

3. Mycotoxin contents (μg/kg) **in the main fraction and impurities of the same batch of sunflower (*Helianthus annuus* L.) seeds depending on storage conditions** (Kursk Region, 2016)

Mycotoxin	Main seeds	Impurities	
		organic impurities	small part
T-2	–/–	–/3	3/5
DAS	–/–	–/–	215/400
DON	–/–	–/–	–/–
ZEN	–/–	–/–	49/45
FUM	–/–	–/–	–/–
EA	–/–	–/–	–/–
AOL	26/240	955/245	480/390
AB_1	–/–	–/–	–/–
STE	–/–	–/–	–/15
CPA	–/–	–/–	–/250
EMO	–/41	735/600	355/1000
OA	–/–	–	–/–
CIT	–/–	26/-	–/–
MPA	53/2630	125/1000	345/6310
PR	–/–	–/–	–/–

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, AB_1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid, PR — PR-toxin. Estimates for due and violated storage condition (self-warming foci) are indicated through a slash. A dash means that no positive samples were found.

For organic residues in the impurities, as a result of self-warming, there was an increase in the contamination of MPA and the appearance of STE and CPA, but the same clear tendency towards AOL, EMO and fusariotoxins (T-2, DAS, ZEN) was not observed (see Table 3). It is probable that higher accumulation of MPA on the dead sunflower tissues is related to the fact that under these conditions *P. stoloniferum* gets a chance to realize the potential of a mycophilic fungus of physiological necrotrophs [27]. The fact regarding its habitat on the stroma of the phytopathogen *Helminthosporium sativum* Pam. and the inhibiting effect against the pathogens of common sunflower diseases *Botrytis cinerea* Fr. and *Verticillium dahliae* are described [31].

The sharp accumulation of mycotoxins, not only in seeds but also in the impurities, serves as another argument in favor of the increased attention to cleaning batches before storage and processing.

When examining the production batches of seeds, as well as the samples from self-warming foci, we did not note a single case of AB_1 detection given the sufficiently high sensitivity of the applied method (0.002 mg/kg). Nevertheless, AB_1 is officially recognized as the only indicator of safety (not more than 0.005 mg/kg) for food oilseeds (including sunflower), as well as for unrefined vegetable oils of all types and their products [32-34]. The apparent inconsistencies between the mycotoxicological criteria for the control of sunflower oil cakes and meals,

and the prevalence of real carriers of the threat to animal health we examined in detail earlier [4].

Thus, in sunflower oil seeds, the main contaminants of mycogenic origin are represented by alternariol, mycophenolic acid, and emodin. The impurities that get into the batch during harvesting and contain the remnants of the herbage of plants are characterized by a more intensive accumulation of these mycotoxins and the appearance of a number of others, i.e. fusariotoxins, as well as cyclopiazonic acid and citrinin. If the storage conditions are violated, higher humidity and temperature lead to sharply increased accumulation in seeds of alternariol and mycophenolic acid which have toxic effects, including genotoxicity and immunosuppressive activity. This first information on the nature of contamination by mycotoxins of commercial sunflower oil seed batches is a step towards the creation of a Russian replenishable database to improve the hygienic and sanitary requirements of food and fodder safety.

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CULTIVAR SPECIFICITY OF THE RHIZOBACTERIAL EFFECTS ON NITROGEN-FIXING SYMBIOSIS AND MINERAL NUTRITION OF SOYBEAN UNDER AGROCENOSIS CONDITIONS

Yu.V. BEREGOVAYA[1], I.L. TYCHINSKAYA[1], S.N. PETROVA[1], N.V. PARAHIN[1], J.V. PUHALSKY[2], N.M. MAKAROVA[2], A.I. SHAPOSHNIKOV[2], A.A. BELIMOV[2]

[1]*Parakhin Orel State Agrarian University*, 69, ul. Generala Rodina, Orel, 302019 Russia, e-mail juliemons@yandex.ru, pridatko1990@mail.ru, svet-orl@yandex.ru;
[2]*All-Russian Research Institute for Agricultural Microbiology,* Federal Agency for Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail jankiss88@gmail.com, nm_makarova@yahoo.com, ai-shaposhnikov@mail.ru, belimov@rambler.ru (✉ corresponding author)
ORCID:
Beregovaya Yu.V. orcid.org/0000-0001-7853-326X
Tychinskaya I.L. orcid.org/0000-0002-9805-8432
Petrova S.N. orcid.org/0000-0003-4482-3458
Puhalsky J.V. orcid.org/0000-0001-5233-3497
Makarova N.M. orcid.org/0000-0003-4781-328X
Shaposhnikov A.I. orcid.org/0000-0003-0771-5589
Belimov A.A. orcid.org/0000-0002-9936-8678
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Abstract

Stimulation of nitrogen-fixing symbiosis by is an important mechanism of interaction between rhizobacteria and leguminous plants. At the same time, little is known about intraspecific (varietal) variability of leguminous when responding to inoculation with rhizobacteria. Our recent model studies of hydroponic soybean seedlings showed that rhizobacteria *Pseudomonas* oryzihabitans Ep4 can better stimulate growth and colonize the roots of Nice Mecha and Swapa soybean plants when compared to Bara variety. The purpose of this work was to study the variety-specific responses of soybeans plants to inoculation with rhizosphere bacteria (rhizobacteria) producing auxins and containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase at various levels of plant mineral nutrition under agrocenosis conditions. The subject plants were three early ripening soybean *Glycine max* (L.) Merr. varieties of the northern ecotypes Nice Mecha, Swapa and Bara. Rhizobacterial strains *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 were used for inoculation. Biopreparation rizotorfin containing a nodule bacterium *Bradyrhizobium japonicum* strain 634b was used for the formation of nitrogen-fixing symbiosis. Three-year field experiments were conducted in 2013-2015 years in the northernmost area of soybean cultivation (Orel region) on a dark gray forest medium-loamy soil. Mineral fertilizer 'diamofoska' was applied 7 days before sowing. Two mineral nutrition levels of $N_{30}P_{81}K_{81}$ and $N_{44}P_{116}K_{116}$ were used. In all treatments with rhizotorfin there was an increase in nodule biomass and nodule number, except the treatment of cultivar Bara at $N_{30}P_{81}K_{81}$. In using lower mineral nutrition with risotorphin, the strain *Ps. oryzihabitans* Ep4 increased number (by 140 %) and weigh (by 176 %) of nodules and nitrogen-fixing activity (by 69 %) of Swapa plants at flowering. At a higher mineral nutrition the influence of *Ps. oryzihabitans* Ep4 on the legume-rhizobia symbiosis manifested by the increased nodule number on Swapa roots (by 55 %) and nitrogen-fixing activity of Bara variety (by 205 %), whereas the strain *V. paradoxus* 3-P4 increased nitrogen fixation of Nice Mecha (by 231 %) and Bara (by 205 %). The positive effects of both rhizobacterial strains on the plant growth at the flowering stage, as well as on the content of nutrients (Mg, Ca, B, Fe, Zn and Mo) in leaves were more pronounced on varieties Nice Mecha and Swapa at lower and/or higher mineral nutrition. At $N_{30}P_{81}K_{81}$ the increase of shoot biomass at the flowering stage in cultivars Nice Mecha and Swapa was obtained after inoculation with monocultures of the studied rhizobacteria and after combination of rhizobacteria with rhizotorfin as well.

However cultivar Bara has a positive response to mono-inoculation with rhizotorfin. At $N_{44}P_{116}K_{116}$ a combined inoculation of cultivar Bara with rhizotorfin and strain *Ps. oryzihabitans* Ep4, as well as cultivar Nice Mecha with rhizotorfin and strain *V. paradoxus* 3-P4, was significantly more efficient as compared to mono-inoculation with rhizotorfin. As a rule, the positive effects of both rhizobacterial strains on plant growth at flowering, as well as on the content of nutrient elements (Mg, Ca, B, Fe, Zn and Mo) in leaves, were more pronounced on cultivars Nice Mecha and Swapa at a lower and/or a higher level of mineral nutrition. The maximum effect of rhizobacteria on seed yield and seed quality (protein and fat content) is also obtained by inoculation of varieties Nice Mecha and Swapa. However, variety Bara has the highest response to mineral fertilizers. The differences found between soybean varieties in the response to inoculation with plant growth-promoting rhizobacteria indicate a higher degree of integration between associative microorganisms and varieties Nice Mecha and Swapa compared to variety Bara. The results of this study indicate the promise for creating plant-microbe systems that combine a high degree of symbiotrophy and assimilation of nutrients from fertilizers and soil.

Keywords: *Glycine max*, *Pseudomonas, Variovorax*, intraspecies variability, mineral nutrition, rhizosphere, symbiotic nitrogen fixation, phytohormones, agrocenosis

At present time, considerable experimental data has been accumulated on the positive effect of associative rhizosphere bacteria (rhizobacteria) on the growth, nutrition, and adaptation of agricultural plants to unfavorable climatic and soilborne factors by fixing atmospheric nitrogen, production or utilization of biologically active substances, mobilization of nutrients in the rhizosphere, induction of system stability and biocontrol of phytopathogens [1-5]. The ability to stimulate the formation of nitrogen-fixing symbiosis with nodule bacteria serves as an important mechanism for leguminous plants to interact with rhizobacteria. Thus, the number of nodules on soybean roots increases upon combined inoculation with nodule bacteria *Bradyrhizobium japonicum* and rhizobacteria *Pseudomonas fluorescens* [9-11], *Azospirillum brasilense* [12], *Bacillus subtilis* [13-17], *B. thuringiensis* [13, 14, 18], *B. megaterium* [19], *Paenibacillus lautus* [19], as well as with unidentified phosphate-mobilizing bacteria. The action of rhizobacteria can be associated with a decrease in the biosynthesis of ethylene, stress phytohormone (to which the process of nodulation is highly sensitive) due to the utilization of its precursor 1-aminocyclopropane-1-carboxylate (ACC) by rhizobacteria which use the ACC deaminase enzyme [19, 21-23]. It is supposed that the production of auxins by rhizobacteria, stimulating root growth and nodulation, is involved in the activation of nitrogen-fixing symbiosis [4, 8, 24]. However, in most of the studies enumerated, the mechanisms of the positive impact of rhizobacteria on the soybean symbiosis with nodule bacteria were not studied.

It is also little known about the intraspecific (varietal) variability of leguminous plants in response to rhizobacteria inoculations. Four pea varieties have demonstrated significant differences in the *Ps. brassicacearum* Am3 strain effects on the absorption of nutrients (N, P, K, Ca, S, Fe) from the soil contaminated with heavy metals [25]. The influence of the *Az. brasilense* rhizobacteria, which fix nitrogen and produces auxins, on the growth of sorghum [26], wheat [27] and bean [28] varied significantly depending on the variety. Similar results were obtained for the related species, *Az. lipoferum,* in the experiments with corn varieties [29]. Varietal specificity in the effects of rhizobacteria on legume-rhizobia symbiosis in soybean [15, 30], pea [31-32] and chick pea [33] has been revealed. Differences in the expression of the genes associated with auxin and ethylene signaling are described for two rice varieties upon *Az. lipoferum* inoculation [34]. The variation in the ability of the introduced rhizobacteria populations to colonize plant roots depending on the variety has been also described in other studies [27, 35]. Nevertheless, the reasons for the intraspecific variability of plant-bacteria interactions have not been studied yet, which restrains significantly the application of the latter as biopreparations [36].

Soybean is the main leguminous crop in the Russian Federation. Its

growth and nutrition depend to a significant extent on symbiosis with nodule bacteria and rhizobacteria. To expand the area of this crop, varieties resistant to cold and adapted to nutrient-poor soil are needed. For the northernmost area of soybean cultivation in Russia, the Orel Region, early ripening varieties of the northern ecotypes were created: Nice Mecha, Swapa and Bara. However, little is known about their symbiotic characteristics and potential. It can be assumed that rhizobacteria producing auxins and containing ACC deaminase should facilitate soybean adaptation to unfavorable climatic and soil conditions.

The authors have previously shown in model studies of hydroponic soybean seedlings that rhizobacteria *Ps. oryzihabitans* Ep4, producing auxins and containing ACC deaminase, perform stimulation of the growth and root colonization at a higher degree in Nice Mecha and Swapa varieties compared to the Bara variety. This is due to the intense root exudation of organic acids, sugars, and amino acids and their effective utilization and transformation by rhizobacteria [32]. The close integration of *Ps. oryzihabitans* Ep4 strain with the Nice Mecha and Swapa varieties was partially confirmed by the results of the field experiment at a combined inoculation of plants with nodule bacteria [37].

In this report, the authors describe for the first time the positive effect of the rhizosphere bacteria, containing ACC deaminase, on different varieties of soybean northern ecotypes and their symbiosis with nodule bacteria.

The purpose of this study was to reveal the correlation of the variety-specific responses of soybean plants with rhizobacterial inoculation and mineral nutrition under agrocenosis conditions.

Techniques. The highest quality seeds of early ripening released soybean *Glycine max* (L.) Merr. varieties Nice Mecha and Swapa were obtained from the Federal Scientific Center of Legumes and Cereal Crops (Orel), the seeds of the Bara variety from Soybean Complex LLC (Krasnodar).

At inoculation, the used strains of associative bacteria containing ACC deaminase and producing auxins were *Pseudomonas oryzihabitans* Ep4 [38] and *Variovorax paradoxus* 3-P4 [39]. To prepare the inoculum, the associative bacteria were cultured in previously formulated liquid nutrient medium [40] for 4 days at 28 °C and 180 rpm. The resulting suspension was diluted with sterile faucet water to a final concentration 10^7 cells/ml. To generate nitrogen-fixing symbiosis, biopreparation rhizotorfin (ECOS, Saint Petersburg) containing nodule bacteria *Bradyrhizobium japonicum* 634b in sterilized turf as a carrier was used (10^9 cells/g turf). The purity of the inocula and the biopreparation was confirmed by microbiological methods. All the strains were deposited in the Institutional Collection of Beneficial Microorganisms of Agricultural Designation (All-Russia Research Institute for Agricultural Microbiology, Saint Petersburg).

The soil of the plots on which the field experiments were conducted (APC Integration, Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015), dark gray forest medium-loamy, humus content 3.4±0.1%, mobile nitrogen 57±8 mg/kg, labile phosphorus 100±8 mg/kg, mobile potassium 136±9 mg/kg, pH 5.0±0.06 (average data for 3 years). Soil characteristics were determined by standard methods.

The plants were cultivated in a seven-field crop rotation system of the grain type with full plot randomization (the plot square 10 m^2) in a fourfold analysis for each variant, the crop was preceded by bare fallow. Mineral fertilizer (diammophoska) was applied 7 days before sowing ($N_{30}P_{81}K_{81}$ and $N_{44}P_{116}K_{116}$, 70 and 100%, respectively, for planned crop yield 3 t/ha). Immediately prior to sowing, the seeds were pretreated with Maxim KS fungicide (fludioxonil, 2 ml/kg seeds; Syngenta LLC, Russia) at a working concentration to which the strains under study are resistant according to preliminary tests. A part of the

seeds was pretreated with rhizotorfin (strain *B. japonicum* 634b, 2g/kg seeds). The seeding rate for all the seeds is 70 pcs/m^2 (selection seeding-machine Plotseed XL, Wintersteiger, Austria). The inocula of *Ps. oryzihabitans* Ep4 and *V. paradoxus* 3-P4 (200 ml/m^2) were used to treat root zone on the day 10 and day 27 after sowing (seedlings and 2-3 true leaves, respectively), samples without inoculation were used as controls (for the evaluation of rhizobacteria effects by integral parameters upon co-inoculation, the sample with rhizotorfin served as a control). For all the variants, the soil preparation Dual Gold, EC (1.6 l/ha; Syngenta LLC, Russia) was applied before sowing and Bazagran, AS (2 l/ha; BASF LLC, Russia) at three true leaves. Insecticide Karate Zeon, MCS (0.4 l/ha; Syngenta LLC, Russia) was used for pest control. Two weeks before harvesting, the plants were desiccated with Reglon Super, AS (1.5 l/ha; Syngenta LLC, Russia). The seeds were harvested by a combine harvester TERRION-SAMPO SR2010 (Agrotekhmash, Russia).

Nitrogenase (nitrogen-fixing) activity was evaluated in roots at flowering by acetylene method [42]. The roots of 5 plants from each plot were washed with water, nodules were counted up, roots were placed into the airproof flasks (250 ml) and incubated in 10% acetylene atmosphere for 1 h at 25 °C. The reaction was terminated by 2.5% formalin solution, the ethylene concentration was determined (gas chromatography system FGC-1, ECAN LLC SPE, Russia). The nodules were separated from the roots, desiccated and weighed. The leaves of the plants of each plot were desiccated and used for elemental analysis.

The seed protein and lipids were measured using the infrared grain analyzer Infratec™ 1241 (FOS", Denmark) according to the manufacturer's guidelines. The quantity of B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Ni, P, S, Zn in leaves was assessed using an emission spectrometer ICPE-9000 (Shimadzu, Japan). For this, dry leaves were milled and burnt in the mixture of concentrated HNO_3 and 38% H_2O_2 (1:1) at 70 °C (DigiBlock system, LabTech, Italy). The total nitrogen content in leaves was determined (an automatic analyzer Kjeltec 2300, FOSS Analytical, Denmark).

Statistical processing was performed with STATISTICA 10 software (StatSoft, Inc., USA). The dispersion analysis (Fisher minimal mean difference test), Student *t*-criterion and correlation analysis were used. The mean values (*M*) and mean values errors (±SEM) were calculated.

Results. Rhizotorfin with $N_{30}P_{81}K_{81}$ increased the number of nodules for all the varieties, and with $N_{44}P_{116}K_{116}$ for Nice Mecha and Swapa as compared to control (Table 1). Combined inoculation with rhizobacteria also led to more intensive nodule formation; moreover, the Swapa variety demonstrated a statistically higher number of nodules compared to rhizotorfin mono-inoculation (see Table 1). Rhizotorfin increased the weight of nodules. Combined treatment with rhizotorfin and *Ps. oryzihabitans* Ep4 increased nodule weight for Swapa plants at both levels of mineral nutrition, as well as nitrogen-fixing activity at $N_{30}P_{81}K_{81}$ as compared to control and rhizotorfin application. With *V. paradoxus* 3-P4 at 70% level of mineral nutrition, the Swapa and Bara plants statistically significant increased the nodule weight ($p < 0.001$ and $p = 0.044$, respectively). Higher nitrogen fixation for all the varieties at the 100% mineral nutrition was scored only at a combined inoculation with nodule and associative bacteria (see Table 1).

$N_{30}P_{81}K_{81}$ increased overground plant weight at flowering of Nice Mecha and Swapa varieties both with bacterial monocultures and in the case of combination with rhizotorfin. However, the Bara variety demonstrated positive response only to rhizotorfin mono-inoculation (see Table 2). At $N_{44}P_{116}K_{116}$ rhizotorfin increased the overground plant weight only for Nice Mecha and Swapa varieties. For Bara variety, combined inoculation with *Ps. oryzihabitans*

1. Symbiotic indicators of soybean *Glycine max* (L.) Merr. varieties during flowering as influenced by nodule bacteria *Bradyrhizobium japonicum* 634b (rhizotorfin) and associative bacteria *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 at different levels of mineral nutrition (*M*±SEM, APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015)

Variant	Nodule number, pcs./plant			Nodule weight, mg/plant			Nitrogenase activity, μmol C_2H_4/(plant · h)		
	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara
				$N_{30}P_{81}K_{81}$ (70 %)					
No inoculation	3±1^{a}	2±1^{a}	2±1^{a}	56±23^{a}	41±11^{a}	28±13^{a}	0.34±0.14^{a}	0.57±0.16^{a}	0.08±0.03^{a}
B. japonicum 634b	18±3^{b}	10±2^{b}	16±4^{b}	603±120^{bc}	339±86^{b}	288±78^{b}	1.15±0.27^{bc}	1.13±0.21^{ab}	0.37±0.05^{b}
Ps. oryzihabitans Ep4	4±1^{a}	4±1^{a}	1±0^{a}	124±49^{a}	124±58^{ab}	41±15^{a}	0.43±0.21^{a}	0.63±0.13^{a}	0.20±0.07^{ab}
V. paradoxus 3P-4	12±5^{ab}	2±1^{a}	3±1^{a}	288±121^{ab}	108±43^{ab}	48±23^{a}	0.80±0.32^{ab}	0.40±0.16^{a}	0.41±0.15^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	21±3^{b}	24±3^{c}	12±4^{b}	707±145^{c}	937±166^{c}	357±115^{bc}	0.88±0.15^{b}	1.91±0.32^{c}	0.55±0.13^{b}
B. japonicum 634b + *V. paradoxus* 3P-4	15±2^{b}	23±3^{c}	12±2^{b}	696±137^{c}	581±81^{c}	469±90^{c}	1.69±0.46^{c}	1.68±0.35^{bc}	0.61±0.16^{b}
				$N_{44}P_{116}K_{116}$ (100 %)					
No inoculation	4±1^{a}	1±0,3^{a}	1±0^{a}	144±45^{a}	44±17^{a}	22±7^{a}	0.05±0.01^{a}	0.43±0.19^{a}	0.22±0.10^{a}
B. japonicum 634b	20±6^{b}	11±2^{b}	5±1^{ab}	685±170^{bc}	363±107^{bc}	173±42^{b}	0.64±0.04^{b}	1.03±0.21^{ab}	0.35±0.06^{a}
Ps. oryzihabitans Ep4	11±4^{ab}	3±1^{a}	3±1^{ab}	629±230^{bc}	156±69^{b}	43±18^{a}	0.47±0.08^{ab}	0.78±0.27^{ab}	0.37±0.14^{a}
V. paradoxus 3P-4	7±4^{a}	1±0^{a}	1±0^{a}	344±224^{ab}	51±14^{a}	13±4^{a}	0.47±0.10^{ab}	0.42±0.14^{a}	0.47±0.20^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	19±6^{b}	17±3^{c}	8±1^{b}	1025±379^{c}	439±113^{c}	189±25^{b}	1.07±0.27^{bc}	1.15±0.24^{b}	1.07±0.31^{c}
B. japonicum 634b + *V. paradoxus* 3P-4	13±2^{b}	11±2^{b}	6±1^{b}	835±117^{c}	373±94^{bc}	173±24^{b}	2.12±0.58^{c}	1.33±0.26^{b}	0.77±0.17^{bc}

N o t e. For each fertilizer dosage, statistically significant differing variants are designated by Latin letters (Fisher test, MMD, $p < 0.05$, $n = 12$).

Ep4 strain, and for Nice Mecha variety with *V. paradoxus* 3-P4 were statistically more effective ($p = 0.015$ and $p = 0.039$, respectively) (see Table 2) than rhizotorfin mono-inoculation. *Ps. oryzihabitans* Ep4 and *V. paradoxus* 3-P4 strains statistically significantly increased the root weight (p between 0.045 and 0.0012) in Nice Mecha variety at mono-inoculation and at a combined inoculation with rhizotorfin regardless of mineral nutrition levels (see Table 2). The inoculation with *V. paradoxus* 3-P4, as well as use of this strain or *Ps. oryzihabitans* Ep4 in combination with rhizotorfin, positively influenced the root weight in Swapa and Bara varieties.

2. Plant biomass of soybean *Glycine max* (L.) Merr. varieties during flowering as influenced by nodule bacteria *Bradyrhizobium japonicum* 634b (rhizotorfin) and associative bacteria *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 at different levels of mineral nutrition ($M \pm$SEM, APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015)

Variant	Dry weight, g/plant					
	overground part			roots		
	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara
	$N_{30}P_{81}K_{81}$ (70 %)					
No inoculation	39 ± 2^{a}	35 ± 4^{a}	44 ± 2^{a}	3.5 ± 0.1^{a}	3.9 ± 0.2^{a}	3.8 ± 0.3^{a}
B. japonicum 634b	45 ± 2^{b}	46 ± 3^{b}	55 ± 4^{b}	4.1 ± 0.3^{ab}	5.1 ± 0.4^{ab}	4.1 ± 0.2^{ab}
Ps. oryzihabitans Ep4	49 ± 3^{bc}	45 ± 2^{ab}	43 ± 2^{a}	4.3 ± 0.2^{bc}	5.1 ± 0.4^{ab}	4.6 ± 0.5^{b}
V. paradoxus 3P-4	48 ± 1^{bc}	47 ± 5^{b}	44 ± 4^{a}	4.5 ± 0.1^{bc}	5.8 ± 0.6^{b}	4.3 ± 0.4^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	48 ± 2^{bc}	46 ± 5^{b}	47 ± 4^{ab}	4.1 ± 0.3^{b}	6.1 ± 0.5^{b}	4.2 ± 0.2^{ab}
B. japonicum 634b + *V. paradoxus* 3P-4	52 ± 1^{c}	50 ± 3^{b}	41 ± 4^{a}	4.8 ± 0.2^{c}	5.0 ± 0.3^{ab}	4.7 ± 0.4^{b}
	$N_{44}P_{116}K_{116}$ (100 %)					
No inoculation	27 ± 3^{a}	46 ± 1^{a}	47 ± 2^{a}	3.5 ± 0.1^{a}	4.8 ± 0.3^{a}	3.7 ± 0.2^{a}
B. japonicum 634b	36 ± 1^{b}	57 ± 3^{b}	49 ± 3^{ab}	3.9 ± 0.3^{ab}	6.1 ± 0.6^{ab}	4.0 ± 0.2^{ab}
Ps. oryzihabitans Ep4	35 ± 1^{b}	48 ± 4^{ab}	59 ± 4^{bc}	4.2 ± 0.2^{b}	5.5 ± 0.5^{ab}	3.9 ± 0.2^{ab}
V. paradoxus 3P-4	33 ± 1^{ab}	52 ± 4^{ab}	53 ± 5^{abc}	4.5 ± 0.2^{b}	5.2 ± 0.4^{ab}	3.8 ± 0.2^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	39 ± 0^{bc}	57 ± 5^{b}	61 ± 3^{c}	4.2 ± 0.2^{b}	6.4 ± 0.8^{b}	4.5 ± 0.1^{b}
B. japonicum 634b + *V. paradoxus* 3P-4	42 ± 1^{c}	52 ± 4^{ab}	58 ± 5^{bc}	4.1 ± 0.1^{b}	6.3 ± 0.8^{b}	4.0 ± 0.2^{ab}

N o t e. For each fertilizer dosage, statistically significant differing variants are designated by Latin letters (Fisher test, MMD, $p < 0.05$, $n = 12$).

N content in leaves increased in all cases with rhizotorfin (Table 3). At the 70% level of mineral nutrition, both strains of rhizobacteria increased the P content in the leaves of Nice Mecha variety at mono- and combined inoculation (see Table 3). This indicator was higher in Swapa plants upon inoculation with rhizotorfin and *Ps. oryzihabitans* Ep4 regardless of the level of mineral nutrition, whereas in Bara plants solely at the 70% level. Mg, Ca, B, Fe, Zn and Mo content (see Table 3) in the leaves of Nice Mecha and Swapa plants when using rhizotorfin and *Ps. oryzihabitans* Ep4 mixture, was generally higher. A similar, but less pronounced effect result was obtained for two these varieties upon rhizotorfin and *V. paradoxus* 3-P4 inoculation: at $N_{30}P_{81}K_{81}$ the bacteria did not affect Mg, Ca, B and Zn, and at $N_{44}P_{116}K_{116}$ the content of P did not increase. Both control and inoculated plants of Bara variety (in contrast to the rest two varieties) differed insignificantly in elemental composition of leaves, which manifested for Mg, B, Zn, Mo at 70% nutrition level and for P, Fe and Zn at the 100% level (see Tables 3, 4). The positive effect of the combined inoculation on patterns of macro- and microelements was in many cases statistically significant compared to control plants without inoculation, as well as to rhizotorfin application (see Tables 3, 4).

Rhizotorfin and rhizobacteria did not increase Co, Cu, K, Mn, Ni and S accumulation in leaves. The exceptions were rise of Co level in Nice Mecha (by 15%, $p = 0.015$, $n = 12$) and Swapa (by 26%, $p = 0.015$, $n = 12$), as well as S content increase in Swapa variety (by 23%, $p = 0.016$, $n = 12$) in response to rhizotorfin and *Ps. oryzihabitans* inoculation (data not shown).

3. Macroelements in leaves of soybean *Glycine max* (L.) Merr. varieties during flowering as influenced by nodule bacteria *Bradyrhizobium japonicum* 634b (rhizotorfin) and associative bacteria *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 at different levels of mineral nutrition (*M*±SEM, APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015)

Variant	N, mg/g			P, mg/g			Mg, м mg/g			Ca, mg/g		
	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara
$N_{30}P_{81}K_{81}$ (70 %)												
No inoculation	25.5±0.6^{a}	26.3±0.6^{a}	26.3±1.0^{a}	10.1±0.4^{a}	10.7±0.4^{a}	10.3±0.3^{a}	5.0±0.4^{ab}	4.0±0.2^{a}	4.2±0.2^{a}	18.0±0.9^{a}	16.2±0.9^{a}	17.6±0.8^{a}
B. japonicum 634b	29.4±07^{c}	31.2±1.0^{bc}	30.9±1.1^{b}	10.8±0.3^{a}	11.8±0.3^{ab}	10.4±0.3^{a}	4.9±0.4^{a}	4.4±0.2^{ab}	4.7±0.2^{a}	19.0±0.9^{a}	16.6±1.0^{a}	19.1±0.4^{ab}
Ps. oryzihabitans Ep4	27.6±0.8^{bc}	27.8±1.0^{a}	27.6±0.9^{a}	11.3±0.5^{b}	11.7±0.5^{ab}	11.0±0.6^{ab}	5.2±0.4^{ab}	4.5±0.3^{ab}	4.5±0.3^{a}	20.2±0.8^{ab}	17.2±1.3^{a}	17.8±0.8^{ab}
V. paradoxus 3P-4	27.1±0.7^{ab}	28.1±0.8^{ab}	27.6±0.9^{a}	11.1±0.4^{b}	11.2±0.5^{ab}	10.7±0.3^{a}	5.3±0.4^{ab}	4.4±0.2^{ab}	4.2±0.2^{a}	18.5±0.8^{a}	16.0±0.5^{a}	18.5±0.8^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	30.7±0.6^{c}	32.6±1.0^{c}	32.6±0.9^{b}	11.8±0.4^{b}	12.5±0.4^{b}	12.0±0.5^{b}	5.9±0.4^{b}	5.0±0.3^{c}	4.7±0.2^{a}	21.7±1.1^{b}	20.1±1.3^{b}	19.6±0.7^{b}
B. japonicum 634b + *V. paradoxus* 3P-4	29.9±0.9^{c}	31.2±1.2^{bc}	31.3±1.2^{b}	11.7±0.3^{b}	12.0±0.6^{ab}	10.7±0.5^{a}	5.1±0.4^{ab}	4.9±0.2^{bc}	4.7±0.2^{a}	19.3±1.0^{ab}	17.2±1.0^{a}	19.2±0.5^{ab}
$N_{44}P_{116}K_{116}$ (100 %)												
No inoculation	28.9±0.7^{a}	30.4±1.3^{a}	29.4± 1.3^{a}	11.7±0.2^{a}	12.3±0.5^{a}	11.6±0.4^{a}	4.4±0.1^{a}	4.7±0.3^{ab}	4.2±0.1^{a}	17.9±1.0^{a}	16.1±0.6^{a}	15.8±0.3^{a}
B. japonicum 634b	32.5±0.8^{bc}	33.9±1.2^{bc}	32.2±1.0^{b}	12.1±0.2^{a}	12.2±0.4^{a}	11.8±0.4^{a}	4.9±0.1^{ab}	4.6±0.3^{a}	4.1±0.2^{a}	21.6±0.9^{bc}	16.9±0.6^{a}	17.3±0.6^{ab}
Ps. oryzihabitans Ep4	30.0±1.1^{a}	32.2±1.5^{ab}	31.3±1.1^{a}	12.6±0.3^{ab}	13.2±0.6^{ab}	11.8±0.5^{a}	5.3±0.2^{ab}	4.6±0.2^{a}	4.3±0.2^{ab}	22.7±1.1^{c}	17.1±0.6^{a}	16.4±0.8^{a}
V. paradoxus 3P-4	30.5±1.1^{ab}	31.7±1.7^{a}	30.1±1.1^{a}	12.1±0.5^{a}	12.4±0.6^{a}	11.9±0.4^{a}	4.9±0.2^{ab}	4.7±0.2^{ab}	4.1±0.2^{a}	19.5±0.9^{ab}	16.8±0.4^{a}	17.0±0.7^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	33.5±0.6^{c}	35.4±1.1^{c}	34.1±0.9^{b}	13.4±0.1^{b}	14.5±0.6^{b}	12.8±0.6^{a}	5.5±0.3^{b}	5.3±0.2^{bc}	5.1±0.4^{c}	24.1±1.4^{c}	21.7±1.7^{b}	18.9±0.7^{b}
B. japonicum 634b + *V. paradoxus* 3P-4	32.0±0.6^{bc}	35.2±1.2^{bc}	33.5±0.9^{b}	12.0±0.4^{a}	13.1±0.6^{ab}	12.0±0.6^{a}	5.4±0.5^{ab}	5.5±0.2^{c}	4.9±0.5^{bc}	22.4±1.3^{bc}	17.3±1.1^{a}	18.6±0.7^{b}

N o t e. For each fertilizer dosage, statistically significant differing variants are designated by Latin letters (Fisher test, MMD, $p < 0.05$, $n = 12$).

4. Microelements in leaves of soybean *Glycine max* (L.) Merr. varieties during flowering as influenced by nodule bacteria *Bradyrhizobium japonicum* 634b (rhizotorfin) and associative bacteria *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 at different levels of mineral nutrition (*M*±SEM, APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015)

Variant	B, μg/g			Fe, μg/g			Zn, μg/g			Mo, μg/g		
	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara
					$N_{30}P_{81}K_{81}$ (70 %)							
No inoculation	45±2^{a}	39±2^{a}	50±5^{a}	109±3^{ab}	112±4^{a}	108±7^{a}	30±3^{a}	35±3^{a}	39±3^{a}	9.3±0.4^{a}	8.7±0.2^{a}	9.4±0.3^{a}
B. japonicum 634b	54±4^{ab}	45±3^{a}	52±5^{a}	102±5^{a}	112±5^{a}	122±11^{a}	31±3^{a}	41±3^{ab}	37±2^{a}	9.6±0.3^{a}	9.1±0.3^{a}	9.7±0.2^{a}
Ps. oryzihabitans Ep4	49±5^{ab}	46±4^{a}	50±4^{a}	125±8^{b}	137±17^{b}	130±10^{ab}	35±2^{ab}	40±3^{a}	37±3^{a}	10.1±0.3^{ab}	8.8±0.3^{a}	9.7±0.3^{a}
V. paradoxus 3P-4	55±4^{b}	46±4^{a}	52±4^{a}	123±13^{b}	126±13^{ab}	112±7^{a}	34±2^{ab}	37±3^{a}	35±3^{a}	9.8±0.3^{ab}	8.9±0.3^{a}	9.4±0.3^{a}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	53±3^{ab}	58±4^{b}	56±5^{a}	143±8^{b}	137±7^{b}	145±12^{b}	39±3^{b}	49±4^{b}	38±3^{a}	10.6±0.3^{b}	10.3±0.5^{b}	10.0±0.4^{a}
B. japonicum 634b + *V. paradoxus* 3P-4	54±5^{ab}	48±4^{a}	51±4^{a}	126±8^{b}	126±4^{ab}	139±12^{b}	34±3^{ab}	36±3^{a}	36±3^{a}	10.2±0.4^{b}	9.3±0.4^{a}	9.6±0.2^{a}
					$N_{44}P_{116}K_{116}$ (100 %)							
No inoculation	55±1^{a}	42±1^{a}	42±2^{a}	121±4^{a}	103±5^{a}	112±6^{a}	42±1^{a}	34±3^{a}	39±2^{a}	9.7±0.1^{a}	8.6±0.3^{a}	9.0±0.2^{a}
B. japonicum 634b	55±2^{a}	44±2^{ab}	55±5^{b}	130±8^{ab}	117±8^{ab}	118±10^{a}	45±2^{a}	45±3^{b}	40±2^{a}	10.7±0.2^{b}	9.1±0.3^{ab}	8.7±0.3^{a}
Ps. oryzihabitans Ep4	57±3^{a}	47±3^{ab}	55±4^{b}	132±6^{ab}	116±5^{ab}	114±5^{a}	42±3^{a}	39±3^{ab}	37±2^{a}	11.1±0.2^{bc}	9.0±0.2^{ab}	8.6±0.2^{a}
V. paradoxus 3P-4	56±2^{a}	50±2^{ab}	55±3^{b}	123±4^{a}	128±6^{bc}	115±5^{a}	41±2^{a}	41±2^{ab}	41±3^{a}	10.2±0.3^{ab}	9.2±0.1^{ab}	9.0±0.2^{a}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	62±5^{a}	60±5^{c}	74±6^{c}	164±8^{c}	144±12^{c}	133±4^{a}	47±2^{a}	47±3^{b}	43±2^{a}	11.9±0.4^{c}	9.3±0.2^{ab}	10.1±0.4^{b}
B. japonicum 634b + *V. paradoxus* 3P-4	61±4^{a}	53±4^{bc}	52±3^{b}	150±9^{bc}	130±6^{bc}	131±5^{a}	45±2^{a}	43±3^{b}	40±2^{a}	11.6±0.4^{c}	9.5±0.4^{b}	10.0±0.4^{b}

N o t e. For each fertilizer dosage, statistically significant differing variants are designated by Latin letters (Fisher test, MMD, $p < 0.05$, $n = 12$).

5. Plant productivity of soybean *Glycine max* (L.) Merr. varieties during flowering as influenced by nodule bacteria *Bradyrhizobium japonicum* 634b (rhizotorfin) and associative bacteria *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 at different levels of mineral nutrition (*M*±SEM, APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015)

Variant	Crop productivity, g/m^2			Protein content in seeds, %			Lipid content in seeds, %		
	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara	Nice Mecha	Swapa	Bara
				$N_{30}P_{81}K_{81}$ (70 %)					
No inoculation	217±10^a	200±14^a	204±6^a	34.2±2.0^a	31.6±0.7^a	33.0±0.8^a	21.0±0.8^a	23.2±0.9^a	23.7±1.2^a
B. japonicum 634b	262±8^b	264±16^b	258±9^{bc}	38.1±0.7^b	34.4±0.4^b	34.6±1.0^a	22.8±0.4^b	24.5±0.8^b	24.0±0.8^{ab}
Ps. oryzihabitans Ep4	250±11^{ab}	258±10^b	240±10^{bc}	38.1±0.9^b	33.7±0.9a	34.5±1.7^a	21.8±0.5^{ab}	24.7±0.6^{ab}	23.3±0.9^a
V. paradoxus 3P-4	260±14^b	229±6^{ab}	226±11^{ab}	38.2±0.6^b	35.9±0.3^b	34.6±0.3^a	21.7±0.7^{ab}	23.9±0.8^{ab}	24.3±1.1^{ab}
B. japonicum 634b + *Ps. oryzihabitans* Ep4	249±12^{ab}	313±15^c	251±10^c	39.0±1.0^b	41.1±0.7^c	35.1±0.8^{ab}	23.2±0.7^b	23.9±0.3^{ab}	25.9±1.1^b
B. japonicum 634b + *V. paradoxus* 3P-4	270±14^b	280±17^{bc}	270±15^c	38.3±0.6^b	36.4±0.9^b	37.8±0.9^b	21.8±0.9^{ab}	24.9±0.6^b	22.5±0.3^a
				$N_{44}P_{116}K_{116}$ (100 %)					
No inoculation	204±26^a	237±11^a	266±21^a	34.6±2.4^a	32.7±1.3^a	33.3±0.8^a	19.8±0.5^a	23.0±0.3^a	22.6±0.4^a
B. japonicum 634b	249±12^b	299±10^b	312±6^{bc}	39.3±1.2^b	35.4±1.1^{bc}	35.8±1.4^{ab}	21.8±0.7^b	22.5±0.3^a	22.7±0.9^a
Ps. oryzihabitans Ep4	219±26^{ab}	267±12^{ab}	290±13^a	40.1±1.1^b	35.6±0.9^c	35.0±0.8^a	21.4±0.7^{ab}	23.3±0.3^{ab}	23.0±0.2^a
V. paradoxus 3P-4	235±18^{ab}	272±17^{ab}	293±15^a	40.3±0.3^b	33.2±1.3^{ab}	35.7±0.7^{ab}	21.8±0.3^b	24.7±0.4^b	23.2±0.2^a
B. japonicum 634b + *Ps. oryzihabitans* Ep4	251±13^b	277±9^b	343±7^c	39.7±0.8^b	37.9±0.5^d	36.9±1.8^b	22.0±0.5^b	23.9±0.5^{ab}	24.2±1.0^a
B. japonicum 634b + *V. paradoxus* 3P-4	253±10^b	301±12^b	313±15^c	38.1±1.0^b	36.3±0.6^c	36.9±0.5^b	22.9±0.3^b	24.8±0.5^b	23.4±0.4^a

N o t e. For each fertilizer dosage, statistically significant differing variants are designated by Latin letters (Fisher test, MMD, $p < 0.05$, $n = 12$).

At both levels of mineral nutrition, rhizotorfin has increased the seed yield of all varieties (maximum level at combined inoculations with rhizobacteria) (Table 5). Swapa plants statistically significant increased crop productivity ($p = 0.008$) compared to rhizotorfin application upon its combination with *Ps. oryzihabitans* Ep4 at 100% mineral nutrition. The protein content in seeds increased in Nice Mecha variety upon any inoculation, as well as in Swapa variety in all tests (except for mono-inoculations with *Ps. oryzihabitans* Ep4 and *V. paradoxus* 3-P4 at $N_{30}P_{81}K_{81}$ and $N_{44}P_{116}K_{116}$, respectively) (Table 5). For Bara variety, this effect was observed only for rhizotorfin and *V. paradoxus* 3-P4 combination at $N_{30}P_{81}K_{81}$, as well as for both rhizosphere strains at $N_{44}P_{116}K_{116}$. The lipid content in seeds of inoculated plants was more frequently higher in Nice Mecha plants, less commonly in Swapa and only in one case (for rhizotorfin and *Ps. oryzihabitans* Ep4 co-inoculation at 70% level) in Bara variety (see Table 5).

Field tests conducted by the authors of the present study have shown that rhizobacteria positively affect nitrogen-fixing symbiosis, growth, and nutrition of early ripening varieties of soybean northern ecotypes. This data expands the concept of interaction between rhizobacteria and leguminous plants at co-inoculation [11, 12, 14, 16], as compared to other varieties [15-19], at different types of nitrogen and phosphorus nutrition [20] in traditional soybean cultivating regions. The data of response specificity of varieties under study at the rhizobacteria inoculation has been confirmed. These findings indicate more effective integration of *Ps. oryzihabitans* Ep4 strain with Nice Mecha and Swapa varieties conditioned by root exudation patterns [37]. In these varieties the roots extract more organic acids and sugars, the nutrients for rhizobacteria, thus promoting rhizosphere colonization. Rhizobacteria, in turn, use Bara variety root exudates less (possibly due to the presence of antibacterial components). Another important component of exudates, tryptophane (the precursor of auxin biosynthesis in bacteria) [34] is not absorbed in Bara variety rhizosphere [37]. Our findings are in line with data pointing to the important role that the root exudates play in varietal specificity of rhizobacterial effects described for pea growth [32], soybean legume-rhizobia symbiosis [15, 30], as well as *Arabidopsis thaliana* [45] and sorghum [46] growth.

More effective interactions between rhizobacteria under study and Nice Mecha and Swapa plants compared to Bara variety are also confirmed by integrated parameters of bacterial effects (Fig. 1). These parameters allow summing up the factors related to different aspect of specific plant response to inoculation but do not depend on the effect of rhizotorfin. In all the cases (except *V. paradoxus* 3-P4 which can influence symbiosis), the minimal rhizobacteria effect values were characteristic of Bara variety (see Fig. 1). Additionally, the effects of both strains on mineral nutrition and of *V. paradoxus* 3-P4 strain on Swapa plant biomass turned out to be statistically higher than that on Bara variety. It should be noted that the maximum relative values of rhizobacterial effects were obtained for nitrogen-fixing symbiosis parameters (see Table 1, Fig. 1). This fact indicates the importance of interactions between microorganisms in the rhizosphere not only for enhanced growth due to effective symbiosis but also for better mineral nutrition of plants. Indeed, additive and synergetic effects on the nutrient levels in leaves appear upon combined inoculation with rhizobacteria and rhizotorfin.

The positive effect of rhizobacteria on nitrogen-fixing soybean symbiosis (the increase in nodule formation and acetylene-reductase activity) could be associated with the presence of ACC deaminase enzyme [38, 39]. It has been established that ACC-utilizing rhizobacteria reduce the ACC content in the roots, contributing to a decrease in plant biosynthesis phytohormone ethylene, which

acts as an inhibitor of symbiotic nodule formation [23, 48]. In our experiments the stimulation of nodule formation in pea by *V. paradoxus* 5C-2 [21], as well as in the soybean Swapa variety by *Ps. oryzihabitans* Ep4 [37] strain has also been described.

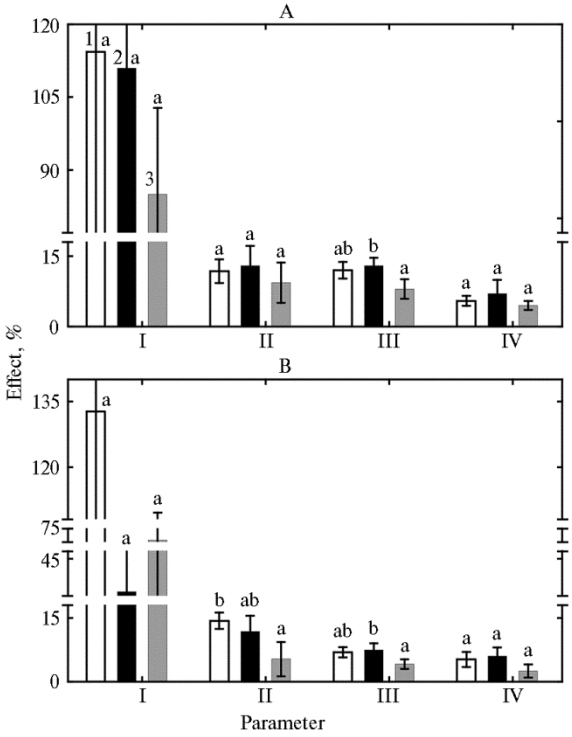

Fig. 1. Evaluation of *Pseudomonas oryzihabitans* Ep4 (A) and *Variovorax paradoxus* 3P-4 (B) effects depending on soybean *Glycine max* (L.) Merr. Variety, by integrated parameters: I — symbiosis (nodule number, nodule weight, nitrogenase activity), II — biomass (overground weight, root weight at flowering, crop productivity), III — N, P, K, Mg, Ca, B, Fe, Zn and Mo content in leaves, IV — quality (proteins and lipids in seeds); 1 — Nice Mecha, 2 — Swapa, 3 — Bara. The rhizobacterial effect is calculated as a median value of the effect of components compared to the corresponding control (with or without *B. japonicum* 634b inoculation) against the background of each level of the two fertilizers ($N_{30}P_{81}K_{81}$ or $N_{44}P_{116}K_{116}$). Median values errors are designated by vertical intervals (±SEM). Statistically significant differences between the varieties for each parameter are designated with different Latin letters (*t*-Student test, $p < 0.05$) (APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015).

The obtained results are in line with the data of the researchers who studied the effects of combined inoculation with nodule and rhizosphere bacteria on the nitrogen-fixing symbiosis in different leguminous crops. Thus, it was shown that chick-pea inoculation with nodule bacteria *Mesorhizobium ciceri* and *Ps. fluorescens* [49] or *Bacillus* sp. [50] led to increased nodule number and nodule weight. These factors were also increasing upon combined inoculation of bean plants with nodule bacteria *R. leguminosarum* bv. *phaseoli* and different species of rhizobacteria of *Bacillus* genera [51-52], *Ps. fluorescens* or *Az. lipoferum* [53]. Rhizobacteria *Az. brasilense*, *Azotobacter chroococcum*, *B. cereus*, *Ps. putida* and *Ps. fluorescens* stimulated nodulation and nitrogen-fixing activity of pigeon-pea (*Cajanus cajan*) upon application of nodule bacteria *Rhizobium* sp. biopreparation [54]. Similar results were obtained with rhizobacteria and nodule bacteria for pea [55, 56], lentil [56], Chickasano pea [57] and medick [58]. As a rule, in these experiments stimulation of nodule formation led to increased biomass of overground parts and seed harvests.

The affection of the strains under study on the soybean plants was to a large extent similar as evidenced by the positive correlation between *Ps. oryzihabitans* Ep4 and *V. paradoxus* 3-P4 effects on the parameters measured at $N_{30}P_{81}K_{81}$ ($r = +0.60$, $p < 0.001$, $n = 48$) and $N_{44}P_{116}K_{116}$ ($r = +0.70$, $p < 0.001$, $n = 48$). This could be due to similarity of the in mechanisms of their interaction with plants, as both strains exhibit high ACC deaminase activity and produce auxins and siderophores [37-39]. Nevertheless, *Ps. oryzihabitans* Ep4 increased the content of nutrients in leaves to a greater extent compared to *V. paradoxus* 3-P4, especially at combined inoculation with nodule bacteria (see Tables 3, 4, Fig. 1). It is known that rhizobacteria of *Pseudomonas* genus can

mobilize soil nutrients, and their positive effect on mineral nutrition is important for plant growth improvement [4, 25, 43, 44]. However, only fragmentary data are available on the ability of rhizobacteria of the *Variovorax* genus to affect the plant consumption of nutrients. Thus, the authors had previously shown that the inoculation of pea with *V. paradoxus* 5C-2 strain increased the plant consumption of N, P, K, Ca and Mg [25, 59]. The results presented in the current study indicate that *V. paradoxus* increases the consumption of nutrients by soybean, and this effect depends on the plant variety and mineral fertilizer dosage.

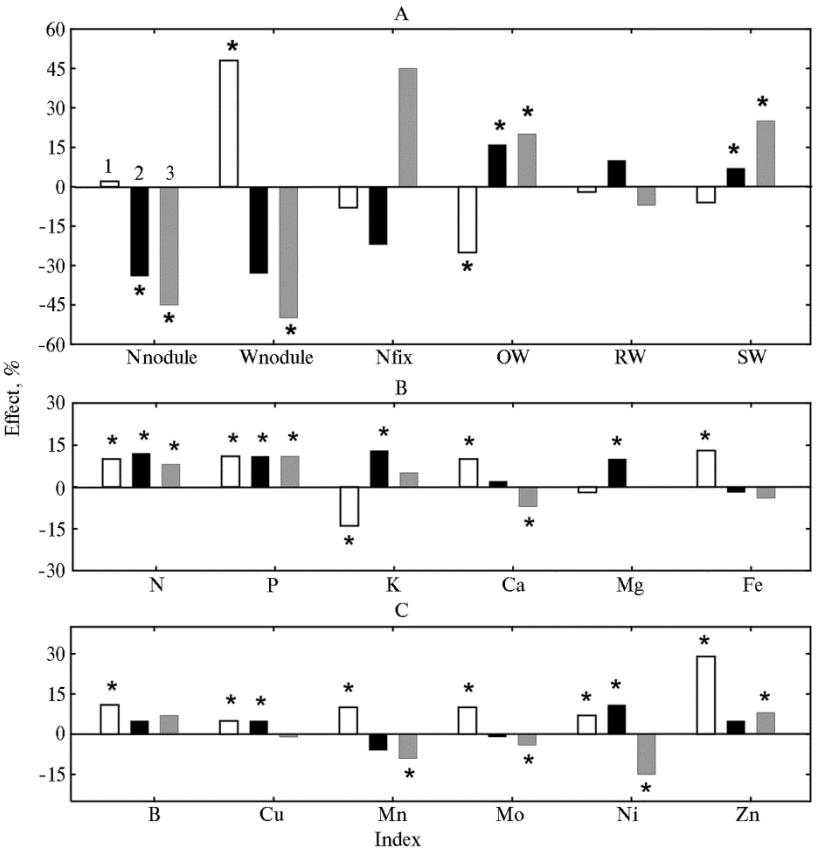

Fig. 2. Soybean *Glycine max* (L.) Merr. varieties Nice Mecha (1)**, Swapa** (2) **and Bara** (3) **response to mineral fertilizers**: Nnodule — nodule number, Wnodule — nodule weight, Nfix — nodule acetylene-reductase activity, OW — overground weight of dry plants at the flowering stage, RW — dry roots weight at the flowering stage, SW — seeds weight at the firm ripe stage; N, P, K, Ca, Mg, Fe, B, Cu, Mn, Mo, Ni and Zn — elemental content in leaves at flowering. The effect of fertilizer level is represented as the ratio of mean absolute values of the factor for all the inoculation variants at $N_{30}P_{81}K_{81}$ to the mean value of this factor at $N_{44}P_{116}K_{116}$. Statistically significant differences between the fertilizer levels are marked with the asterisks (Fisher MMD test, $p < 0.05$, $n = 72$) (APC Integration of Orel State Agricultural University, Lavrovo, Orel Province, 2013-2015).

The observed effects on the plants for each of two rhizobacteria strains were similar at both mineral nutrition levels. The authors observed a positive correlation of the effects at the given levels by measured parameters for *Ps. oryzihabitans* Ep4 ($r = +0.61$, $p < 0.001$, $n = 48$) and *V. paradoxus* 3-P4 ($r = +0.49$, $p < 0.001$, $n = 48$). The results give the evidence of the ability of rhizobacteria under study to mediate stable and positive affection on growth and nutritional parameters of soybean plants at different mineral nutrition dosages. At the same time, responses of soybean varieties to mineral significantly differ, which are shown as averaged effects of the fertilizer factor for all the inoculation variants (Fig. 2). Firstly, at 100% level of mineral nutrition, the number and weight of nodules were

less for Swapa and Bara varieties, whereas for Nice Mecha variety the second factor increased. That means that the optimal dosage of the fertilizer (most likely nitrogenic) for nitrogen-fixing symbiosis formation is individual for each variety. Secondly, the positive growth response to the increase in the mineral fertilizer dosage was to a greater extent indicative for the Bara variety with the relatively low rhizobacterial symbiotic potential. This led to the maximum increase in the overground plant part and seed weight (see Fig. 1). Responsiveness to mineral fertilizers and symbiosis effectivity may be interdependent, as far as the plants have an evolutionary determinant aimed to compensate for low adaptation to unfavorable soil conditions by means of intensification of symbiotic interactions with microorganisms. This phenomenon was described by the authors of the present study for the first time while investigating correlations between the effectiveness of interaction of 99 pea genotypes with the arbuscular-mycorrhiza fungi and their resistance to heavy metals [60]. Thirdly, only the Bara variety has demonstrated the decrease in the leaf content of nutrients (Ca, Mn, Mo, and Ni) upon the fertilizer dosage increase (also K content reduction occurred in Nice Mecha variety) (see Fig. 1). This effect was possibly associated with the dilution of elements by biomass which the most significantly increased in Bara variety at 100% level of mineral nutrition.

Therefore, rhizobacteria *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* 3-P4 which produce auxins and exhibit ACC-deaminase activity can activate legume-rhizobia symbiosis, consumption of nutrients from the soil by the roots, and increase in seed quality of early ripening soybean varieties of the northern ecotype. This indicates the stability and significance of the effects observed, and therefore the prospects of using such microorganisms as biopreparations to improve soybean adaptation to the northern regions of cultivation. Significant genotypic differences between soybean varieties in their response to rhizobacterial inoculation point to the higher extent of integration of rhizobacteria with Nice Mecha and Swapa plants in agrocenosis as compared to Bara variety. It is possible that the insufficiency of Bara plant symbiotic potential is compensated by its ability to use mineral fertilizers more effectively. In this regard, creation of varieties and plant-microbial systems combining a high level of symbiotrophy and an ability to actively assimilate nutrients from fertilizers and soil is of interest.

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PHYLOGENETIC STRUCTURE OF COMMUNITY OF PROCARIOTS OF SODDY-PODZOLIC SOIL UNDER THE COVER OF WINTER RYE IS NOT INFLUENCED BY AGROTECHNICS

V.A. FILIPPOVA, Yu.V. KRUGLOV, E.E. ANDRONOV

All-Russian Research Institute for Agricultural Microbiology, Federal Agency of Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail: doumova@mail.ru (✉ corresponding author), yuvkruglov@yandex.ru, eeandr@gmail.com
ORCID:
Filippova V.A. orcid.org/0000-0001-8789-9837
Kruglov Yu.V. orcid.org/0000-0001-9259-3701
Andronov E.E. orcid.org/0000-0002-5204-262X
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Abstract

Soil microbial communities are complex multicomponent systems that form under the influence of a wide range of factors, among them — soil type, plant species, climate, agricultural technology — in general, determining the physical and chemical characteristics of the environment. The plant, according to many researchers, is the main factor determining the structure of the soil microbial community, due to the extensive number of compounds released into the soil. There is still a discussion about the specific nature of the action of various plants on soil microbiome, which is very important both for understanding the mechanism of interaction of microorganisms and plants, and for building optimal crop rotations, as well as organizing measures to protect agricultural crops from phytopathogenic microorganisms and pests. Winter rye is one of the few crops that can grow continuously for decades. It has a powerful root system, comparable to the biomass of the above-ground part of plants. The root excretions of winter rye reach 21 % of the synthesized plant mass. This paper presents the results of research aimed at studying the phylotypical structure and diversity of prokaryotic microorganisms in rye crops grown in permanent culture and six-field crop rotation for almost 100 years, in the long-term multifactorial field experiment of the Moscow Timiryazev Agricultural Academy. The aim of our work was to study the influence of various agricultural technicians such as crop rotation and liming, under the conditions of a long field experiment on the phylogenetic structure of prokaryotic micro-organisms in rye crops. The results of the high-throughput DNA sequencing of the soil microbiome and the subsequent analysis of the phylogenetic structure and diversity of the prokaryotic microorganisms of the sod-podzolic soil under the conditions of perennial rye culture showed that the plant is one of the key factors in the formation of the prokaryotic community. Regardless of the agrotechnical methods under the cover of winter rye, the same core structure of prokaryotes, including a small number of types of proteobacteria and actinobacteria, develops in the earing phase. The dominant position among them is occupied by the bacteria of the *Rhizobiaceae* family, which in this case is to some extent related to the history of the experimental field. Apparently, the bacteria of this family and, above all, the nodule bacteria, find favorable conditions for their development in the rye rhizosphere. It is possible that a kind of associative symbiosis is formed between them, which was observed by some authors with other cereal crops. In this connection, studies of the viability of *Rhizobiaceae* in winter rye crops, and their evolution to associative endosymbiotic relationships with rye in the course of a long coexistence are of undoubted interest. The effect of liming on the genetic structure of the prokaryote community of acidic soils may be different. At the same time, apparently, the specific type of plants, as well as the history of the field (crop rotation, permanent culture, fertilizer system, etc.) are of significant importance.

Keywords: phylogenetic structure, biodiversity of prokaryotes, *Rhizobium* sp., *Proteobacteria*, sod-podzolic soil, winter rye.

The soil is a complex ecological system, where a critical role is played by

microbial communities, which ensure the normal functioning of the biosphere [1, 2]. For agriculture, the taxonomic and functional structure of the microbial community, which is formed under a variety of physical and chemical environmental conditions under the cover of different plant species, is of special interest. Most researchers are of the opinion that the main factor in the formation of the rhizosphere microbiota, and especially the rhizoplane, are plants, but, given the complexity of the interaction in the soil—microorganism —plant system, many of them recognize the significant influence of the soil type, agrotechnical measures, and climate on the structure and diversity of rhizosphere microorganisms [3-5].

Until now, there is a discussion about the specific nature of the effect on various plants on the soil microbiome, which is very important for understanding the mechanism of interaction between microorganisms and plants, building optimal crop rotations, as well as organizing measures to protect agricultural crops from phytopathogenic microorganisms and pests. In this regard, a special role is given to the study of soil microbial flora under the conditions of long field experiments in which the same crop is under cultivation in the field for many years. Such experiments are conducted in Russia, Germany, Great Britain, the USA, Canada, and France [6]. Total studies of the phylogenetic structure of the soil microbiome in the multifactorial long-term (more than 100 years) field experiment (Moscow Timiryazev Agricultural Academy) showed [7] that the key factor in the microbiome phylogenetic diversity is the type of cultivated plant, liming is in the second place. The systematic use of mineral fertilizers had no noticeable effect on the phylogenetic structure of the soil microbiome. At the same time, microbiomes of the soil sown with various plants respond differently to liming [7].

The structure of the soil microbiome under winter rye is of special interest. It is one of the few crops that can grow continuously for decades. It has a powerful root system, comparable to the biomass of the above-ground part of plants and reaching 6 t/ha [8]. The total area of the roots is about 6 thousand m^2 and their surface exceeds the surface of the above-ground part by 130 times [9]. According to some authors, the root excretions reach 21% of the synthesized plant mass. The root excretions of winter rye and plant tissues include organic acids, sugars, cyclic hydroxamic acid glucosides, as well as the products of their secondary transformation, α-glucans and benzoxyzolinone derivatives [11-13]. Hydroxamic acid derivatives and their transformation products have herbicidal, fungicidal and insecticidal properties, providing protection of the crop from phytopathogenic fungi and its high competitiveness with weeds [14-16]. This suggests a significant effect of winter rye on the soil microbiome.

E. Kurek et al. [17] showed that the number of prokaryotes in the rhizosphere of winter rye is higher than in the soil. According to their data, gram-positive bacteria prevail in the soil and in the rhizosphere of plants. Other researchers note that gram-negative bacteria prevail in the rhizosphere of plants of almost all field crops, especially at an early age [2, 9]. According to A.O. Zverev et al. [18], the phylogenetic structure of prokaryotes and their diversity in the rhizosphere of winter rye at the age of 42 days and in the fallow soil almost do not differ. I.G. Shirokikh et al. [19] found in the rye rhizosphere a significant number of actinomycetes; their species composition and number changed during the ontogeny of plants. The dominant position was occupied by streptomycetes. Data on the long-term effect of rye cultivation on soil microbial flora are not available.

This paper for the first time presents data on the phylotype structure and diversity of prokaryotic microorganisms in the soil when growing rye as a permanent crop in six-field crop rotation for almost 100 years (long-term multifactorial field experiment of the Moscow Timiryazev Agricultural Academy). The

results indicate that the plant is a major factor in the formation of a soil prokaryotic community.

The purpose of this study was to assess the effect of different agricultural technologies (crop rotation, liming) under the conditions of a long-term field experiment on the phylogenetic structure of microorganisms in rye crops.

Techniques. Soil samples were collected in 2010 at the site of the long-term experiment of the Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, which was located on an area of about 1.5 hectares with a slope of 1° to the north-west on the morainic plain in the southern part of the Klin-Dmitrov Upland. The altitude above the sea level was 162 m, the average precipitation was about 600 mm per year, about half of which occurred in May-August; the average annual temperature was 4.1 °C. The soil was sod-podzolic, sandy large-silt loam, old-arable (over 200 years in tillage) [20]. Plots planted with winter rye (*Secale cereale* L.), which has been cultivated as a permanent crop in six-field crop rotation since 1912, were used for phylogenetic analysis of the prokaryotic microorganisms system in the soil. Crop rotation included bare fallow, winter rye, potatoes, barley with clover undersowing, first-year clover, and flax. Mineral fertilizers were applied to experimental plots annually. The total volume of these fertilizers over the years of study (1912-2009) was 5820 kg of nitrogen, 7990 kg of phosphorus, 6716 kg/ha of potassium [20].

Soil samples were collected during the winter rye paniculation phase to a depth of arable horizon A1 (0-20 cm) in 5 replications, of which an average sample was made, which was thoroughly mixed.

When isolating DNA from a soil sample, a weighed portion (0.2 g) was placed in an Eppendorf tube with a volume of 2 ml, then an equal volume of glass beads with a diameter of 0.1 mm (Innomed, Hungary), 350 μl of A solution (20 mM of sodium phosphate buffer, 240 mM of guanidine isothiocyanate, pH 7.0), 350 μl of B solution (500 mM of Tris-HCl, 1% SDS, pH 7.0) and 400 μl of a phenol and chloroform mixture were added. The tube was placed in a FastPrep-24 homogenizer (MP Biomedicals, USA) and the sample was destroyed within 10-15 min. Then it was centrifuged at 10,000-15,000 g for 5 min, the aqueous phase was collected. After homogenization, 400 μl of chloroform was added into the sample, then it was vigorously shaken using a vortex for 1 min, centrifuged under the same conditions as in the previous stage, the aqueous phase was collected. An equal volume of isopropyl alcohol was added to the extracted DNA; then it was vortexed, centrifuged, washed twice with 70% ethanol, and dried in air. The precipitate was dissolved in 100 μl of water at 65 °C for 15 min.

DNA was purified from impurities using electrophoresis in 1% agarose gel. A cut agarose block containing DNA was placed in an Eppendorf tube (1.5 ml), 2 volumes of C solution (3 M of guanidine isothiocyanate, 20 mM of Tris-HCl, 20 mg/ml of Triton X-100, pH 7.0) were added and incubated at a temperature of 65 °C until complete dissolution of the block. 20 μl of D solution (C solution with the addition of silica, 40 mg/ml) was added to the solution, then it was stirred and incubated for 5 min at room temperature and shaken occasionally. Then it was centrifuged at 10000-15000 g for 1 min, the supernatant was completely removed, the precipitate was suspended in 200 μl of D solution (25% of ethanol, 25% of isopropanol, 100 mM of NaCl, 10 mM of Tris-HCl, pH 7.0), centrifuged at 10,000-15,000 g for 1 min, then the supernatant was removed, the precipitate was resuspended in ethanol, centrifuged again for 1 min, and then the supernatant was removed. The precipitate was dried in air for 15 minutes, 50 μl of elution buffer (10 mM of Tris-HCl, 1 mM of EDTA, pH 8.0) was added and vortexed for 30 minutes. After that, the samples were centrifuged and the supernatant was collected, avoiding the ingress of silicon oxide into the

purified DNA sample.

The isolated total soil DNA was used as a template for sequencing nucleotide sequences. Universal primers for the V4 variable region of the 16S rRNA gene (F515 – 5′-GTGCCAGCMGCCGCGGTAA-3′, R806 – 5′-GGATACVSGGGTATCTAAT-3′) were used with the addition of oligonucleotide identifiers for each sample and service sequences necessary for high-throughput DNA sequencing using the Roche protocol (Switzerland). Samples preparation and sequencing were performed using GS Junior (Roche, Switzerland) in accordance with the manufacturer's recommendations. Taxonomic identification of DNA sequences and comparative analysis of microbial communities were performed using VAMPS (Visualization and Analysis of Microbial Population Structure) (http://vamps.mbl.edu/). In addition, the RDP database (Ribosomal Database Project, http://rdp.cme.msu.edu/) was used for the extended phylogenetic characteristics of sequences.

Results. Agrochemical characteristics of the soil as of the date of soil sampling are presented in the table.*Результаты.* Агрохимическая характеристика почвы на дату отбора почвенных образцов представлена в таблице.

Agrochemical characteristics of sod-podzolic soil in the plots of the long-term experiment under the crops of winter rye (experimental field of the Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow, 2010)

Experiment variant	N_{total}, %	P_2O_5, mg/100g	K_2O, mg/100g	C_{total}, %	$pH_{sal.}$	The amount of exchange bases, mEq/100 g
Rye crop rotation	0.090	31.45	4.25	0.79	4.2	8.00
Rye crop rotation + lime	0.098	31.85	4.63	0.93	5.7	7.75
Permanent rye	0.112	53.80	24.70	1.28	4.6	8.63
Permanent rye + lime	0.095	53.70	21.51	0.98	6.1	8.25

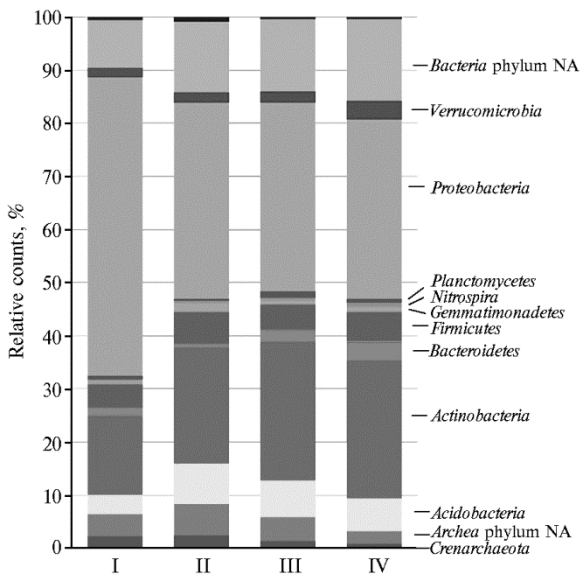

Fig. 1. Taxonomic diversity of prokaryotic microorganisms of sod-podzolic soil (at the phyla level) in the plots of the long-term experiment under the crops of winter rye depending on the cultivation technology: I – in crop rotation with soil liming, II – in crop rotation without soil liming, III – permanent crop with soil liming, IV – permanent crop without soil liming (experimental field of the Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow, 2010)

High-throughput pyrosequencing of amplified DNA from soil samples as part of the microbial community of the sod-podzolic soil under winter rye crops revealed 16 phyla of bacteria and 2 phyla of archaea. The dominant position was occupied by two bacterial phyla — *Proteobacteria* (from 34 to 56%) and *Actinobacteria* (from 15 to 26%). The *Acidobacteria* and *Firmicutes* phyla were from 3.5 to 7.5%, and archaeans were from 3.0 to 8.5% of the total number of prokaryotic microorganisms (Fig. 1).

About 300 genera of microorganisms were found in of the soil prokaryotic community. Among them, only 41 (which did not exceed 13% of the total number of taxa) had a frequency of more than 1% (Fig. 2). 12 genera were encountered in all variants of the

experiment, regardless of the winter rye growing technology (rotation, permanent crop, liming). Apparently, they were the core system of prokaryotes, characteristic of the studied soil type under winter rye crops. The system was dominated by bacteria belonging to the *Proteobacteria* and *Actinobacteria* phyla, as well as unidentifiable bacteria (see Fig. 2). The dominant development of these taxa in the rhizosphere of winter rye was observed by other authors [2, 18], which is consistent with the results of this study.

Microorganism	I	II	III	IV
Acidobacteria Gp6	0.99	1.85	0.37	1.42
Acidobacteria Gp16	0.58	1.50	0.87	2.24
Acidobacteria Gp1	0.58	0.45	3.29	0.67
Acidobacteria Gp4	0.58	1.00	0.31	0.89
Acidobacteria Gp3	0.25	0.65	1.49	0.37
Actinobacteria genus NA18	4.47	7.44	5.21	6.18
Actinobacteria genus NA17	1.66	2.70	2.73	2.76
Solirubrobacter sp.	1.41	1.45	1.43	1.86
Actinobacteria genus NA16	1.08	2.15	2.42	1.86
Arthrobacter sp.	0.91	1.50	0.93	1.12
Actinobacteria genus NA11	0.58	0.70	0.74	0.97
Streptomyces sp.	0.50	1.05	0.37	1.04
Conexibacter sp.	0.50	0.35	1.05	0.60
Nocardioides sp.	0.25	0.95	0.50	1.19
Actinobacteria genus NA5	0.17	1.40	0.37	0.60
Bacteroidetes genus NA2	0.50	1.55	0.37	1.04
Crenarchaeota genus NA	2.24	0.85	2.42	1.42
Firmicutes genus NA1	1.08	1.15	1.12	1.19
Firmicutes genus NA5	0.83	1.10	1.24	1.56
Paenibacillus sp.	0.75	0.70	1.05	0.07
Bacillus sp.	0.58	0.85	0.93	0.67
Gemmatimonas sp.	0.66	1.10	1.74	0.89
Bacteria genus NA	9.02	15.43	13.08	13.56
Archaea genus NA	4.22	2.50	5.95	4.47
Rhizobium sp.	25.83	6.04	13.14	6.33
Proteobacteria genus NA7	14.90	3.55	3.60	3.80
Proteobacteria genus NA24	2.15	4.20	2.36	3.13
Proteobacteria genus NA30	1.57	1.55	3.35	2.76
Proteobacteria genus NA6	1.41	0.05	0.12	0.15
Proteobacteria genus NA28	0.75	1.35	0.43	1.04
Pseudomonas sp.	0.66	0.45	0.74	1.34
Bradyrhizobium sp.	0.58	0.85	0.93	0.60
Proteobacteria genus NA15	0.50	1.00	1.43	1.27
Sphingomonas sp.	0.41	0.95	0.50	1.27
Proteobacteria genus NA36	0.41	1.45	0.68	1.27
Proteobacteria genus NA34	0.33	1.00	0.93	0.97
Hyphomicrobium sp.	0.17	1.05	0.12	1.42
Verrucomicrobia genus NA2	1.08	2.20	1.12	1.19

Fig. 2. Heat map of the dominant prokaryotic microorganisms of sod-podzolic soil (at the genus level) in the plots of the long-term experiment under the crops of winter rye depending on the cultivation technology: I — in crop rotation with soil liming, II — permanent crop with soil liming, III — in crop rotation without soil liming, IV — permanent crop without soil liming; white to dark gray color gradations — abundance of microorganisms (in percent), respectively, ≤1; 1.01-5; 5.01-10 and > 10 (experimental field of the Russian State Agrarian University − Moscow Timiryazev Agricultural Academy, Moscow, 2010).

According to the dominance (diversity) curves of the prokaryotic system in the soil, the greatest species wealth of prokaryotic microorganisms was found under the permanent rye crop (Fig. 3). It was significantly lower in the crops of rye grown under the conditions of six-field crop rotation. The low species wealth in the latter case, apparently, is due to the fact that rye precursors were flax and fallow field, where the delivery into the soil of fresh organic matter as a source of nutrition and energy-yielding material for microorganisms is extremely limited. These observations lead to the conclusion about a certain effect of the precursor on the species wealth and diversity of the prokaryotic system of soil microorganisms in winter rye crops..

In all variants of the experiment, the *Rhizobium* genus occupied the dominant position among proteobacteria. In terms of phylogenetics, the *Rhizobium* genus is very close to the *Agrobacterium* and *Allorhizobium* genera, which are the members of the *Rhizobiaceae* family, and which are currently united in the *Rhizobium — Agrobacterium* phylogenetic group. Moreover, J.M. Young et al. [21], on the basis of phylogenetic similarity, proposed to combine these bacteria into one genus — *Rhizobium*. The results obtained on the dominant position of the *Rhizobium* genus bacteria in the soil under the cover of winter rye should be considered, at least, as the dominant position of the *Rhizobium − Agrobacterium* group bacteria, taking into account the degree of their relationship and the resolution of the method used. A significant number of microorganisms from this group in the soil and rhizosphere of plants was noted by many authors. According to M. Sadowski et al. [22], nodule bacteria (*Rhizobium* sp. and *Bradyrhizobium* sp.) are wide-

spread and are up to 8.0% of the total number of bacteria in the soil. The presence of *Rhizobiaceae* representatives, including nodule bacteria, in the rhizosphere and the roots of cereal crops has been reported in a number of papers [18, 23, 24]. Some researchers call nodule bacteria endophytes of cereal crops [25-27]. Zverev *et al.* [18], on the basis of phylogenetic analysis, found nodule bacteria of the *Mezorhizobium* genus belonging to this family in the rhizosphere of a 42-day-old winter rye crop. The pathogenesis of winter rye caused by *Agrobacterium tumefaciens* was identified in no case [28].

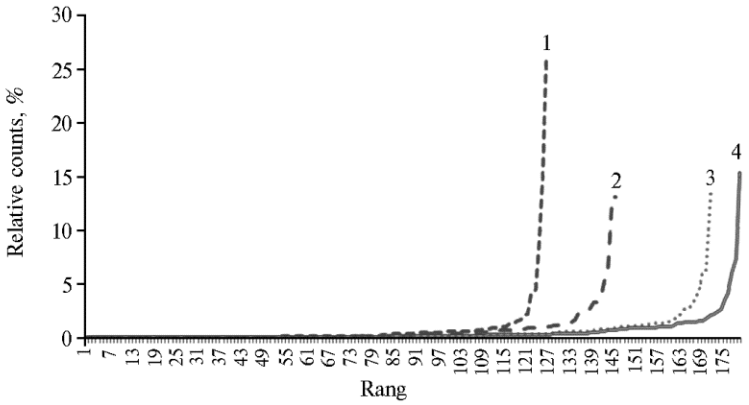

Fig. 3. Dominance (diversity) curves of the prokaryotic system in sod-podzolic soil in the plots of the long-term experiment under the crops of winter rye depending on the cultivation technology: 1 — in crop rotation with soil liming, 2 — in crop rotation without soil liming, 3 — permanent crop with soil liming, 4 — permanent crop without soil liming (experimental field of the Russian State Agrarian University —Moscow Timiryazev Agricultural Academy, Moscow, 2010).

The presence of the *Rhizobiaceae* family in the soil under the winter rye crops in long-term field experiments, in the authors' opinion, is due to the fact that in the conditions of six-field crop rotation two fields were occupied by clover. The nodule bacteria present in the clover crops survived the period when the soil was occupied by the precursors of winter rye (flax, clean fallow) and found favorable conditions for their development under the cover of this crop. The latter, in terms of biology, is of interest for assessing the adaptation and survival of nodule bacteria in agrocenoses.

As for the permanent rye crop, it should be noted that before establishing the experiment, 100 years ago this field was occupied by clover for several years [20]. In addition, bacteria of the *Rhizobium* genus can exist in the soil without fixing atmospheric nitrogen. It is possible that nodule bacteria adapted and strike roots in the rhizosphere of plants as associative endosymbionts. The presence of such association of spiked cereals of the *Bradyrhizobium* sp., *Agrobacterium* sp., *Rhizobium* sp. nodule bacteria is noted in other papers [25, 27]. It should be noted that the permanent rye crop has formed its own microbial flora, an important component of which was bacteria of the *Rhizobiaceae* family. This raises the interest for a deep analysis of the evolution of *Rhizobiaceae* and cereals relationship, in particular, winter rye, and the practical significance of these studies. Thus, biopreparations on the basis of non-pathogenic *Agrobacterium radiobacter* have already been created and are successfully used in cereal crops planting [29].

Liming almost did not affect the species wealth in the permanent rye crops, but sharply reduced it in crop rotation (see Fig. 3). A direct dependence of prokaryotes biodiversity on the pH value was not found. Large taxa at the phyla level were present in all variants of the experiment. Changes in the phylogenetic structure of prokaryotes were observed at the level of the genus, species,

and strain of microorganisms. Thus, while liming, the abundance of some *Acidobacteria* species decreased, but the abundance of others increased, which indicates a rearrangement of the taxonomic composition of prokaryotes. This contradicts the point of view about the unconditional positive effect of liming acidic soils on the microbial flora [30, 31]. However, it should be noted that material, which gives a controversial assessment of the effect of liming on the soil microorganisms community, has been accumulated in recent years. If according to some authors [32, 33], liming increased the biomass of microorganisms and the intensity of soil respiration, other researchers [34] showed that changes in the pH of the red soils, both towards acidic and alkaline, led to a decrease in the biomass of microorganisms. Kennedy *et al.* [35], along with an increase in microbiological activity during liming, show a change in the phylogenetic structure and a decrease in the diversity of the soil bacterial community. Different effects of liming on the phylogenetic diversity of prokaryotes under the cover of various plants were noted by Korvigo *et al.* [7]. In particular, liming led to a decrease in the diversity of prokaryotes in the link of crop rotation of potatoes and flax (precursors of winter rye), which corresponds to the results of this research.

Thus, the analysis of the phylogenetic structure and diversity of prokaryotic microorganisms in sod-podzolic soil under the conditions of a perennial rye crop showed that the plant is the main factor in the formation of the prokaryotic community. Regardless of the agrotechnical methods, the core system of prokaryotes with the same structure, including a small number of *Proteobacteria* and *Actinobacteria* species, is formed under the cover of winter rye during the paniculation phase. A dominant position among them is occupied by bacteria of the *Rhizobiacea*e family, in particular, the *Rhizobium* genus, which is a member of the *Rhizobium* − *Agrobacterium* group, which to a certain extent is related to the history of the experimental field. Apparently, the bacteria of this family, first of all, nodule bacteria, find favorable conditions for development in the rye rhizosphere. The effect of liming on the structure of the prokaryotic community of acid soils may be different. Apparently, the specific type of plants, as well as the history of the field (crop rotation, permanent crop, fertilizing system, etc.), are essential. Further studies of the *Rhizobiaceae* viability in winter rye crops and their evolution towards associative endosymbiotic relationships with rye plants in the process of long-term coexistence are of undoubted scientific interest.

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THE USE OF LOCAL FERTILIZERS SUPPLEMENTED WITH *Trichoderma koningii* Oudem. AT NO-TILL vs. CONVENTIONAL TILLAGE OF AGROCHERNOZEM IN SOUTHERN URAL

I.M. GABBASOVA[1], R.R. SULEIMANOV[1], T.T. GARIPOV[1], M.A. KOMISSAROV[1], L.V. SIDOROVA[1], N.F. GALIMZYANOVA[1], P. LIEBELT[2], E.V. ABAKUMOV[3], G.A. GIMALETDINOVA[1], Z.G. PROSTYAKOVA[1]

[1]*Ufa Federal Research Center RAS, Institute of Biology,* Federal Agency for Scientific Organizations, 69, pr. Oktyabrya, Ufa, 450054 Russia, e-mail gimib@mail.ru, soils@mail.ru, timurgar@gmail.com, mkomissarov@list.ru, galnailya@yandex.ru;
[2]*Martin-Luther-Universität Halle-Wittenberg,* Germany, 06120, Halle (Saale), Von-Seckendorff-Platz 4, e-mail peter.liebelt@geo.uni halle.de;
[3]*Saint-Petersburg State University,* 29, 16 Liniya, St. Petersburg, 199178 Russia, e-mail e_abakumov@mail.ru (✉ corresponding author)
ORCID:
Gabbasova I.M. orcid.org/0000-0002-9238-9011
Suleimanov R.R. orcid.org/0000-0002-7754-0406
Garipov T.T. orcid.org/0000-003-4942-8203
Komissarov M.A. orcid.org/0000-0001-6135-7212
Sidorova L.V. orcid.org/0000-0002-1648-1457
Galimzyanova N.F. orcid.org/0000-0001-6137
Liebelt P. orcid.org/0000-0002-3203-0285
Abakumov E.V. orcid.org/0000-0002-5248-9018
Gimaletdinova G.A. orcid.org/0000-0002-0197-9017
Prostyakova Z.G. orcid.org/0000-0001-6784-3318
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Abstract

Local agrominerals and fertilizers, including marsh plants, which are the waste from cleaning lakes and reservoirs, have definite prospects in preventing soil erosion and restoring fertility alongside with the traditional anti-erosion technologies. Given this, we studied local fertilizers (manure, zeolite, sodium humate) and plant residues (marsh plants, straw) with the addition of $(NP)_{60}$ and suspensions of microscopic fungus *Trichoderma koningii* strain IB G-51 (*T. koningii*) when used at no-till (NT) and conventional tillage (CV) of the weakly eroded Chernozem (Mollisol). The effects were estimated based on the key agrochemical properties (humus content, mobile phosphorus, potassium, and alkaline hydrolyzed nitrogen), assay of activity of soil hydrolases and oxidoreductases and the yield of agricultural crops (spring wheat, barley and sugar beet). A three-year investigation was conducted in the Ural steppe zone. Single application of local fertilizers was shown to promote increasing in soil humus content, improvement of nutrient regime, and increment of enzymatic activity and crops yields. Thus, the increase of humus content in the 0-30 cm soil layer for 3 years is reliable for both types of tillage except the cases when zeolite and sodium humate were applied. These values increase by 3.5-5.6 % for NT, and by 1.8-4.1 % for CV as compared to the control. The soil phosphorus reserve increases from low to medium level due to manure and crop residues with mineral fertilizers added at no-till, whereas at CV a significant increase is observed due to the manure only. Potassium content elevated significantly, from 32 to 45 %, only at application of manure and marsh plants with the addition of $(NP)_{60}$ and *T. koningii*. The content of alkaline hydrolyzed nitrogen varies in a narrow range, and the significant increase is observed only at no-till with manure and marsh plants. Soil enzymatic activity is higher when manure and plant residues are introduced, in contrast to variants with sodium humate and zeolite. Among enzymes, protease and polyphenoloxidase show the closest correlation with agrochemical properties ($r = 0.53$-0.75, $p < 0.05$). The change of agrochemical properties and enzymatic activity of soil is more apparent in 0-10 cm layer under NT and in 0-30 cm layer under CT. The profitability of fertilizers under NT is higher, as compared to CV, only in arid conditions. Biologization of agricultural technology by introduction of the microscopic fungi *T. koningii* IB G-51 causes the faster humification of the marsh plants than the straw that must be especially accounted for at NT. The effect of marsh plants + *T. koningii* on soil properties is commensurate with that of manure.

Keywords: biologization of agriculture, agrochernozem, no-till, local natural fertilizers, marsh plants, *Trichoderma koningii*, soil enzymatic activity, humification, crop yields

Water and wind soil erosion remains one of the agronomic soil science problems in all natural and climatic zones of the planet. Along with traditional anti-erosion farming technologies [1-3], local agronomical ores [4, 5] and fertilizers are used to prevent soil erosion and restore fertility. It is possible to use floating mat [6, 7] as a basis for organic fertilizers, which is formed in large quantities when overgrowing of lakes and water reservoirs and requires disposal after their cleaning (RF patents No. 2524376 and No. 2531167). The methods of farming biologization are particularly effective in combination with soil-protective treatment, especially no-till [8-12]. In this case, not only the hydrophysical, agrochemical, but also the biological properties of the soil are improved, e.g. microbial biomass [13, 14], enzymatic activity [15, 16], as well as germination of seeds [17], are increased.

As is well known, long-term use of the no-till technology results in the accumulation of slowly decomposing plant residues [18], the destruction of which under anaerobic conditions can lead to an increase in soil phytotoxicity. It is advisable to use various species of microscopic fungi of the *Trichoderma* genus to speed up the humification of residues, which allows obtaining valuable organic fertilizer that has the ability to limit the development of diseases [19-21]. The use of *T. harzianum* and *T. viride* strains in the composting of post-harvest residues (rice, wheat straw) reduced the C:N ratio and formed the compost with nutrient content favorable for plants [22]. In these studies, composting was carried out under special conditions – in pits, composters, storage clamps, which required additional costs for transportation and specially designated areas. It would be much more profitable to carry out this process in the field, but only a few papers are related to the study of the possibility to use microorganisms of the *Trichoderma* genus in the field. It was shown that the introduction of *T. reesei* to accelerate the decomposition of straw in the field provided an increase in the activity of soil enzymes and an increase in the humus content in the soil [23]. The use of *T. viride* for the treatment of fields with sugar cane allowed increasing the content of nutrients in the soil, activating microbial respiration and increasing the crop yield [24]. These data indicate that various strains of the *Trichoderma* genus can survive under natural conditions for a sufficient time and accelerate the decomposition of plant residues, which are likely to accumulate excessively under the climatic conditions of the Southern Pre-Ural region; however, such studies have not been conducted in this region.

The effectiveness of local fertilizers and plant residues with the addition of *Trichoderma koningii* Oudem. at soil-protective treatment of slightly eroded agrochernozem was studied for the first time and it was shown that introduction of microscopic fungi *Trichoderma koningii* strain IB G-51 enhances humification of the floating mat and, to a lesser extent, of the straw, which is especially important under the no-till technology conditions. Combination of the floating mat with *T. koningii* in terms of the effect on the soil properties is comparable to manure.

The objectives of this paper included a comparison of the effect of fertilizers on the agrochemical properties, the enzymatic activity of the soil and the crops yield at no-till and traditional soil treatments, as well as the effect of microscopic fungus *Trichoderma koningii* on the decomposition of plant residues.

Techniques. A 3-year study was carried out in the southern forest-steppe zone of the Republic of Bashkortostan on a clay-illuvial, slightly eroded agrochernozem. Against the background of no-till and traditional soil treatment, a small-plot experiment was established, which included a single introduction of 10 kg of fertilizers according to the following variants: 1 (control, without fertiliz-

er); 2 — floating mat + $(NP)_{60}$; 3 — floating mat + *T. koningii* + $(NP)_{60}$; 4 — litter manure of cattle; 5 — straw + $(NP)_{60}$; 6 — straw + *T. koningii* + $(NP)_{60}$; 7 — zeolite (Tuzbek deposit); 8 — Na humate (brown coal powder, Bashinkom, Russia). The area of the plots was 4 m^2 (2×2 m), 3-fold replications. The floating mat was a plant mass (cattail, reed, sedge), extracted during the cleaning of a nearby pond. The floating mat was crushed together with the roots to 3-5 cm units and introduced into the soil in a wet state. Wheat straw was crushed to the same size. To accelerate the humification process, plant residues were treated with a suspension of microscopic fungus *T. koningii* strain IB G-51 grown in the Czapek medium (2% sucrose) for 14 days at 28 °C (the strain was previously isolated from the agrochernozem and is maintained in the collection of microorganisms (Ufa Institute of Biology, the Ufa Federal Research Center RAS).

In 2011, soft spring wheat (Omskaya 36 variety) was grown on experimental plots, in 2012 spring barley (Chelyabinsky 99 variety) was grown, in 2013 this was sugar beet (Masha hybrid, OOO KWS RUS; bred by KWS SAAT SE, Germany).

The moisture supply in 2011 and 2013 was close to the average perennial values, 2012 was extremely dry, the temperature for all three years corresponded to the average perennial values.

Soil samples were collected in the spring and autumn of each year from 0-10, 10-20 and 20-30 cm layers. Agrochemical studies were carried out using standard methods: humus content was determined according to Orlov and Grindel, alkaline hydrolyzable nitrogen content by Cornfield, mobile phosphorus and exchangeable potassium content by Chirikov [25], invertase activity by Shcherbakova with ending according to Samner, peroxidases and polyphenol oxidases content by Karyagina and Mikhailova, proteases and dehydrogenases content by Galstyan, cellulases content by Kong with ending according to Sumner, urease content by Shcherbakov [26].

The MS Excel software package was used for statistical processing of the obtained results. The tables show mean values (*M*) and their standard deviations (±SEM). The statistical significance of differences was evaluated using the smallest significant difference at 5% significance level (HCP_{05}). The effect of soil agrochemical indicators on its enzymatic activity was evaluated using correlation analysis (the *r* values at $p < 0.05$ are given).

Results. The thickness of the humus-accumulative horizon of the experimental plot soil was on average 29 cm less than that of the nearby deposit, which was the basis for considering that the agrochernozem is clay-illuvial slightly eroded. The introduction of manure and plant residues (Table 1) led to a change in the agrochemical properties of the soil. The content of humus in the arable horizon has increased over 3 years, and not only at soil overturning but also under no-till conditions. Compared to control, it increased by 3.5-5.6% at the no-till and by 1.8-4.1 at the traditional soil treatment. At the same time, multidirectional tendencies were observed in the humus content dynamics: a slight decrease in this indicator was observed for the 3rd year of using manure and straw, a gradual increase was observed when using floating mat. This was most noticeable in the upper layer (0-10 cm) under the no-till. Similar results for the 0-5 cm layer were shown when using plant residues [27]. In the same layer, the introduction of *T. koningii* suspension to the floating mat contributed to a significant increase in the humus content compared with not only the control but also with the variant without its introduction. This effect was less pronounced at the introduction of straw.

In general, after 3 years, the increase in the humus content in the 0-30 cm layer was significant in all variants with fertilizer (except for the

introduction of zeolite and sodium humate) regardless of the type of treatment, and the efficiency of the floating mat straw and manure was almost the same.

Along with the humus state, the content of nutrients has changed. Mobile phosphorus availability in the experiment soil was low. When introducing the manure and plant residues with the addition of mineral fertilizers against the background of no-till, the content of mobile phosphorus for 3 years increased to the average category, and in the case of traditional soil treatment, a significant increase was observed only on the variant with manure introduction.

1. Agrochemical properties of the soil in the 0-30 cm layer at different fertilizing depending on the treatment technology (*M*±SEM, Republic of Bashkortostan, 2011-2013)

Variant	Humus, %	R_{mobile}, Mg/100 g	$K_{exch.}$, mg/kg	$N_{alk.}$, mg/kg
No-till				
Control	7.53±0.03	4.7±0.1	95.7±0.3	197.4±1.2
Manure	7.95±0.03	6.8±0.3	127.6±5.0	213.2±2.5
Floating mat + *Trichoderma koningii* + $(NP)_{60}$	7.87±0.03	6.3±0.5	126.5±15.0	212.0±3.8
Floating mat + $(NP)_{60}$	7.77±0.05	5.6±0.3	109.1±4.6	212.6±3.1
Straw + *T. koningii* + $(NP)_{60}$	7.82±0.03	5.5±0.3	98.8±1.0	209.4±5.3
Straw + $(NP)_{60}$	7.79±0.03	5.3±0.2	97.8±2.1	200.1±5.8
Zeolite	7.59±0.02	4.8±4.6	103.5±0.9	197.2±3.8
Na humate	7.66±0.02	5.0±0.2	100.6±5.7	200.6±6.0
Tillage				
Control	7.73±0.01	4.5±0.1	89.1±5.6	204.2±2.4
Manure	8.04±0.02	6.8±0.4	129.0±2.5	213.1±3.7
Floating mat + *T. koningii* + $(NP)_{60}$	8.05±0.03	5.4±0.7	123.1±2.9	206.9±1.6
Floating mat + $(NP)_{60}$	7.90±0.02	4.8±0.3	105.5±8.1	203.9±3.6
Straw + *T. koningii* + $(NP)_{60}$	8.00±0.02	4.6±0.2	99.1±2.4	206.2±4.1
Straw + $(NP)_{60}$	7.87±0.02	4.4±0.2	92.9±4.2	205.6±2.8
Zeolite	7.78±0.01	4.6±0.3	89.3±0.4	207.3±2.4
Na humate	7.76±0.02	5.6±0.7	98.8±4.4	203.8±5.0
LSD_{05}	0.11	1.1	8.2	10.9

Unlike mobile phosphorus, the content of exchangeable potassium was initially increased (see Table 1). Regardless of the treatment type, its significant (LSD_{05}) increase to a high degree of availability occurred only when manure and floating mat were introduced with the addition of $(NP)_{60}$ and *T. koningii*. In the first case, the amount of exchangeable potassium gradually decreased during the experiment, and in the second case, it increased with the decomposition of the floating mat. The content of alkaline hydrolyzable nitrogen varied in a narrow range, and an increase in its amount by 7-8% compared with the control was observed only with the introduction manure and floating mat under no-till. This is in good agreement with shown in the paper losses of nitrogen compounds exposed to leaching under no-till [28].

2. Activity of soil enzymes in the 0-10 cm layer by years of study at different fertilizing and treatment technologies (*M*±SEM, Republic of Bashkortostan)

Variant	Treatment	2011	2012		2013	
		autumn	spring	autumn	spring	autumn
Peroxidase, mg benzoquinone/g of soil for 30 min at 30°C						
1	No-till	80.1±2.2	202.4±14.2	208.1±12.0	225.1±8.8	134.5±5.5
	Tillage	83.2±3.1	202.7±10.8	218.6±13.0	188.2±10.2	115.8±4.8
2	No-till	85.8±3.8	214.7±11.0	236.3±9.5	233.8±14.3	173.3±10.4
	Tillage	109.8±5.1	237.5±15.2	247.4±6.1	210.9±7.2	132.6±9.8
3	No-till	92.5±3.6	215.9±9.1	228.9±9.7	254.2±7.4	151.1±7.7
	Tillage	98.1±3.8	242.4±8.0	254.8±14.2	209.1±5.7	150.5±8.3
4	No-till	86.9±1.9	210.4±13.8	208.5±14.5	238.7±9.6	143.7±11.2
	Tillage	88.2±1.9	230.7±14.1	243.1±15.3	204.2±6.7	125.2±10.8
5	No-till	91.3±2.8	218.9±9.7	243.7±7.2	259.1±13.8	148.0±12.5
	Tillage	99.3±3.0	241.8±15.2	259.7±10.5	257.2±14.5	167.8±14.7
6	No-till	82.6±1.7	214.1±14.0	240.6±8.3	235.7±9.5	136.3±9.7
	Tillage	85.1±2.0	220.7±13.7	253.5±15.1	214.7±10.0	141.8±11.3
LSD_{05}		6.1	25.1	23.2	19.7	12.7
Polyphenol oxidase, mg benzoquinone/g of soil for 30 min at 30°C						
1	No-till	80.0±3.1	101.2±7.8	93.2±4.4	107.6±7.9	90.8±4.2
	Tillage	88.6±2.8	87.1±5.7	94.7±3.9	93.9±6.5	95.0±5.7

Continued Table 2

2	No-till	95.9±4.5	116.0±8.1	116.1±6.7	134.4±12.5	125.2±9.7
	Tillage	100.7±5.2	93.5±7.8	109.0±7.9	134.1±11.6	99.7±8.4
3	No-till	103.8±6.7	117.4±6.6	105.1±4.7	128.9±8.8	114.8±7.6
	Tillage	112.4±11.0	94.1±4.2	119.4±10.6	104.4±6.2	117.9±9.2
4	No-till	92.5±9.8	106.5±8.8	104.2±9.9	114.8±10.6	109.9±8.6
	Tillage	91.0±9.8	89.8±10.3	112.7±12.1	95.3±9.7	98.0±6.7
5	No-till	91.3±7.4	107.5±7.6	104.4±10.1	109.9±12.4	100.2±3.8
	Tillage	115.4±12.2	89.1±9.9	105.5±5.7	144.1±15.3	123.9±5.7
6	No-till	82.6±5.6	102.4±5.9	96.5±8.2	110.2±8.9	89.8±10.4
	Tillage	108.3±7.6	86.7±6.2	97.7±8.7	131.9±13.4	101.4±8.8
LSD_{05}		7.3	6.8	6.6	9.7	8.1
		Invertase, mg glucose/g of soil for 24 h				
1	No-till	4.8±0.3	19.6±0.9	14.5±1.3	16.0±1.4	2.6±0.7
	Tillage	5.4±0.3	22.8±1.2	25.5±2.0	23.8±2.5	7.2±0.9
2	No-till	6.9±0.5	22.5±1.2	19.8±2.3	16.7±1.9	4.5±0.4
	Tillage	5.2±0.4	26.9±1.4	29.0±2.5	23.1±2.2	8.3±0.5
3	No-till	6.9±0.7	22.9±1.1	22.7±2.9	18.7±1.1	6.4±0.7
	Tillage	5.0±0.6	24.7±1.6	24.8±1.8	20.2±1.2	6.2±0.7
4	No-till	5.8±0.4	21.2±1.0	20.4±1.5	18.3±2.0	2.6±0.3
	Tillage	5.1±0.7	24.9±0.8	29.4±2.9	22.6±2.5	8.1±0.8
5	No-till	6.7±1.0	22.3±1.2	18.6±2.7	21.9±2.8	5.2±0.7
	Tillage	4.8±0.3	22.3±0.9	26.6±3.3	16.6±2.9	6.1±0.5
6	No-till	6.5±0.7	17.9±1.3	18.2±1.5	19.1±1.7	2.5±0.3
	Tillage	4.8±0.3	19.6±0.9	14.5±1.3	16.0±1.4	2.6±0.7
LSD_{05}		0.7	2.1	3.3	2.8	0.5
		Protease, mg histidine/g of soil for 24 h				
1	No-till	10.0±1.3	6.3±0.3	7.9±0.8	8.0±0.9	6.5±0.2
	Tillage	7.6±0.9	4.7±0.8	5.9±0.7	8.5±1.1	4.6±0.3
2	No-till	15.8±1.2	8.3±0.4	9.4±0.7	11.5±1.0	10.4±0.8
	Tillage	15.7±1.2	7.0±0.4	7.7±0.4	11.5±1.0	5.9±0.5
3	No-till	15.2±0.8	9.2±1.0	10.9±0.9	11.4±1.0	8.8±0.7
	Tillage	13.3±0.7	6.2±0.7	8.4±0.8	14.0±1.2	7.1±0.3
4	No-till	13.5±0.7	7.3±0.9	8.7±0.8	8.1±0.6	7.4±0.6
	Tillage	11.3±0.6	5.9±0.5	4.2±0.6	11.7±0.7	5.4±0.2
5	No-till	15.4±0.8	10.1±1.1	7.2±0.4	9.2±0.4	6.9±0.2
	Tillage	13.9±0.7	5.7±0.2	7.5±0.4	10.4±0.6	5.3±0.4
6	No-till	11.3±0.5	9.7±0.9	6.3±0.5	9.8±0.3	6.4±0.5
	Tillage	11.3±0.6	5.8±0.4	5.6±0.3	13.9±0.8	5.1±0.5
LSD_{05}		1.1	0.6	0.7	1.0	0.7

Note. 1 — control, 2 — manure, 3 — floating mat + *Trichoderma koningii* + $(NP)_{60}$, 4 — floating mat + $(NP)_{60}$, 5 — straw + *T. koningii* + $(NP)_{60}$, 6 — straw + $(NP)_{60}$.

On year 3 of the experiment, plant residues morphologically became indistinguishable due to their transformation. It is known that soil enzymes [29] and components of plant litter [30] play an important role in the humification of plant residues. When introducing manure and plant residues, in contrast to the variants with the use of sodium humate and zeolite, the enzymatic activity of the soil was higher than in the control (Table 2). The dynamics of the activity of the studied enzymes was multidirectional, which, on the one hand, may be due to the transformation of organic matter, and on the other, due to the change in the agrophysical properties of the soil. Thus, the maximum activity of peroxidase, cellulase and invertase was recorded in the 2nd year of the study, the activity of dehydrogenase consistently increased, and the activity of protease decreased. Dynamics of the polyphenol oxidase and urease activity was weakly expressed (data not shown). The closest correlation relationship ($p < 0.05$) was found for the protease with the content of mobile phosphorus ($r = 0.75 \pm 0.12$), potassium ($r = 0.69 \pm 0.12$) and nitrogen ($r = 0.53 \pm 0.14$), for polyphenol oxidase with the content of humus ($r = 0.62 \pm 0.13$), potassium ($r = 0.56 \pm 0.14$) and nitrogen ($r = 0.62 \pm 0.13$). For other enzymes, the correlation coefficients, as a rule, did not exceed 0.4. Addition of the microscopic fungus *T. koningii* suspension to the floating mat and straw promoted the growth of the soil enzymatic activity regardless of the treatment technology.

The yield of agricultural crops grown in the crop rotation system of the experimental farm depended not only on the use of fertilizers but also on the

methods of tillage, which largely determine the moisture content. In contrast to manure and plant residues, zeolite and sodium humate were almost ineffective under the experimental conditions (Table 3). Probably, this was due to the lack of irrigation [31] and organic fertilizers, which increase the effect of zeolite [32], as well as due to the form of the introduced sodium humate (powder) [33]. In the 1st (wet) year, against the background of tillage, the yield of wheat was higher than in similar no-till variants. Obviously, this is due to the increased availability of nutrients with the addition of $(NP)_{60}$ and faster mineralization of the manure organic matter. The highest yield was ensured by use of manure and straw when tilling, and at no-till by the introduction of floating mats with the addition of microscopic fungi suspension. During the vegetative period of the extremely dry 2012, the moisture content at no-till was higher than during tillage [30], which predetermined a higher yield of barley. In 2013, the yield of sugar beet at moldboard tillage was higher than at no-till. This time, the limiting factor was the increase in soil density [34], to which sugar beet is very sensitive.

3. Yields of agricultural crops at different fertilizing and treatment technologies (*M*±SEM, Republic of Bashkortostan)

Variant	Wheat, g/m^2 (2011)		Barley, g/m^2 (2012)		Sugar beet, kg/m^2 (2013)	
	tillage	no-tillage	tillage	no-tillage	tillage	no-tillage
1	406.8±33.8 2000.0±51.7	283.5±30.5 1950.0±70.6	95.1±6.0 385.3±12.8	146,8±10,7 393,3±13,7	2,9±0,3	2,3±0,2
2	723±42.1 3500.0±52.8	582.8±49.4 2316.7±120.3	231.3±10.4 510.0±13.5	254,1±12,3 783,3±15,1	3,6±0,3	2,6±0,3
3	651±43.2 3066.7±93.7	621.4±39.0 2433.3±115.7	165.3±15.4 471.7±19.3	192,6±13,6 570,0±22,3	3,9±0,5	2,7±0,2
4	425.0±28.6 2900.0±88.3	546±35.7 2733.3±87.4	120.9±9.4 463.3±12.9	150,3±12,4 466,7±17,5	3,7±0,3	3,0±0,3
5	705.0±48.3 3666.7±114.7	566.4±60.3 2000.0±103.0	115.8±11.6 483.3±16.4	166,7±13,4 568,3±23,5	3,6±0,3	2,8±0,3
6	686.6±65.0 3566.7±106.8	432±53.6 2483.3±98.3	128.6±11.0 413.3±14.5	167,2±12,8 470,0±16,7	3,5±0,2	2,7±0,1
7	368±38.9 2466.7±87.5	330±37.1 2250.0±90.3	122.6±16.7 343.3±20.4	104,9±11,4 380,2±23,1	2,8±0,4	2,0±0,3
8	424.1±39.7 3300.0±106.5	369.8±36.8 2283.3±123.5	125.4±14.6 383.3±22.7	129,6±14,7 386,7±19,7	3,0±0,3	2,3±0,2
LSD_{05}	43.1 450.8		9.2 57.8		0.2	

N o t e. 1 — control, 2 — manure, 3 — floating mat + *Trichoderma koningii* + $(NP)_{60}$, 4 — floating mat + $(NP)_{60}$, 5 — straw + *T. koningii* + $(NP)_{60}$, 6 — straw + $(NP)_{60}$, 7 — zeolite, 8 — Na humate. The grain weight is above the line, g/m^2, the mass of the sheaf is below the line.

Thus, on average over 3 years of the study, the yield of crops under the traditional soil treatment was higher, but in the dry 2012, it was greater under no-till, when profitability was 257% versus 116% under tillage. The yield increase was on average 40-72% (for manure 67%, for floating mat + *T. koningii* 47%, for floating mat 40%, for straw + *T. koningii* 72%, for straw 66%) under tillage and 21-38% (for manure 32%, for floating mat + *T. koningii* 28%, for floating mat 36%, for straw + *T. koningii* 10%, for straw − 26%) under no-till.

So, in the conditions of the Southern Pre-Ural region on the clay-illuvial, slightly eroded agrochernozem, introduction of manure and plant residues under tillage and no-till promotes the increase in the humus content, improvement in the supply of nutrients, increase in the enzymatic activity of the soil and crop yields. Under no-till, the changes in agrochemical indicators are more pronounced in the 0-10 cm layer, under the traditional treatment − in the 0-30 cm layer. The profitability of fertilizers with no-till technology is higher than with traditional tillage only in dry conditions. The use of microscopic fungi *Trichoderma koningii* in biologized technologies leads to an increase in the processes of plant residues humification, especially floating mats. In terms of affecting the soil properties, this fertilizer is close to the variant with the introduction of manure.

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APPLE TREE (*Malus domestica* Borkh) NITROGEN SUPPLY OPTIMIZATION BY FERTIGATION AND BACTERIAL FERTILIZERS

A.I. KUZIN[1, 2], Yu.V. TRUNOV[1], A.V. SOLOVYEV[1]

[1]*Michurinsk State Agrarian University,* 101, ul. Internatsionalnaya, Michurinsk, Tambov Region, 393760 Russia, e-mail info@mgau.ru, kuzin@mgau.ru (✉ corresponding author), trunov.yu58@mail.ru, vladsoloviev1611@yandex.ru;
[2]*Michurin Federal Scientific Centre,* Federal Agency for Scientific Organizations, 30, ul. Michurina, Michurinsk, Tambov Region, 393774 Russia, e-mail info@fnc-mich.ru
ORCID:
Kuzin A.I. orcid.org/0000-0002-0446-0085
Trunov Yu.V. orcid.org/0000-0001-9406-3501
Solovyev A.V. orcid.org/0000-0002-3186-9767
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Abstract

Nitrogen is a special macronutrient as it comes into soil only with rainfall, remains of living organisms and fertilizers. Changes of apple (*Malus domestica* Borkh) nitrogen supply faster affect the fruit yield and quality as compared to other nutrients. Our paper it the first multivariate study of soil, leaf and fruit mineral status of apple trees on leached chernozem at different ways and sources of nitrogen supply which shows high environmental safety and efficiency of fertigation. The aim of the research was development of a model for optimal nitrogen provision to improve apple tree yielding and fruit quality. Tests with cv. Zhigulevskoe grafted on rootstock 62-396 were carried out in 2014-2016 in the experimental apple orchard planted in 2007 (Michurin Federal Scientific Centre, Tambov Region) at 4.5×1.0 m planting spacing. Each plot comprised 5 trees; all tests were arranged in triplicate. Obtained data were statistically processed by dispersion, correlation and regression analysis. Humus content and soil acidity, abundant of rhizosphere microorganisms, apple tree productivity, and the levels of essential elements in leaves and soil were determined, as well as vitamin C, sugars and organic acids content in fruits. Experimental variants were control 1 (no fertilizers and no irrigation), control 2 (no fertilizers, drip irrigation); subsoil placing of N_{60}, N_{90}, N_{120}, $N_{60}P_{20}K_{60}$, $N_{90}P_{30}K_{90}$, $N_{120}P_{60}K_{120}$; fertigation of N_{15}, N_{25}, N_{35}, $N_{15}P_{12}K_{15}$, $N_{25}P_{20}K_{25}$, $N_{35}P_{25}K_{35}$; Azovit, 4 l/h (cells and spores of *Azotobakter chroococcum* B-9029, 5×10^9 CFU/g), Azovit, 4 l/h + Phosphatovit, 4 l/h (cells and spores of *Bacillus mucilaginosus* B-8966, 0,129 CFU/g). In deep fertilizer placement, the complex NPK but not N increases the apple tree yielding. Fertigation with $N_{35}P_{25}K_{35}$ provides optimal level of all major nutrients in leaves and maximum fruit yield averaged 396.3 c/ha for three years. Single application of N fertilizers unbalances soil nutrient composition by increasing the content of easily hydrolyzed nitrogen, which, in turn, reduces the increase in yield. Complex fertilizing by fertigation and using bacterial preparations ensures the optimum N/P ratio in fruits (6.8), as well as K/N ratio (1.8-1.9). Fertilization by fertigation and in the tree trunk strips reduces the concentration of ascorbic acid in fruits at harvest maturity. N variants, despite the mode of fertilizer application, have the worst impact on vitamin C concentration which was 15-20 % lower compared to the complex fertilization, and the sugar-acid ratios also decreased to 10.7-12.8. Fertigation and use of bacterial fertilizers increase microbiological activity of the soil. As a result, we suggest the model for apple tree yield optimization based on proper use of nitrogen fertilizers. This study shows that the use of bacterial cultures as a temporary alternative to chemical fertilizer improves productivity in intensive apple orchards (up to 327.5 c/ha on average over 3 year experiment).

Keywords: *Malus domestica* Borkh, apple tree, nitrogen nutrition, fertigation, deep fertilizer placement, drip irrigation, yield, fruit quality

Nitrogen deficiency inhibits the growth of plant roots and aerial parts, which reduces their photosynthetic capacity and productivity [1] while optimal nitrogen intake activates enzyme proteins. It is therefore important to note that the nitrogen status regulates the ability to generate heat-shock proteins, which improves the heat resistance of plants [2]. Nitrogen intake activates photosynthesis and improves productivity [3]; it affects the growth of trees, their bodies, shoots, and leaves via protein synthesis [4, 5]. Optimal nitrogen intake has positive

effects on roots, growth, root morphology and branching [6, 7]. According to Trunov [6], increased nitrogen dosage improves the intake of nitrogen, phosphorus, and potassium via roots. Nitrogen increases the number and longevity of fruit-bearing formations, causes more abundant flowering and blossoming of fruits, decreases the ovary reduction, and contributes to fruit growth, increase in size and yields [8-10]. Nitrogen surplus induces excessive shooting at the expense of productivity, causing larger but looser leaves to grow while also entailing protracted growth, slower ripening, and inhibited resistance to cold [11, 12]. Zn, Cu, and Fe deficit ensues, which results in physiological diseases [13]. Fruit ripening is delayed, which worsens their taste, shelf life, and appearance while making fruits more susceptible to physiological and infectious diseases during storage [14-16].

Apples contain sugars, necessary acids, and nearly all the vitamins and micronutrients the human needs [17]; however, excess nitrogen intake reduces the consumer quality of fruits. Meanwhile, appropriate dosing and application of nitrogen fertilizers help control the productivity of plantations and the quality of yield while also reducing costs and negative environmental impact [10, 18].

Vitamin C content is what largely determines the nutritional value of apple fruits, especially in winter. Besides, ascorbic acid is important for long-term storage. Being an antioxidant, it is involved in oxidation and reduction processes, in the synthesis of hormones and sundry essential compounds [19]. Ascorbic acid is also needed to overcome and reduce the effects of oxidative stress [20, 21]. For example, sufficient amounts of ascorbic acid inhibit pulp browning to a great extent [21]. The mechanisms behind these processes are yet to be explained; however, it has been shown that the acid inhibits negative oxidation processes in membranes and prevents peroxides from destroying fats [22]. Ascorbic acid is crucial for curbing salinity stress [23]. For the best fruit taste, the sugar-acid ratio (SAR) must be within 15 to 25. At SAR > 25, fruits become blank-tasted and useless for further processing [24].

Shelf life and preservation of fruits are two important indicators that depend not only on the storage conditions but also on the content and ratio of minerals in fruits and leaves [25], i.e. they are also related to nutrient intake.

The authors hereof are the first to have evaluated how altering mineral and water intake by fertigation and drip irrigation affects the mineral status and productivity improvements observed in large-scale leached-chernozem apple plantations. The research team was able to describe particularities of using bacterial fertilizers (Azovit and Phosphatovit) and to establish approximate recommended region-specific fertigation rates.

The purpose of this research was to model apple yield and to optimize nitrogen intake for better productivity and fruit quality while using different fertilizers.

Techniques. Experiments were carried out in a test orchard of Michurin Federal Research Center, Michurinsk, Tambov Region, in 2014-2016 on *Malus domestica* Borkh trees, cv. Zhigulevskoye grafted on rootstock 62-396. The orchard was planted in 2007 with a 4.5 m×1 m planting layout and equipped with a fertigation and drip irrigation system; each plot comprised 5 trees in triplicates. The test-plot soil was leached, low-humus, medium-loam meadow chernozem on sands with pseudofibers. The humus content was 2.6 to 3.2 percent, the alkaline saturation was 70 to 90 percent, and the humus horizon depth was 40 to 50 cm on average. Upper-layer reaction was low-acidic at pH 5.7 to 5.9. It was a dusty, crumby, and grainy soil. Upper-horizon porosity reached 65%. Field moisture capacity of the arable layer was about 30%, the easily hydrolyzed nitrogen content was 152.8 mg/kg as found by I.V. Tyurin and M.M. Kononova's method; mobile phosphorus content was 146.0 mg/kg, while exchangeable potassium content was

167.6 mg/kg as found by the Chirikov method.

Fertilizers were incorporated annually in early spring at 10 to 15 cm below the surface; fertigation was performed throughout the vegetation period to meet the nutrient-specific needs of plants. Annual fertigation rates were divided into 10 irrigations. Sampling was as follows: Control 1 (no fertilizers, no irrigation); Control 2 (no fertilizers, drip irrigation); application of N_{60}, N_{90}, N_{120}, $N_{60}P_{20}K_{60}$, $N_{90}P_{30}K_{90}$; application of bacterial fertilizers: Azovit (4 l/ha, living cells and spores of *Azotobacter chroococcum* B-9029, 5×10^9 CFU/g), Azovit (4 l/ha) plus Phosphatovit (4 l/ha, living cells and spores of *Bacillus mucilaginosus* B-8966, 0.129 CFU/g). The bacterial fertilizers were produced by Industrial Innovations Limited, Russia. In case of drip irrigation, bacterial fertilizers were applied by irrigating the roots in spring.

The following preparations were incorporated in the soil: ammonium nitrate containing at least 34.4% of ammonium- and nitrate-nitrogen; huminified superphosphate, a $Ca(H_2PO_4)_2 \cdot H_2O$ water-soluble phosphorus-based fertilizer with 26% P_2O_5, 5% N; and potassium sulfate, a concentrated chlorine-free fertilizer (46-50% K_2O). All the preparations were produced by OAO Buy Chemicals Factory, BHZ, Russia. For fertigation, the researchers used Amofoska, a complex NPK fertilizer (12% N, 15% P_2O_5, 15% K_2O, 14% S, 0.5% MgO) produced by OOO Mettorg, Russia; potassium monophosphate, a phosphorus and potassium compound fertilizer (52% P_2O_5, 34% K_2O) produced by OAO BHZ; and Master, a complex water-soluble fertilizer with chelated micronutrients ($N_{13}P_{40}K_{13}$, 0.070% Fe, 0.030% Mn, 0.010% Zn, 0.005% Cu, 0.020% B, 0.001% Mo) produced by Valagro SpA, Italy. The fertilizer composition for making a fertigation solution was adjusted to vegetation phase-specific needs.

The soil was sampled in late August (in June and August to measure microbial population) at a depth of up to 40 cm. Data included humus quantification, easily hydrolyzed nitrogen quantification by the Kjeldahl method [26]; mobile phosphorus quantification by a KFK-3-01 photometer (Zagorsk Optical and Mechanical Plant, Russia) and exchangeable potassium quantification by a Jenway PFP 7 flame photometer (Bibby Scientific, UK) by the Chirikov method [27]; soil pH in KCl extraction; total exchangeable bases by the Kappen-Hilkovic method; and the microbial population of the rhizosphere by the Krasilnikov method, using beef extract agar (BEA) [28].

Leaves were sampled in mid-August. Total potassium, phosphorus, and nitrogen (by the Kjeldahl method) content were evaluated in a single batch [27].

Monosaccharide and disaccharide content was determined by the Bertrand method; ascorbic acid content was measured by iodometry; total acidity was measured by titrometry [29]; dry content was measured after drying to constant weight [27]. Only picking-maturity fruits were used for these measurements. The yield was accounted when weighing fruits from accountable trees [30].

Data were processed statistically by analysis of variance, correlation and regression analysis [31, 32] using Microsoft Excel 2007 with the AgCStat add-in. Below are the mean values (M) with standard errors of means (±SEM), pairwise correlation coefficients (r), and least significant difference values at CI 95% ($t_{0.05}$).

Results. Nitrogen is extremely mobile in plants and soils; nitrate-nitrogen is easily leached into groundwater. Active drip irrigation and excessive application of nitrogen fertilizers may have negative effects, which is why clarifying the particularities of nitrogen intake is important for reducing environmental pollution [33].

In this experiment, nitrogen fertilizers applied without irrigation had no significant effect on the mean triennial yield (LSD_{05} 36.4 centers/ha), except the sample that had the maximum rate of N_{120} (see Fig. 1). Application of nitrogen fertilizers at 120 kg/ha, as well as incorporation of complex fertilizers in the near-

trunk areas resulted in a significant increase in yield against the controls: the mean triennial yield rose from 260.2 to 317.0 (N_{120}), 321.2 ($N_{90}P_{30}K_{90}$) and 321 centners/ha ($N_{120}P_{60}K_{120}$), i.e. by 56.8 to 60.0 centners/ha (or by 21.8 to 23.1%) due to raising the soil nitrogen, phosphorus, and potassium content to the optimal levels. Fertigation enabled lowering the application rates; however, a significant increase in productivity was only noted in the case of complex fertilizers, as yield peaked when using the maximum dosage. Over 2014-2016, drip irrigation increased this indicator from 260.2 (Control 1) to 295.4 (Control 2) centners/ha on average, i.e. by 35.2 centners/ha or by 13.5%.

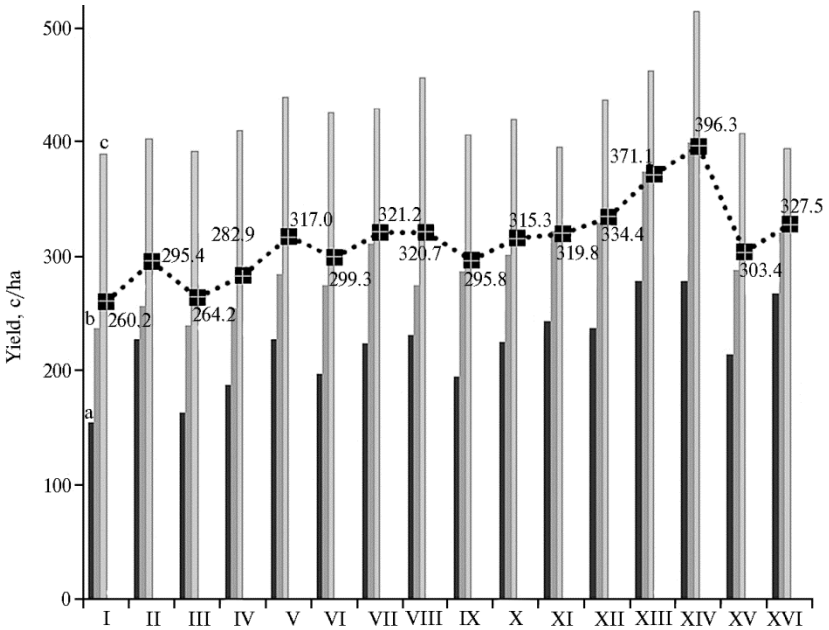

Fig. 1. ***Malus domestica*** **Borkh, cv. Zhigulevskoye yield on rootstock 62-396: results obtained by using different nitrogen fertilizing methods in 2014** (a)**, 2015** (b)**, and 2016** (c)**:** I for Control 1 (no fertilizers, no irrigation); II for Control 2 (drip irrigation); III for N60, IV for N_{90}, V for N_{120}, VI for $N_{60}P_{20}K_{60}$, VII for $N_{90}P_{30}K_{90}$, VIII $N_{120}P_{60}K_{120}$ (fertilizers); IX for N_{15}, X for N_{25}, XI for N_{35}, XII for $N_{15}P_{12}K_{15}$; XIII for $N_{25}P_{20}K_{25}$; XIV for $N_{35}P_{25}K_{35}$ (fertigation); XV for Azovit, XVI for Azovit + Phosphatovit. The figure shows the mean yield. LSD_{05}: 25.8 in 2014; 34.0 in 2015; 49.3 in 2016; the triennial mean is 36.4 centners/ha (Michurinsk, Tambov Province).

Azovit and Phosphatovit are preparations intended to replace or limit the use of mineral fertilizers while optimizing the assimilation of the essential nutrients, which is expected to produce eco-friendly products of better quality and yield. Yield increase in bacterial-fertilized samples was evaluated against Control 2. Azovit placement in the soil did not have a significant effect on this indicator. A combination of bacterial fertilizers did increase the yield, but the increase was commensurate with that resultant from a minimum concentration of a complex mineral fertilizer. Simultaneous application of Azovit and Phosphatovit increased the yield from 295.4 to 337.5 centners/ha, i.e. by 42.1 centners/ha or by 14.3%; this was due to increasing the bacterial colonization of the apple tree's rhizosphere and improving the availability of minerals. Fertigation with a mineral complex increased the mean triennial yield in 2014-2016 from 295.4 to 334.4 ($N_{15}P_{12}K_{15}$), 371.1 ($N_{25}P_{20}K_{25}$), and 396.3 centners/ha ($N_{35}P_{25}K_{35}$), i.e. by 39.0 to 100.9 centners/ha or by 13.2 to 34.2 percent, which is statistically significant (LSD_{05} = 36.4 centners/ha).

Fertilizing positively affected the soil nutrient content, see Table 1. Nitrogen and phosphorus fertilizing was of utmost significance in this experiment,

as the content of these elements was suboptimal in the controls. Exchangeable-potassium content in the soil was higher, although, in some years, plants were deficient in potassium as well, perhaps due to precipitation-induced leaching and redistribution of root-absorbed potassium into the ripening fruits [34]. Such response greatly depends on the experimental conditions, primarily on the soil type. In a study by D. Malaguti et al. [35], potassium content was increased by fertigation, while nitrogen content was increased by incorporation in alluvial soils, Italy. However, high-dose fertigation may increase the heterogeneity of soils in an orchard [36]. In this experiment, unifactorial application of nitrogen increased its content to optimal levels (177.9 to 201.6 mg/kg of soil); however, phosphorus and potassium content remained unchanged (121.7 to 137.3 P_2O_5 and 128.9 to 150.7 K_2O, values in mg/kg of soil). The multifactorial application only increased yield when the nitrogen application rates were at max. Complex fertilizing optimized the soil nutrient content in nearly all incorporated-fertilizer samples. Comparing fertigation at down to 40 cm below surface against incorporation at 10 to 15 cm below surface, easily hydrolyzed nitrogen content was only 3 to 5 percent lower; meanwhile, such fertigation used 70 to 75 percent less nitrogen fertilizer(s).

1. Soil and leaf nutrient content in apple trees *Malus domestica* Borkh (cv. Zhigulevskoye, rootstock 62-396) for different nitrogen fertilizing methods (*M*±SEM, Michurinsk, Tambov Region, 2014-2016)

Variant	Content					
	in the soil, mg/kg			in leaves, % wet		
	easily hydrolyzed N	P_2O_5	K_2O	N	P	K
No fertilizers						
Control 1 (no irrigation)	107,2	131,4	138,8	1,17	0,32	0,76
Control 2 (irrigated)	87,4	114,5	114,6	1,37	0,21	0,98
Mean	97,3±4,3	123,0±4,2	126,7±4,7	1,27±0,067	0,27±0,008	0,87±0,041
Incorporation of fertilizers						
N_{60}	127,4	121,7	150,7	1,48	0,24	1,21
N_{90}	177,9	137,3	128,9	1,55	0,22	1,09
N_{120}	201,6	123,2	135,6	1,77	0,26	1,25
$N_{60}P_{20}K_{60}$	146,5	176,4	173,9	1,87	0,33	1,34
$N_{90}P_{30}K_{90}$	184,6	185,5	206,1	1,95	0,41	1,26
$N_{120}P_{60}K_{120}$	196,8	193,1	219,7	2,14	0,39	1,48
Mean	172,5±8,1	156,2±9,4	169,2±8,8	1,79±0,094	0,31±0,011	1,27±0,57
Fertigation						
N_{15}	156,3±7,8	102,9	118,4	1,60	0,28	1,09
N_{25}	173,6	107,7	128,9	1,69	0,18	1,19
N_{35}	177,4	115,7	107,7	1,96	0,32	0,95
$N_{15}P_{12}K_{15}$	156,3	142,5	168,9	1,88	0,44	1,48
$N_{25}P_{20}K_{25}$	166,4	154,9	175,8	2,07	0,49	1,51
$N_{35}P_{25}K_{35}$	182,7	169,7	171,2	2,41	0,55	1,30
Mean	168,8±7,7	132,2±7,3	145,2±8,1	1,94±0,114	0,38±0,012	1,25±0,055
Bacterial fertilizers						
Azovit, 4 l/ha	162,4	103,9	141,3	1,70	0,31	1,20
Azovit, 4 l/ha + Phosphatovit, 4 l/ha	175,3	172,7	182,2	2,27	0,43	1,33
Mean	168,9±8,2	172,7±8,9	182,2±9,2	2,0±0,115	0,37±0,09	1,3±0,057
LSD_{05}	22,2	18,3	15,1	0,18	0,07	0,12
Optimal content (according to literature))						
	151-200 [37]	151-200 [38]	121-180 [38]	1,8-2,5 [39]	0,3-0,5 [39]	1,2-1,8 [39]

The need for nitrogen and its availability depend on the water intake as well as on the presence and content of other nutrients in the soil. Leaves indicate the availability of nutrients to plants. However, nutrient content in leaves does not correlate directly with that in the soil; it is only nitrogen that displays explicit correlations [40]. Despite more easily hydrolyzed nitrogen being present in the soil in case of unifactorial nitrogen fertilizing, leaf nitrogen content was suboptimal. This was typical for incorporation and for fertigation alike. Only complex fertilizing

2. Chemical composition and some quality indicators of apple fruits (*Malus domestica* Borkh, cv. Zhigulevskoye, rootstock 62-396) depending on fertilizing methods (*M*±SEM, Michurinsk, Tambov Province, 2014-2016)

Variant	Basic nutrients, % wet			Ratio in fruits		Content			SAR
	N	P	K	N/P	N/K	ascorbic acid, mg%	total sugars, %	organic acids, %	
No fertilizers									
Control 1 (no irrigation)	0.19	0.046	0.69	4.1	3.6	15.70	10.5	0.66	15.9
Control 2 (irrigated)	0.23	0.034	0.79	6.8	3.4	14.36	10.1	0.76	13.3
Mean	0.21±0.01	0.040±0.02	0.74±0.04	5.5±0.3	3.5±0.2	15.03±0.75	10.3±0.6	0.71±0.04	14.6±0.8
Incorporation of fertilizers									
N_{60}	0.19	0.033	0.85	5.8	4.5	13.48	9.2	0.83	11.1
N_{90}	0.42	0.059	0.67	7.1	1.6	12.78	8.6	0.73	11.8
N_{120}	0.55	0.053	1.04	10.4	1.9	11.85	8.1	0.76	10.7
$N_{60}P_{20}K_{60}$	0.28	0.073	1.06	3.8	3.8	13.20	10.0	0.68	14.7
$N_{90}P_{30}K_{90}$	0.39	0.070	1.24	5.6	3.2	14.83	11.6	0.77	15.1
$N_{120}P_{60}K_{120}$	0.48	0.078	1.14	6.2	2.3	14.07	11.7	0.82	14.3
Mean	0.39±0.02	0.061±0.003	1.00±0.05	6.5±0.4	2.9±0.1	13.37±0.71	9.6±0.6	0.77±0.04	13.0±0.8
Fertigation									
N_{15}	0.33	0.062	0.69	5.3	2.1	13.64	8.7	0.68	12.8
N_{25}	0.40	0.033	0.76	12.1	1.9	12.83	8.7	0.82	10.6
N_{35}	0.57	0.044	0.73	13.0	1.3	11.94	8.6	0.77	11.2
$N_{15}P_{12}K_{15}$	0.40	0.069	1.07	5.8	2.6	15.41	12.4	0.74	16.8
$N_{25}P_{20}K_{25}$	0.41	0.055	1.13	7.5	2.8	14.75	12.5	0.93	13.4
$N_{35}P_{25}K_{35}$	0.48	0.071	1.15	6.8	1.9	14.87	12.9	0.76	17.0
Mean	0.43±0.02	0.056±0.03	0.92±0.005	8.4±0.05	2.1±0.1	13.91±0.68	9.6±.05	0.74±0.05	13.6±0.7
Bacterial fertilizers									
Azovit, 4 l/ha	0.34	0.052	0.69	6.5	2.3	14.09	9.6	0.84	11.4
Azovit, 4 l/ha + Phosphatovit, 4 l/ha	0.48	0.071	0.86	6.8	1.8	15.72	11.8	0.74	15.9
Mean	0.41±0.02	0.062±0.003	0.78±0.04	6.7±0.4	2.1±0.1	14.91±0.91	10.7±0.7	0.79±0.05	13.7±0.8
LSD_{05}	0.05	0.007	0.12	0.9	0.3	1.92	1.3	0.08	1.76
Comparative figures (according to literature)									
	0.3-0.5 [37]	0.07-0.10 [37]	0.8-1.2 [37]	6.5-7.0 [unp]	1.8-2.2 [но]	10.1-15.0 [41]	9.2-12.2 [41]	0.51-0.80 [41]	13.0-20.0 [41]

Note. SAR stands for the sugar-acid ratio. The Table presents optimal N, P, and K content [37]; fruit means of total sugars, organic acids, and SAR [41]; unp is the results of the authors' unpublished studies.

aimed to optimize the content of all the nutrients under analysis was able to raise leaf nitrogen content to optimal levels.

Compared to leaves, the concentration of elements in fruits has a weaker correlation with that in the soil. However, fruit chemistry data enable quality analysis and shelf-life forecasts. Too high fruit nitrogen content coupled with relatively low calcium content may result in numerous physiological diseases during storage [21].

Increasing the nitrogen fertilizer application rates resulted in accumulating excessive amounts of nutrients, primarily nitrogen itself, see Table 2. Unifactorial nitrogen fertigation raised its quantity above optimum. Meanwhile, such high nitrogen content was coupled with suboptimal concentrations of other elements, especially phosphorus. Exchangeable-potassium availability in the soil was sufficiently high; however, its content in fruits largely depended on the fruit load.

Complex fertilizing can optimize the availability of elements, the ratios whereof are crucial for quality. Such ratios largely depend on the variety and on the region of cultivation; unpublished data suggest that N/P = 6.5 to 7.0 and N/K = 1.2 to 2.2 is optimal for Central Chernozemye. Optimal N/P ratios were observed at peak fertilizing rates in both incorporation and fertigation, in Control 2 and in both bacterial-fertilized samples. When applying the maximum amount of complex mineral fertilizers by incorporation, when applying nitrogen fertilizers and maximum complex by fertigation, as well as when applying bacterial fertilizer, N/K was optimal or close to optimal. Both N/K and N/P were only optimized by maximum-rate incorporation ($N_{120}P_{60}K_{120}$) and fertigation ($N_{35}P_{25}K_{35}$). Apparently, this was resultant from the improved microbial activity of the soil, which enhanced the absorption of nitrogen and sundry elements alike.

3. Test-site soil characterization (layer 0 to 40 cm) **in experiments with *Malus domestica* Borkh (cv. Zhigulevskoye, rootstock 62-396) under various fertilizing methods** (*M*±SEM, Michurinsk, Tambov Province, 2014-2016)

Variant	pH	Humus content, %	Total exchangeable bases, mmol/100 g of soil
No fertilizers			
Control 1 (no irrigation)	5.5	3.0	26.2
Control 2 (irrigated)	5.5	2.9	26.0
Mean	5.50±0.14	2.95±0.11	26.10±1.14
Incorporation of fertilizers			
N_{60}	5.6	2.9	26.1
N_{90}	5.5	3.0	24.4
N_{120}	5.6	2.8	23.8
$N_{60}P_{20}K_{75}$	5.5	2.9	25.9
$N_{90}P_{30}K_{90}$	5.4	3.1	26.8
$N_{120}P_{60}K_{120}$	5.4	3.0	26.8
Mean	5.50±0.23	2.95±0.14	25.63±1.46
Fertigation			
N_{15}	5.5	3.0	24.2
N_{25}	5.4	3.1	27.0
N_{35}	5.4	2.9	25.1
$N_{15}P_{12}K_{15}$	5.4	3.0	27.3
$N_{25}P_{20}K_{25}$	5.4	2.8	26.9
$N_{35}P_{25}K_{35}$	5.4	2.9	23.4
Mean	5.42±0.18	2.95±0.07	25.65±1.62
Bacterial fertilizers			
Azovit, 4 l/ha	5.5	2.8	26.1
Azovit, 4 l/ha + Phosphatovit, 4 l/ha	5.6	2.9	25.8
Mean	5.55±0.17	2.95±0.11	25.95±1.38
LSD_{05}	0.14	0.19	1.19

Fertilization had negative effects on the ascorbic acid content in the picking-maturity fruits. The mean triennial peak in this indicator was noted in Control 1 and when applying the complex bacterial fertilizers to the soil. $N_{90}P_{30}K_{90}$, $N_{120}P_{60}K_{120}$ and complex fertigation samples had slightly lesser (yet within statistical error) vitamin C content regardless of the application rate. Fertilizing the soil

with nitrogen had a significant negative effect on the ascorbic acid content, which was reduced at greater fertilizing rates.

Introduction and fertigation with complex mineral and bacterial fertilizers resulted in greater sugar content compared to the controls. Meanwhile, unifactorial application of nitrogen fertilizers resulted in a significant decrease in this indicator. The degree of the negative effect did not correlate with the application rates or methods; only in the case of Azovit, the effect did not occur. Organic-acid content in picking-maturity fruits was greater in case of using mineral fertilizers; however, no unambiguous correlation with the unifactorial application of nitrogen was identified. Since the unifactorial application of nitrogen fertilizers had negative effects on fruit sugars content, the increased organic-acid content in these samples entailed a considerable drop in the sugar-acid ratio (SAR).

Soil humus content and acidity varied insignificantly from sample to sample. No unambiguous correlation was identified in such variations. Total absorbed bases in the 0 to 40 cm layer did not depend on the fertilizer type or on whether irrigation was used.

Complete NPK fertigation and application of bacterial preparations led to a significant increase in the microbial population of the rhizosphere, as compared to the controls, see Figure 2; NPK fertigation increased the microbial population in June, while bacterial fertilizers did so in August. Microbial population was comparable to the controls in case of top-dressing application and incorporation of complete NPK or N-only fertilizers. Nitrogen fertigation resulted in a significant increase in the microbial activity of soils, but only in June. Unifactorial application of mineral nitrogen fertilizers by incorporation (N_{120}) did not have a significant effect on the microbial population; however, unifactorial application of Azovit did increase the microbial activity.

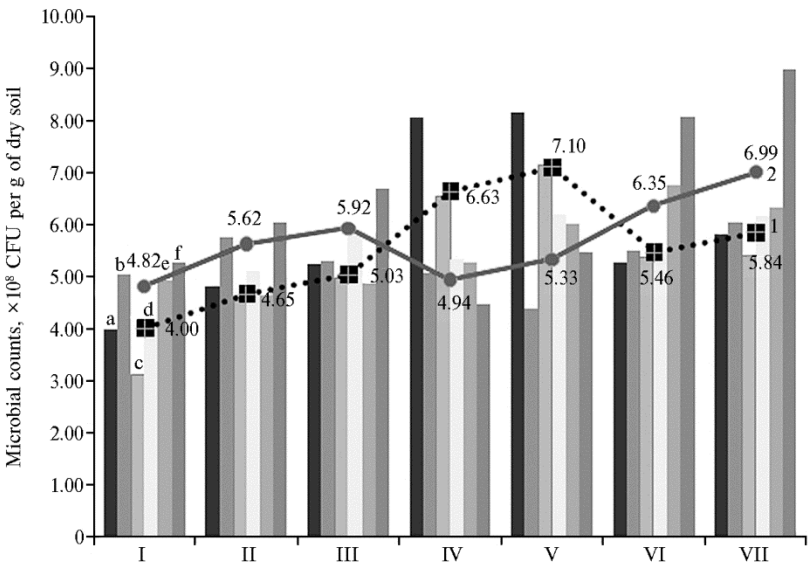

Fig. 2. Microbial population of *Malus domestica* Borkh (cv. Zhigulevskoye, rootstock 62-396) rhizosphere as affected by nitrogen fertilizers in June (a) **and August** (b)**, 2014; in June** (c) **and August** (d)**, 2015; and in June** (e) **and August** (f)**, 2016:** I for Control 1 (no fertilizers, no irrigation), II for N_{120}, III for $N_{120}P_{60}K_{120}$, IV for N_{35}, V for $N_{35}P_{25}K_{35}$; VI for Azovit, 4 l/ha, VII for Azovit, 4 l/ha + Phosphatovit, 4 l/ha; 1 and 2 for the mean microbial population in June and August, respectively. LSD_{05}: 1.06×10^6 in June 2014; 7.65×10^5 in August 2014; 5.53×10^5 in June 2015; 3.40×10^5 in August 2015; 3.51×10^5 in June 2016; 6.93×10^5 in August 2016; the triennial means were 3.58×10^5 for June and 8.38×10^5 for August, respectively. The figures are given in CFU/g (Michurinsk, Tambov Province, 2014-2016).

The authors hereof had previously indicated that the microbial population of the apple rhizosphere had a direct positive correlation with the plant productivity [42]. Applying a mineral-organic complex fertilizer in combination with Extrasol (*Basillus subtilus* Ch 13 strain, liquid form) improved the apple yield and the microbial activity of soils in the Moscow Region [43]. Using a bioorganic fertilizer in China (Linfen, Shaanxi) brought about a significant improvement in yield and productivity, as well as in the physicochemical and enzymatic activity of soils [44].

Data processing reveals that the leaf nitrogen content positively correlates with the easily hydrolyzed nitrogen in the soil ($r = 0.73$) as well as with yield ($r = 0.72$). The easily hydrolyzed nitrogen content in the soil correlates with that in fruits, albeit such correlation is weaker at $r = 0.61$.

Based on these results, the authors hereof have developed an apple yield model to optimize nitrogen fertilizing in the context of other factors: $Y = 1.37 + 0.0922x_1 - 0.0521x_2 + 9.01x_3 + 108.95x_4 + 1.91x_5 - 1.98x_6 + 0.61x_7 + 0.63x_8 + 0.21x_9$, where Y is yield, centners/ha; x_1 is the nitrogen fertilizer application rate, pn kg/ha; x_2 is the easily hydrolyzed nitrogen content in the soil; x_3 is the leaf nitrogen content, dry matter %; x_4 is the fruit nitrogen content, dry matter, %; x_5 is the soil humidity, %; x_6 is the phosphorus fertilizer application rate, pn kg/ha; x_7 is the potassium fertilizer application rate, pn kg/ha; x_8 is the available phosphorus content in the soil, mg/kg of soil; x_9 is the exchangeable-potassium content in the soil, mg/kg of soil; pn stands for primary nutrient.

Therefore, unifactorial application of nitrogen to soils helps optimize its accumulation in leaves at very high fertilizing rates; however, this causes a nutritional imbalance while exposing the environment to a greater chemical load. Fertigation has the greatest effect on apple yield and fruit quality while being eco-friendlier, as it requires 70 to 75 percent less fertilizer than incorporation. It does improve the microbial activity of soils, which in its turn improves the absorption of nutrients by plants, thus enhancing the physiological status of trees.

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AGE-DEPENDENT MORPHOPHYSIOLOGICAL CHANGES AND BIOCHEMICAL COMPOSITION OF *Lactuca sativa* L. PLANTS INFLUENCED BY Se AND SOLAR RADIATION OF VARYING INTENSITY

I.F. GOLOVATSKAYA, E.V. BOYKO, A.N. VIDERSHPAN, N.I. LAPTEV

National Research Tomsk State University, Biological Institute, 36, pr. Lenina, Tomsk, 634050 Russia, e-mail golovatskaya.irina@mail.ru (✉ corresponding author), caterinasoloveva@gmail.com, van1303@mail.ru, experteco@mail.ru
ORCID:
Golovatskaya I.F. orcid.org/0000-0002-1919-1893
Boyko E.V. orcid.org/0000-0003-3815-872X
Vidershpan A.N. orcid.org/0000-0002-3204-6519
Laptev N.I. orcid.org/0000-0002-4115-3025
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Abstract

Selenium, a micronutrient significantly involved in plant metabolism control, is also essential for human. Se regulates plant growth and protects plants from many adverse factors. The relevance of the issue is particularly high in biogeochemical provinces with selenium deficiency. Improvement of cultivation of greenhouse crops is also largely associated with optimization of the light regime. In this paper, we first reported on how selenite and selenate ions, in combination with intensity of UV-A + PAR, impact on growth and age-associated accumulation of primary and secondary metabolites in *Lactuca sativa* L. plants. These results will contribute to a better understanding of signaling elements involved in metabolic regulation. Prior to sowing, the seeds were treated with 4 % sodium selenite or sodium selenate in test and with water in control. Light intensity and spectral characteristics were changed by covering a greenhouse with polyethylene films F1 and F2 (for F2, the UV-A transmission was 40-50 % higher and PAR was 30-35 % higher as compared to F1). More insolation under F2 led to elevated content of leaf chlorophyll a (Chla), chlorophyll b (Chlb), and sugars in 60-day-old plants, thus promoting shoot development due to formation of more internodes (by 15 %) and higher stem weight compared to F1 ($p < 0.05$). Se + F1 intensified accumulation of carbohydrates and proteins, increased leaf area and caused the decline in ascorbic acid content, while F2 stimulated accumulation of ascorbic acid and flavonoids. Higher accumulation of leaf pigments (carotenoids, flavonoids and anthocyanins), lower carbohydrates in juvenile leaves under F2, and a greater number of leaf layers on a stem, due to synergic effect of light and Se, were peculiar of selenate action. Selenite + F1 led to higher content of carotenoids in juvenile leaves, whereas under selenite + F2 the level of ascorbic acid and flavonoids was higher in aging leaves. The highest content of reducing sugars (RS) and soluble proteins was in the mature leaves (layers 8 to 16) of control plants (F1). When solar radiation going up, a rise of RS level by 30 %, 45 % and 2.3 times occurred in aging leaves (layers 4-7), in adult leaves, and in young leaves (layers 17-21), respectively, while the protein content decreased in aging leaves ($p < 0.05$). Both SeO_3^{2-} and SeO_4^{2-} resulted in a higher level of RS and proteins in young leaves and kept this high in aging ones. The young and aging leaves of the control F1 plants differed in the content of flavonoids (Fla) 6-fold. Both selenium ions reduced the Fla level in mature and aging leaves by 20-30 % ($p < 0.05$), and SeO_4^{2-} led to a 4-fold increase in Fla of young leaves (F1). Increasing solar radiation (F2) resulted in the decline of Fla content. SeO_3^{2-} provided a higher Fla level in aging and mature leaves, whereas SeO_4^{2-} enhanced Fla accumulation in young leaves. At a higher light intensity, SeO_4^{2-} + F2 increased the carotenoids content by 76 %, while SeO_3^{2-} + F1 ensured only a 60 % increase ($p < 0.05$). In increasing insolation, both selenium ions elevated the shoot dry weight and the content of low molecular antioxidants (ascorbic acid and Fla) in plants. Thus, our findings showed the dependence of plant growth and metabolism on specific forms of selenium under varying intensity of solar radiation. These biomarkers should be accounted while growing plants using selenium in different lighting conditions.

Keywords: *Lactuca sativa* L., sodium selenite, sodium selenate, solar radiation, carotenoids,

reducing sugars, proteins, ascorbic acid, flavonoids, anthocyanins, primary and secondary metabolites

Selenium (Se) is an essential element for animals and humans, as well as a regulator of biochemical processes in plants. In some countries, such as China, Egypt, and Thailand, its concentration is reduced [1]. In Russia, the most selenium-deficient soils are located in Buryatia, the Chita Region, and the Khabarovsk Territory [2], where the minimal content of selenium in wheat grains is recorded. Selenium deficiency in the human diet leads to endemic osteopathy, myxomatous endemic cretinism, development of cardiological and oncological diseases, pathologies of the reproduction system, malfunctioning of the thyroid and pancreatic glands, which is related to abnormalities in synthesis and functioning of 25 selenium-dependent proteins [3, 4]. Agricultural plants are biofortified through foliar or soil application of Se compounds [5]. Se regulates plant growth, modifies the carbohydrate composition and increases resistance to abiotic stresses induced by cold, drought, UV-B rays, water shortage, salinity, and heavy metals [1, 6-8]. The positive effects of Se depend on its dose and the plant genotype and are accompanied by the activation of antioxidant protection in the cells [5, 8, 9]. The existence of several inorganic forms of Se including selenites (SeO_3^2) and selenates (SeO_4^2) raises the question about their functions and availability to plants, the impact on the productivity and sustainability of crops to the effects of light.

Light plays an important role in the regulation of plant life. It activates signaling pathways, which are controlled by selective sensory pigments and are involved in the implementation of growth and metabolic processes. Selective light alters plant growth and the amount of absorbed Se [10]. The total efficiency of the mixed stream of solar radiation on the productivity of crops in greenhouses is less studied [11]. It is known that removing UV(A + B) (λ= 280-400 nm) from a stream of solar radiation enhances the growth of terrestrial and subterranean plant organs, increases the concentration of photosynthetic pigments, photosynthetic enzyme activity and efficiency of the photosynthetic systems PSII [12]. At the same time, it is suggested that the role of photosynthetically active radiation (PAR λ = 400-700 nm) is to modify the sensitivity and photo-morphogenetic responses of plants to UV-B radiation (λ = 280-320 nm). Increasing the ratios PAR/UV-B and UV-A/UV-B is important for reducing the damage from UV-B to terrestrial and aquatic plants [13]. However, there are few studies on the interaction of UV-A (A = 320-400 nm) and PAR.

The authors have demonstrated for the first time significant differences in the manifestation of responses to the combined action of selenium and light between lettuce leaves of different ages characterized by the varying intensity of growth processes.

The aim of this work was to examine the effects of different forms of selenium (SeO_3^{2-} and SeO_4^{2-}) on plant growth and the content of primary and secondary metabolites in lettuce leaves (*Lactuca sativa* L.) of different age under different lighting (varying percentage of PAR and UV-A in the light stream).

Techniques. The anthocyanin-containing lettuce variety Gurman (*Lactuca sativa* L.) has been selected as a research object. Plants were grown in a greenhouse (Tomsk Region, 2011 and 2014) during the period from May to June with varying proportions of PAR and UV-A in the light stream. Greenhouses were covered with double (F1) or single (F2) polyethylene film. The emission spectrum and relative intensity were measured using a spectrometer AvaSpec-102/256/1024/2048 version 6.2 (Avantes BV, Netherlands).

Seeds were preliminarily treated with 4% solution of sodium selenite or selenate (Sigma, USA) (experiment) or water (control). At the end of the vegeta-

tive stage of 60-day plants, the following parameters were determined: morphological parameters (dry weight and dimensions of sprout structural elements), biochemical parameters, i.e. the content of reducing sugars [14], proteins [15], photosynthetic pigments [16], ascorbic acid [17], anthocyanins and the amount of flavonoids [18]. The content of substances was measured using a spectrophotometer UV-1650 (Shimadzu Corp., Japan), a cell with 10 mm optical path length.

The content of reducing sugars (RS) in plants was evaluated spectrophotometrically [14]. The sample weight of leaves was extracted three times with distilled water at a temperature of 70-80 °C. An aliquot of supernatant was taken from the combined extract and heated in the presence of an alkaline solution of potassium ferricyanide (15 min at 100 °C). A solution of ferrous sulfate mixed with gelatin was added to the cooled mixture. The optical density (OD) of blue-colored solution was measured at $\lambda = 690$ nm. The control solution was a sample that went through all analysis stages, but without RS extracts. In order to express the relative solution density in mass units, a calibration curve was plotted for glucose (initial solution concentration 1000 μg/ml).

The quantification of protein was made according to M.M. Bradford [15]. This method is based on the direct binding of Coomassie G-250 with amino acid residues (arginine, tryptophan, tyrosine, phenylalanine and histidine) in protein. Extracts of leaves were mixed with the Bradford reagent at the room temperature, allowed for at least 2-3 min, and OD was measured at $\lambda = 595$ nm. The control solution was a mixture of the same reagents without the extract. The protein content was determined by a calibration graph for 0.01 to 0.10 mg of the standard protein samples (bovine serum albumin).

Leaves of the 20th layer were analyzed in order to determine the content of chlorophylls a and b (Chla and Chlb) and the amount of carotenoids (Car). Pigments were extracted with 96% ethanol three times. The extract was centrifuged at 10,000 g for 10 min. The optical density of the supernatant was measured using a spectrophotometer at 470, 648.6, 664.2 and 720 nm (values of the last measurement were subtracted from the previous ones to account for possible diffusion). The following formulas by Lichtenthaler were used for calculation of the concentrations of pigments (C_a, C_b, $C_{car.}$) in 96% ethanol (mg/l):

$$C_a = 13.36 \cdot OD_{664.2} - 5.19 \cdot OD_{648.6};\ C_b = 27.43 \cdot OD_{648.6} - 8.12 \cdot OD_{664.2};$$

$$C_{car.} = (1{,}000 \cdot OD_{470} - 2.13 \cdot C_a - 97.64 \cdot C_b)/209.$$

The pigment content per unit leaf area (mg/dm^2) was calculated basing on the data of spectrophotometric analysis given the surface area of cut-outs taken for study.

The content of ascorbic acid (AsA) was determined spectrophotometrically [17]. Fresh leaves were extracted with 50 mM solution of oxalic acid (OA). The extract in equal volumes was mixed with the phosphotungstic reagent (PTR, pH = 1.0), aged for 30 min at 20-25 °C, and centrifuged (7,000 g, 10 min). The optical density of the supernatant (OD_x) was measured at $\lambda = 700$ nm relative to the control solution PTR:OA = 1:1 (v/v). The AsA content (C_x, rM) was calculated according to the formula: $C_x = (OD_x/OD_s)\ C_s$, where C_s is the concentration of the standard solution (56.8 μM L-Ascorbic acid), OD_s − optical density of the standard solution.

The quantity of anthocyanins (Ant) in plant raw material was evaluated as described in [18]. The sample weight was extracted three times with 1% HCl at a temperature 40-45 °C; the combined extract was centrifuged at 10,000 g for 10 min. The optical density of the supernatant was measured spectrophotometrically at $\lambda = 510$ nm (control solution 1% HCl). The amount of anthocyanin (%) in terms of cyanidin-3,5-diglycoside in absolutely dry raw materials ($X_{ant.}$) was calculated according to the formula: $X_{ant.} = OD \cdot 250 \cdot 100 \cdot 453^{-1} \cdot m^{-1} \cdot (100 - W)^{-1}$,

where OD is the optical density of the experimental solution; 453 is specific absorbtion of cyanidin-3,5-diglycoside in 1% HCl; m is raw weight; W is loss in weight after drying, %.

Flavonoids (Fl) were determined spectrophotometrically [18]. The sample of plant raw material was extracted three times with 70% ethyl alcohol on a boiling water bath for 60 min, the extracts were combined. An aliquot of the extract was aged in the presence of aluminium chloride and acetic acid, and after 40 min, optical density was determined at λ = 415 nm. The control solution did not contain aluminum chloride and was prepared for each sample separately. Scheme for OD measurement of control rutin solution was the same. The total content (%) was re-calculated per rutin and absolutely dry weight ($X_{flav.}$):

$$X_{flav.} = OD_x \cdot K_x \cdot m_x^{-1} \cdot m_p \cdot OD_p^{-1} \cdot K_p^{-1} \cdot 100 \cdot (100 - W)^{-1} \cdot 100,$$

where OD_x is the optical density of the experimental solution; OD_p is optical density of the rutin solution; m_x raw weight of biomaterial, g; m_p is weight of rutin, g; K_x is dilution factor of the experimental solution (1250); K_p is dilution factor of the rutin solution (2500); W is loss in weight after drying, %.

The physiological condition of leaves in different layers was assessed according to changes in growth processes. The leaf length and width on plants with the same number of layers as in test plants were measured 5 days prior to estimation of the main indicators with subsequent plotting of growth curves. The leaf area was measured using photographs (Moticam 3.0 software, Motic, Netherlands).

The results were statistically processed using the Student and Fisher criteria (Microsoft Excel 2007standard software). The figures represent the arithmetical average (M) for the growth (n = 50) and biochemical (n = 5) parameters with double-sided confidence intervals (M±1.96 SEM). The growth parameters were analyzed independently in the same organ or layer of leaves, the contents of pigments in a mixed group. Differences between values marked by different letters are statistically significant at $p < 0.05$. Changes of the studied morphological and physiological indicators of plants have similar dynamics, so the article contains data for one year 2014.

Results. Plants are characterized by long-lasting growth throughout the whole life. The growth of sprouts is ensured by the formation of new metamers, between which donor-acceptor relations remain. The sprout has actively growing (young) leaves, already grown leaves, actively functioning (adult) leaves and aging leaves with elements of chlorosis. Regulation of the size and shape of the sprout is one of the adaptation mechanisms of plants to environmental conditions. The response of the whole plant to an external factor seems to be more complex than the response of a separate organ. Therefore, the authors have conducted studies of a multilayered sprout, rather than a single metamer (layer) of the plant.

Changes of PAR and UV-A during the experiment are illustrated (Fig. 1, A). The optical properties of F2 were characterized by a greater light-permitting ability than F1: in the range λ = 360-390 nm (UV-A), the differences accounted for 40-50%, λ = 400-500 nm (blue light) for 29-35%, λ = 500-600 nm (green light) and λ = 600-700 nm (red light) for 30%. At the initial stages of ontogenesis (on day 27), donor-acceptor relations between the consistently forming structural elements of the sprout, leaves of different layers, were transforming, which was reflected in a change of their dry weight (see Fig. 1, B). Control plants cultivated without selenium have demonstrated earlier completion of growth of the 1st layer leaves under F1, which has caused longer growth of subsequent leaves of the 2nd and 3rd layers (by 4.2 and 3.8 times, $p < 0.05$). Growth inhibition of these layers under F2 has led to the accumula-

tion of dry mass in the 4th and 5th layers (by 4.6 and 3.8 times relative to the 1st layer). This kind of redistribution of the growth processes in plants under F2 resulted in a greater increase in the sprout size, which was accompanied by the development of new metamers while maintaining the total surface area of leaves in control plants compared with those under F1 (see Fig. 1, B). As a result, 60-day plants had 21 layers under F1 and 24 layers under F2.

Pre-seeding treatment of seeds with SeO_3^2 has slowed down the accumulation of dry matter by leaves of the 1st-2nd layers (20% to the control), which contributed to the growth of the leaves of the following layers, while SeO_4^2 has led to a 3.4 times more longer growth of the first leaf and inhibited growth of subsequent leaves (see Fig. 1B, F1). An increase in the percentage of PAR and UV-A in the light stream has changed the sprout response to Se. SeO_3^2 hindered the growth of leaves of the 1st and 4th-5th layers (by 36, 40 and 17%, respectively, $p < 0.05$), whereas SeO_4^2 accelerated the growth of leaves of the 1st-2nd layers by 39 and 88%, respectively, compared to the control under F2 ($p < 0.05$).

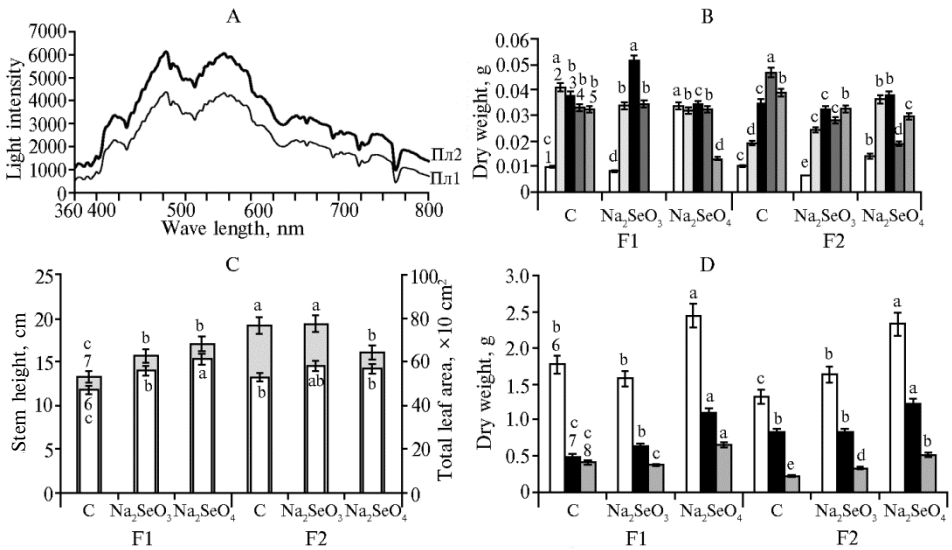

Fig. 1. Light spectra in greenhouses under films F1 and F2 (A) **and the growth parameters of leaves of the 1st-5th layers** (B) **and organs** (C, D) **in 27-day** (B) **and 60-day** (C, D) **lettuce (*Lactuca sativa* L.) plants cv. Gurman depending on light intensity and pre-seeding treatment with Se:** 1-5 — leaves of the 1st-5th layers, 6 — leaves of all layers, 7 — stem, 8 — root (Tomsk Province, average for the year 2014). Vertical bars indicate ±1.96 SEM ($n = 50$). Growth parameters were analyzed independently for the same layer of leaves (B) or organ (C, D). For each parameter, differences in values marked with different letters are statistically significant at $p < 0.05$.

With the completion of the vegetative development stage, SeO_4^2 increased the dry mass of leaves, stems, and roots in experimental 60-day lettuce plants relative to the control (see Fig. 1, D). Ions of SeO_4^2 had an advantage in the regulation of sprout development compared to SeO_3^2. Leaves form more layers under the influence of SeO_4^2 rather than SeO_3^2, 24 and 21 for F1, 24 and 28 for F2, respectively. Treatment with SeO_4^2 provided a stimulating effect (+15%) similar to light under F1 and an additive effect (+33%) after an increase in the percentage of PAR and UV-A under F2. Other authors have shown greater efficiency of SeO_4^2 (2-4 μM) compared to SeO_3^2 (6-10 μM) in the regulation of the leaf area in *Cucumis sativus* L. [19]. The age dependence of growth processes on the concentration of SeO_4^2 has been demonstrated: addition of low concentrations did not affect the raw or dry weight of younger plants *L. sativa*, but signifi-

cantly stimulated growth in aging plants [20].

Differences in growth processes in plants in response to light of different quality could be due to metabolic changes. Sugars as primary exchange products are necessary for growth and differentiation. The sugar content in leaves has been changing depending on their functional status (age) and the intensity of photosynthesis. For control plants *L. sativa* under F1, the authors have identified a higher content of reducing sugars (RS) in grown leaves, where an active synthesis of these compounds took place. Low accumulation of RS has been noted in the actively growing (17th-21th layers) and aging (4th-7th layers) leaves (Fig. 2, A) since the former acted only as acceptors of sugars, and the latter were dying. An increase in the proportion of PAR and UV-A in the light stream (F2) resulted in an increase in RS production by 30%, 45% and by 2.3 times in aging, adult (8th-16th layer) and young leaves in control plants compared with those under F1, which could indicate their different physiological status associated with the activation of photosynthesis or increased transport of sugars. A change in hormonal balance is also possible since light-dependent integration of signaling pathways for sugar and hormones through PIF (phytochrome interacting factor) and DELLA (transcriptional repressors of gibberellin signaling) proteins has been demonstrated [21].

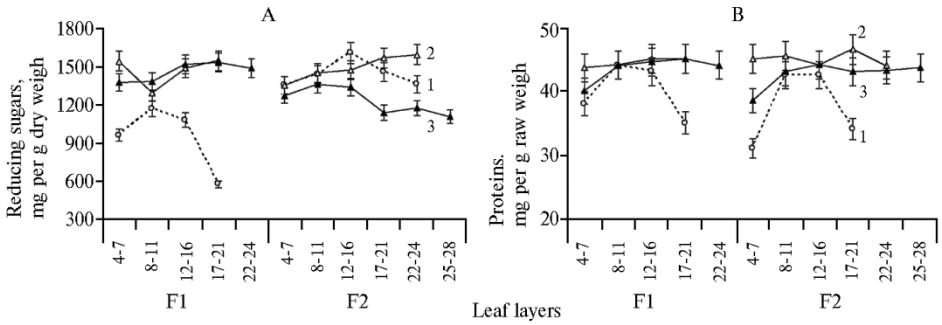

Fig. 2. Reducing sugars (RS, A) **and water-soluble proteins** (B) **in leaves of different layers in 60-day lettuce (*Lactuca sativa* L.) plants cv. Gurman depending on light intensity in greenhouses under films F1 and F2 and pre-seeding treatment with Se:** 1 — control, 2 — sodium selenite, 3 — sodium selenate (Tomsk Province, average for the year 2014). Vertical bars indicate ±1.96 SEM (n = 5).

According to the research results, the amount of RS increased in young lettuce leaves and remained high in aging leaves under F1 under the influence of SeO_3^2 and SeO_4^2 ions. The increase in the RS content in old lettuce leaves (4th-7th layers) could indicate a withdrawal of their aging effects and activation of photosynthetic reactions. Such effect of Se could be connected to the restoration or maintenance of the structure of cell membranes and integrity of cells by reducing the amount of O_2 and H_2O_2 after adding of this element [1]. The role of SeO_4^2 in the acceleration of photosynthesis is confirmed by the results by M. Djanaguiraman et al. [22] obtained for sorghum plants. The increased content of soluble sugars and starch has been also described for potato leaves after treatment with Se [4]. Under F2, the effectiveness of SeO_3^2 increased only in young lettuce leaves, and the effectiveness of SeO_4^2 decreased in adult and young leaves. The observed reactions of lettuce plants showed the influence of light on the accumulation of Se and the influence of the element on the duration of synthetic processes in leaves of different layers. It is known that the travel rate of SeO_3^2 in plants is lower than that of SeO_4^2, and the impact of red and blue light in addition to white light increases the amount of endogenous Se [20, 10].

The authors hereof have shown that in plants *L. sativa* under F1, the highest content of soluble proteins (see Fig. 2, B) was in grown leaves (8th-16th

layers). With an increasing proportion of PAR and UV-A in light (F2), the amount of proteins decreased in aging leaves of control plants, which was accompanied, however, by an increase in carbohydrate metabolism (see Fig. 2, A). Se increased the protein content in young (17th-28th layers) and aging (4th-7th layers) leaves regardless of light spectral composition. SeO_3^2 supported the content of proteins in aging leaves better than SeO_4^2, since it is known that selenite is more effective than selenate as an inductor of the activity of antioxidant enzyme selenium-dependent glutathione peroxidase (GSH-Px) [23].

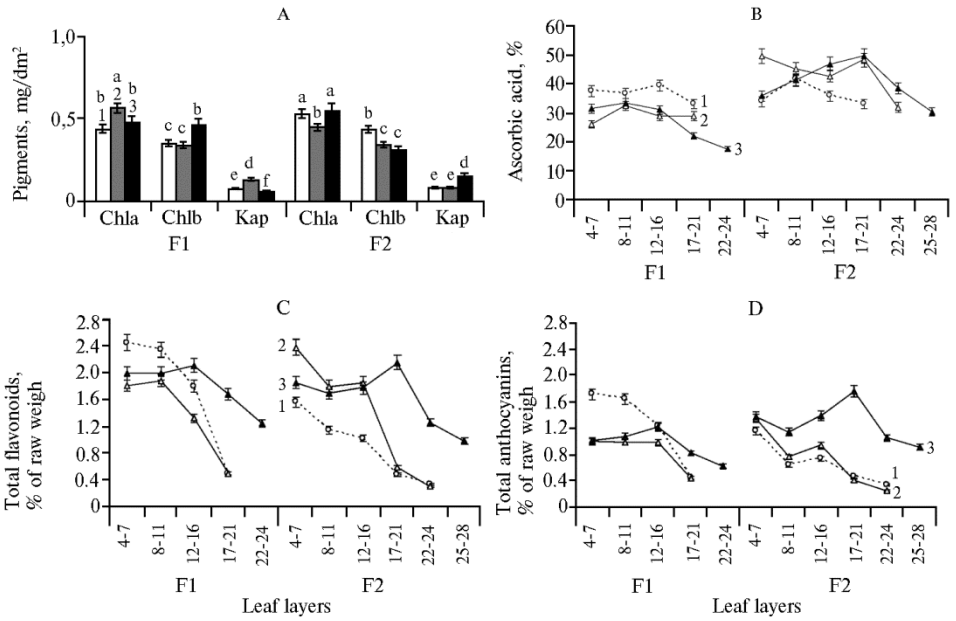

Fig. 3. Photosynthetic pigments in leaves of the 20th layer (A), **distribution of ascorbic acid** (B), **flavonoids** (C) **and anthocyanins** (D) **in leaves of different layers in 60-day lettuce (*Lactuca sativa* L.) plants cv. Gurman depending on the light intensity in greenhouses under films F1 and F2 and preseeding treatment with Se:** 1 — control, 2 — sodium selenite, 3 — sodium selenate, Chla and Chlb — chlorophylls a and b, Car — carotenoids (Tomsk Provice, average for the year 2014). Vertical bars indicate ±1.96 SEM (n = 5). The pigment content was analyzed in a mixed group. For pigments, differences in values marked with different letters are statistically significant at $p < 0.05$.

Accumulation of photosynthetic pigments in leaves of the 20th layer (Fig. 3, A) varied depending on the light intensity and spectral composition. The growing proportion of PAR and UV-A in the light (F2) caused an increase in Chla and Chlb compared to control plants under F1. The total content of all photosynthetic pigments in different variants under F1 and F2 was higher than the control for F1. However, the total number of chlorophylls influenced by SeO_3^2 for F2 stayed within the F1 control and below the F2 control. The individual pigment composition of leaves also depended on the form of selenium. SeO_3^2 under F1 increased the content of carotenoids by 60% ($p < 0.05$) that act as antioxidants and protect Chla from photochemical oxidation [24]. This contributed to the accumulation of Chla under F1, whereas SeO_4^2 decreased the carotenoid content and increased the amount of the oxidized Chlb form. The expression of the antioxidant effect of SeO_4^2 similar to SeO_3^2 under F1 occurred with an increasing proportion of PAR and UV-A radiation in the light stream (F2). Perhaps the latter conditions increased the absorption of selenium or its restoration.

Ascorbic acid is an important antioxidant in plant tissues [25]. Most of its content is located in grown leaves of the 12th-16th layers (F1) and grown leaves of the 8th-11th layers (F2). Both forms of selenium hindered the synthesis

of AsA under F1, whereas with an increasing proportion of PAR and UV-A in the light stream (F2), SeO_3^2 increased the content of AsA in aging and adult leaves, and SeO_4^2 in adult and young leaves (see Fig. 3, B). These results are consistent with the data on the increase in AsA in leaves and chloroplasts during acclimatization to high-intensity light [26]. AsA deficit in *vtc* mutants of *Arabidopsis* reduces the zeaxanthin-dependent non-photochemical quenching supported by violaxanthin de-epoxidase and determines sensitivity to photooxidation. Processing with exogenous AsA reduces the phytotoxic effect of high concentrations of Se that is manifested in relation to the membrane, chlorophyll and PSII functions in plants *Oryza sativa* L. through an increase in the activity of antioxidant and metal-tolerant mechanisms [27]. In the first mechanism, the effect is due to the action of enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as non-enzyme antioxidants, i.e. AsA, glutathione, and proline. The second mechanism is implemented through metallothioneins, thiols, and glutathione-S-transferase (GST). An increase in the content of these molecules reduces the toxic effect of Se through its conjugating and/or removal of reactive oxygen species generated due to selenium stress. Low Se concentrations (1 mg/kg) increase the activity of SOD, CAT, APX, and GR [27].

Flavonoids are essential among the secondary metabolites. The authors have established the age dependency of Fl accumulation in leaves of *L. sativa* plants (see Fig. 3). In adult and aging leaves, flavonoid accumulation was 4 and 6 times higher than in young leaves (17th-21th layers). This meant the strengthening of the synthesis of secondary metabolites with the completion of the active leaf growth. The obtained data are consistent with reports on photoinhibition of flavonoid biosynthesis at an early stage of development of the *Gossypium hirsutum* fiber *in vitro*, but stimulating at later stages [28].

Pre-seeding treatment with SeO_4^2 increased 4-fold the total amount of flavonoids in leaves compared to control leaves and plants treated with SeO_3^2 in variants with F1. With an increasing proportion of PAR and UV-A (F2), the stimulating effect of both Se forms on the accumulation of flavonoids in plants was observed. Other authors also describe Se as a photoprotector from harmful UV-B radiation [27], which manifests itself in *Triticum aestivum* L. primarily as increasing amounts of antioxidants and a decrease in the membrane peroxidation of lipids (MPL) in aboveground parts of plants. Se provides an ambiguous effect on MPL in roots: it inhibited peroxidation at low concentrations and intensified it at high concentrations [19, 29].

Anthocyanins (Ant) as flavonoids have been accumulated in grown leaves of the 4th-11th layers of control plants under F1 (see Fig. 3). A simultaneous increase in the proportion of the visible spectrum and UV-A (F1) has reduced the amount of Ant in the control. Under F1, the treatment with SeO_4^2 has increased its content in young leaves of the 17th-24th layers and reduced in adult leaves compared with the controls. Treatment with SeO_4^2 under F2 has increased the content of Ant in leaves of the most layers (see Fig. 3). At the same time, a decrease in the accumulation of RS has been observed (see Fig. 2, A). The latter fact can be explained by the role of substrate sugars in the synthesis of Ant. However, partial coherence in changes of the content of RS (see Fig. 2, A), Fl (see Fig. 3A) and Ant (see Fig. 3, B) in other circumstances was probably caused by a signal function of sugars regulating the expression of genes that control the biosynthesis of flavonoids and Ant. Other authors have shown that sugars activate the gene *PAP1* (*Production of Anthocyanin Pigmentation 1*) through a sugar-specific signaling pathway [30]. At that, not RS, but sucrose increased the synthesis of *PAP1* mRNA and the expression of genes encoding enzymes of Ant biosynthesis, the *DFR*, *LDOX*,

and *UF3GT* (*Dihydroflavonol-4-Reductase*, *Leucoanthocyanidin Dioxygenase*, *UDP-Glucose: Flavonoid 3-O-Glucosyltransferase*) determining Ant accumulation.

Based on the obtained results, the authors have suggested that differences in the metabolism of *L. sativa* plants depending on lighting are caused by the specific functioning of regulatory photoreceptors. An increase in the proportion of PAR and UV-A in light (F2) has increased the contents of photosynthetic pigments and carbohydrate in the leaves of control plants, which accelerated the development of sprouts. This is consistent with the data on elimination of UV-A-induced negative effects on photosynthesis, PSII activity and the contents of photosynthetic pigments, with preliminary exposure to the red light that is associated with the phytochrome control of these reactions [31]. Specific light-dependent response of plants to Se can be associated with the unequal accumulation of different forms of its ions, because additional exposure to the red and blue light in addition to white increases the amount of endogenous Se in plants [10]. It is known that the growth-enhancing effect of SeO_3^2 exists in a more narrow range of concentrations than in the case of SeO_4^2 (respectively 6, and 6-20 μm) [19]. Another explanation for the different direction and rate of growth processes can be the fact that Se as a pro- or antioxidant changed the accumulation of Fl of various nature. Flavonoids with o-hydroxyls in the nucleus acted as auxin synergists stimulating the growth of plants as a result of inhibition of IAA-oxidase, while Fl with p-hydroxyls acted as cofactors of IAA-oxidase demonstrating the properties of IAA antagonists and, consequently, being growth inhibitors [32]. Se could affect the content of other phytohormones [33], and hormones, in turn, could alter the Se-dependent growth of plants [34].

The differential response of lettuce leaves in different layers depended on age (primarily on the oxidative status, which was determined by the content of metabolic or stress ROS). Other authors [20] have demonstrated the ability of SeO_4^2 to counteract the aging-induced oxidative stress in *L. sativa*. In young and aging plants, the antioxidant effect of Se is associated with the increased activity of glutathione peroxidase (GSH-Px). In aging plants, an increase in the amount of Se enhances the antioxidant ability, preventing a decrease in the concentration of α-tocopherol and increasing SOD activity.

Thus, it has been established that the pre-seeding treatment of *Lactuca sativa* seeds with Se in two ionic forms regulates the intensity of growth and metabolic processes in plants, changing the content of primary and secondary metabolites. The pre-seeding treatment of seeds with Se provided a stimulating effect on the formation of new metamers of *L. sativa* sprouts and seed germination (SeO_4^2). At the same time, the regulation of the light stream spectrum has changed the efficiency of Se. An increased proportion of PAR and UV-A has stimulated growth processes (stretching and thickening of the sprout, formation of new metamers) through the activation of carbohydrate metabolism. The age dependence of the morphological and physiological parameters of leaves in *L. sativa* plants on the forms of selenium and light spectrum has been demonstrated. Under F1, at a lower intensity of UV-A and PAR, after the treatment with Se, the content of reduced sugars and proteins increased in actively growing leaves and remained the same in old ones. SeO_4^2 increases the amount of flavonoids regardless of the light spectrum, whereas SeO_3^2 has the same upon an increase in the proportion of PAR and UV-A. A higher proportion of PAR and UV-A can probably result in Se metabolism acceleration of. Maybe there was also a protective effect of high PAR with increasing UV-A, which resulted in the enhancement of carbohydrate metabolism and an increase in the content of ascorbic acid. Our findings extend the understanding of plant adaptive response to light of varying quality by providing better understanding of Se-dependent mechanisms

defining resistance to increased proportions of PAR and UV-A in light. The obtained data can also be used for diagnosis of the physiological condition of leaves in different layers with and without selenium. Pre-seeding treatment with Se combined with a changing light spectrum increases the nutritional value of lettuce due to the accumulation of primary and secondary metabolites.

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FACTORS WHICH INFLUENCE TOXICITY OF LEGUME SEED DISINFECTANTS TOWARDS BIOLOGICALS BASED ON SYMBIOTIC NITROGEN FIXERS

Yu.V. KOSULNIKOV, Yu.V. LAKTIONOV

All-Russian Research Institute for Agricultural Microbiology, Federal Agency for Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail laktionov@list.ru, kullavayn@gmail.com (✉ corresponding author)
ORCID:
Laktionov Yu.V. orcid.org/0000-0001-6241-0273 Kosulnikov Yu.V. orcid.org/0000-0003-1134-3503
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Abstract

Symbiotic nitrogen fixers of *Rhizobiaceae* family serve as biologicals for agriculture. This is due to the fact that free-living inoculants which are not crop-specific possess much less nitrogen-fixing ability than the legume—rhizobial symbiosis of a plant and its species-specific symbiont. Despite this, the seedbed inoculation and a wider use of biopreparations of nodule bacteria in legumes are hampered by a number of objective deficiencies of such preparations, for example, the relatively low resistance of rhizobia to adverse environmental factors. These factors include direct contact of bacteria with aggressive substances, i.e. chemical fungicides used for seed treatment. This paper is the first to report that the rhizobia survival rate depends on the temperature of tank solutions and may differ under the effect of disinfectants based on the same active ingredient. That is, methods of disinfectant manufacture significantly affect its toxicity towards nodule bacteria. Our goal was to determine the effect of treaters, its concentration in the solution, the time the solution was kept and the temperature mode on the number of nodule bacteria of soybean, lupine, pea and lentils that survived in the solution. Bacterial suspensions studied were root nodule bacteria of soybean (*Bradyrhizobium japonicum* 634b), lupine (*Bradyrhizobium lupini* 367a), pea (*Rhizobium leguminosarum* 261b), lentil (*Rhizobium leguminosarum* 712), and chemical fungicides were Maxim (fludioxonil, 25 g/l; «Syngenta International AG», Switzerland), Protekt, (fludioksonil, 25 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary), Protekt Forte (fludioxonil, 40 g/l + flutriafol, 30 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary). Compatibility was determined by preparing tank solutions of biologicals and disinfectants, followed by determining the percentage of rhizobia that survived, depending on the type of disinfectant, its concentration (10 and 20 %), solution holding time (2, 4, 8 hours) and temperature (2-5, 16-18, 27 °C). Our results show that the resistance of nodule bacteria of various leguminous plants to these pesticides differs and decreases among the nodule bacteria of soybean, lupine, pea, lentils. The pesticide toxicity increases in the order Maxim, Protect, and Protect Forte. The presence of rhizobia in the same solution with disinfectants negatively affects the bacteria survival. The longer the mixture is kept, the less rhizobia remain alive. With increasing temperature of the mixture and the concentration of disinfectants in the solution, their toxicity increases. Low temperatures (2-5 °C) significantly increase the survival rate of rhizobia. The disinfectants Maxim and Protect, prepared on the basis of the same active ingredient with the same concentration, differed sharply in toxicity.

Keywords: symbiotic nitrogen fixers, *Bradyrhizobium*, *Rhizobium,* biologicals, seed dressing agents, treaters, compatibility and toxicity

Leguminous plants are the main source of vegetable protein [1]. The average yield of legumes in Russia is much lower (sometimes by several times) than in Europe and the USA [2, 3]. One of the significant reasons is the low efficiency of the technologies used in the majority of cases. The paradox of the situation is that in the harsh climatic conditions that are characteristic of most of the Russian agricultural land (Ural, Siberia), the need for the most modern

farming practices increases by multiple times [4, 5]. These include, in particular, the use of preparations of symbiotic nodule bacteria, which, populating the plant's root system, provide it with the ability to fix atmospheric nitrogen [6-8]. In the Russian market, they are presented but have not yet been widely distributed. Among other reasons, there is a lack of substantiated regulations for the use of microbiological preparations in conjunction with chemical plant protection products. In practice, this inevitably reduces the efficiency and profitability of biopreparations, leads to direct economic losses and unjustifiably discredits the method, which is recognized as an important element of biologization, ecologization and increasing the sustainability of modern agricultural production.

The composition of disinfectants includes the substances that are toxic to microorganisms; therefore, the bacteria, on the basis of which microbiological preparations are made, face unfavorable conditions. Unfortunately, the study of the compatibility of biopreparations and disinfectants clearly lags behind the emergence of new strains that are potentially suitable for practice, forms of biopreparations [9, 10], and changes in the production technology of disinfectants under the same brand [11, 12]. The disinfectants (herbicides, fungicides, insecticides, etc.) have long been proved to be effective, the technologies for their application were tested [13, 14] and entrenched in domestic agriculture. Therefore, if there are doubts regarding the effectiveness of the joint use of biological and chemical preparations, in practice, the latter is preferred [15, 16]. In other words, the lack of scientific works on the assessment of the compatibility of microbiological and chemical methods for the treatment of seeds of leguminous plants [17, 18] may lead to the rejection of biopreparations, despite their environmental friendliness [19, 20], economic efficiency [21] and effect [22, 23] with an increase in the yield of leguminous plants. It should be noted that there are quite a few domestic publications on this issue [24, 25].

This study presents the first results confirming that the compatibility of inoculants and disinfectants based on the same active substance is significantly influenced by the method of manufacturing the disinfectant, that is, the qualitative and quantitative composition (formulation) of additional components (film-forming polymers, adjuvants, surfactants, etc., which, in the opinion of the manufacturers, improve the manufacturability of a disinfectant), as well as the temperature regime of the tank solution. These data supplement the limited amount of information concerning the compatibility of preparations of nodule bacteria and chemical products of protection of legumes.

The authors' goal was to determine the effect of treaters, their concentration in the solution, the time the solution was kept and the temperature mode on the number of nodule bacteria of soybean, lupine, pea, and lentils that survived in the solution.

Techniques. Strains of root nodule bacteria of soybean (*Bradyrhizobium japonicum* 634b), lupine (*Bradyrhizobium lupini* 367a), pea (*Rhizobium leguminosarum* 261b), and lentil (*Rhizobium leguminosarum* 712) were obtained from the departmental collection of useful microorganisms of agricultural purpose of the All-Russia Research Institute for Agricultural Microbiology (ARRIAM, St. Petersburg). The preparations were made on a semi-synthetic medium (0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l NaCl, 1.0 g/l of yeast extract, 10.0 g/l of mannitol) with subsequent cultivation (28 °C, 170 rpm, orbital shaker incubator ES-20/60, "BioSan", Latvia).

The following chemical fungicides were used: Maxim, SC (fludioxonil, 25 g/l; Syngenta International AG, Switzerland), Protekt, SC (fludioxonil, 25 g/l; Agro Expert Group LLC, Russia, Agro Expert Group Kft., Hungary), Protekt Forte, WSC (fludioxonil, 40 g/l + flutriafol, 30 g/l; Agro Expert Group LLC,

Russia, Agro Expert Group Kft., Hungary).

Fungicides and nodule bacteria were mixed (20% solution of a bacterial suspension with 10% and 20% solutions of the disinfectant of each examined brand). After certain intervals (0, 1, 2, 4, and 8 hours), the titers of bacteria were determined by seeding on Petri dishes with semi-synthetic medium (the composition is shown above) with the addition of 20 g/l of agar-agar. The mixtures of cultures and fungicides were kept in a refrigerating chamber (2-5 °C), at room temperature under the laboratory conditions (16-18 °C) and in a thermostat (27.5 °C). After 10 days (time of growth of nodule bacteria on Petri dishes), the formed colonies (CFU) were counted.

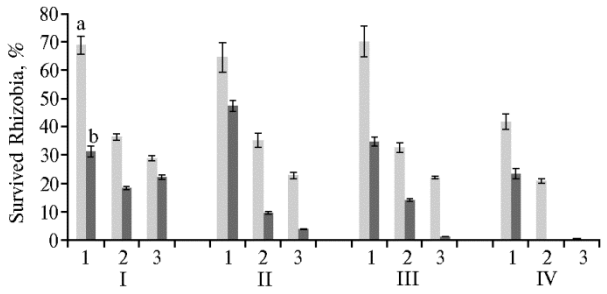

Fig. 1. The proportion of survived rhizobia *Bradyrhizobium japonicum* 634b (I), ***Bradyrhizobium lupini* 367a** (II), ***Rhizobium leguminosarum* 261b** (III) **and *Rhizobium leguminosarum* 712** (IV) **mixed with 10%** (a) **and 20%** (b) **solutions of fungicides Maxim** (1), **Protekt** (2) **and Protekt Forte** (3) (the mixture of cultures and fungicides was kept for 8 h at 16-18 °C).

Data were processed using the Microsoft Excel 10 software. To confirm the reliability of differences between the variants in the figures and in the table, mean values (M) and standard errors of the mean (±SEM) are presented. The differences were assessed by Student's *t*-test and considered statistically significant at $p < 0.05$. The repetition of the experiment is threefold.

Results. The selection of the strains of microorganisms was determined by the greatest practical importance of crops (soybean, lupine, pea, and lentils) in modern Russia and the CIS countries. In Russia, based on these strains, biopreparations are produced for legumes under the commercial name Rizotorfin® (manufactured by ARRIAM).

Mixing chemical disinfectants with rhizobia preparations negatively influenced the survival rate of the latter. The resistance of nodule bacteria of various leguminous plants to pesticides was different and decreased in the following order: nodule bacteria of soybean, lupine, pea, lentils. The toxicity of the pesticides increased in the following order: Maxim, Protekt, Protekt Forte (Fig. 1).

The survival rate of rhizobia in a mixture with disinfectants also significantly depended on the temperature at which the mixture was kept. The more toxic for rhizobia was the disinfectant (Fig. 2), the more clearly the positive effect of low temperatures on the survival of nodule bacteria was manifested. The role of the temperature factor grew with increasing concentration of the disinfectant. Thus, the proportion of surviving nodule bacteria of soybean in a mixture with a 10% Maxim fungicide solution 8 hours after the mixing at 2-5 °C and 16-18 °C was 72.02 and 68.88%, respectively. At the same time, the values of 65.73 and 31.12% were obtained for a 20% solution of the fungicide. The revealed pattern turned out to be valid for each examined biopreparation—disinfectant pair.

In some cases, the active ingredient of the fungicide was not the main factor determining the dynamics of reducing the number of rhizobia. For example, the Maxim disinfectant, which is very low-toxic for all studied rhizobia species, contains the same active ingredient and is in the same concentration as the much more toxic preparation Protekt (Table). At the same time, the toxicity of the Protekt fungicide for soybean and lupine rhizobia was comparable to

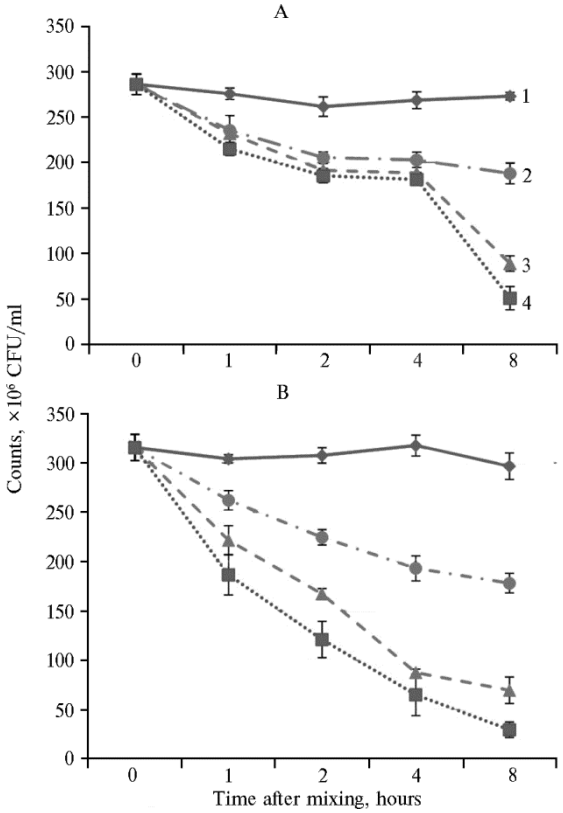

Fig. 2. The number of colonies of *Bradyrhizobium japonicum* 634b in solution with 20% Maxim (A) **and Protekt Forte** (B) **fungicides, depending on the exposure time and temperature of the mixture:** 1 — room temperature (control), 2 — 2-5 °C, 3 — 16-18 °C, 4 — 27.5 °C.

Protekt Forte, despite the fact that the latter has almost 2 times the concentration of fludioxonil and the second active ingredient, flutriafol (see Table). The control was a 20% working solution of bacterial suspensions in tap water; all the differences between the experimental and corresponding control variants are statistically significant at $p < 0.05$.

The analysis of domestic and foreign literature on the factors of toxicity of disinfectants for bacteria showed that the active substances of most disinfectants (in a pure form) were identified by researchers as toxic to rhizosphere microorganisms to some extent [26, 27], including nodule bacteria [28, 29]. It is reported [30] that contact of soybean rhizobia on inoculated seeds with such common fungicidal substances as captan and thiram (contact fungicides), as well as benomyl, carbendazim, difenoconazole and tebuconazole (systemic fungicides), causes a significant reduction in the numbers of viable bacteria.

The proportion of rhizobia surviving in a mixture with 10 and 20% solutions of fungicides, depending on the time from the moment of mixing (the mixture was kept at a temperature of 16-18 °C) (*M*±SEM)

Hours	Rhizobia and fungicide concentration							
	Bradyrhizobium				*Rhizobium*			
	japonicum 634		*lupini* 367a		*leguminosarum* 2616		*leguminosarum* 712	
	10 %	20 %	10 %	20 %	10 %	20 %	10 %	20 %
	Maxim SC							
2	79.02±4.95	67.31±3.56	82.56±5.64	73.76±4.26	83.43±5.23	81.6±5.26	81.67±5.27	63.33±3.21
4	75.52±4.20	65.91±3.24	75.36±4.58	69.92±3.98	74.11±4.13	67.2±3.89	71.67±7.13	60.00±3.14
8	68.88±3.98	31.12±1.72	64.48±3.67	47.36±2.58	70.19±3.98	34.7±1.94	41.67±2.10	23.33±0.79
	Protekt SC							
2	75.20±4.45	50.30±3.12	60.69±3.33	33.49±1.49	62.58±3.09	31.11±1.21	81.67±5.28	13.41±0.26
4	58.57±2.89	32.47±1.32	48.43±2.98	25.63±0.71	52.02±2.27	22.14±0.76	71.67±7.16	3.66±0.19
8	36.25±1.97	18.23±0.45	35.06±1.73	9.43±0.14	32.52±1.67	13.98±1.05	41.67±2.13	0.00
	Protekt Forte WSC							
2	69.73±3.64	52.93±3.16	55.03±3.57	38.76±2.03	39.00±1.99	17.00±0.54	2.62±0.16	0.00
4	46.12±2.31	27.73±0.86	46.64±2.75	18.12±0.41	36.00±1.75	14.00±0.34	1.07±0.12	0.00
8	28.68±0.95	22.03±0.69	22.65±0.74	3.69±0.10	22.00±0.68	1.00±0.12	0.12±0.10	0.00

Note. All differences between the experimental and corresponding control variants are statistically significant at $p < 0.05$.

Not all active ingredients of disinfectants are unequivocally toxic in relation to all species and strains of rhizobia. Thus, in the work by Tariq *et al.* [31], pea rhizobia are defined as benzimidazole-resistant. According to another study [32], fludioxonil has a significant toxic effect on soybean rhizobia. The authors

state [32] that the contact of soya rhizobia with fludioxonil on inoculated seeds significantly reduces the number of surviving bacteria as compared with the control 24 and 48 hours after the inoculation. The addition of alginate polymer to the inoculant significantly increased the survival rate of rhizobia in contact with fludioxonil [32]. It allows suggesting that, in the authors' experiment, the best survival rate of rhizobia mixed with the Maxim disinfectant compared to the Protekt disinfectant is not associated with the greater toxicity of the additional components in the composition of the latter, but with the protective effect on the polymer rhizobia in the Maxim preparation. This assumption is supported by the fact that some water-soluble polymers actually increase the overall resistance of rhizobia to adverse environmental conditions; in particular, the addition of sodium alginate and carboxymethylcellulose to the bacterial suspension significantly increases the shelf life of the bacterial preparation [33]. Apparently, not only the composition and concentrations of the active ingredients of the disinfectant are important but also the composition and concentrations of the additional components (film-forming polymers, surfactants, emulsifiers, antiseptics, etc.), that is, the so-called formulation of the preparative form of the disinfectant. A number of studies have confirmed the strongest influence of film-forming polymers, adjuvants, and surfactants on the survival of bacteria in biological preparations [34].

There are the reports that different brands of disinfectants [35] and different temperature regimes during storage of tank solutions significantly affect the survival of bacteria in such solutions. A number of studies have shown the ability of rhizobia to decompose pesticides [36], which, however, is quite common among rhizosphere microorganisms [37]. According to the available data [38, 39], the slow-growing rhizobia of soybean *Bradyrhizobium japonicum* and the fast-growing rhizobia of soybean *Sinorhizobium fredii* can grow on a mineral and plant agar medium with the addition of the production concentration of the fungicide Maxim. At the same time, the intensity of their growth is either not inferior to that in the control [38] or slightly decreases [39].

It should be noted that the absence of a clear toxic effect of a disinfectant with respect to rhizobia in a joint tank solution does not at all guarantee the prevention of negative consequences for nodulation [40, 41]. A number of works describe the inhibitory effect of the Maxim fungicide on the intensity of nodule formation in inoculated soybean plants [24], while seed disinfecting and inoculation were separated in time. At the same time, some authors highlight [39] that inoculation of soybean seeds with the treatment with Maxim fungicide provides more intensive nodulation, increase in the aerial mass and reliable yield increase, compared with inoculation "in its pure form".

Thus, it can be stated that among the studied rhizobia, the nodule soybean bacteria (*Bradyrhizobium japonicum* 634b) were the most resistant to the disinfectants, and the nodule lentil bacteria (*Rhizobium leguminosarum* 712) were the least resistant. In turn, among the used disinfectants, the least toxic for rhizobia was the fungicide Maxim, and the most toxic – Protekt Forte. The disinfectants Maxim and Protekt, prepared on the basis of the same active ingredient with the same concentration, differed sharply in toxicity. Probably, the toxicity of these fungicides for nodule bacteria is associated not only and not so much with the active substances in their composition but also with those additional components (film-forming polymers, surfactants, emulsifiers, antiseptics, etc.) that the manufacturers add to a disinfectant of this or that brand to improve its technological properties (formulation of the preparative form of the disinfectant). The presence of rhizobia in the same solution with disinfectants negatively affects the survival of bacteria: the longer the mixture was kept, the less amount of

viable rhizobia remained. With increasing temperature of the mixture and the concentration of disinfectants in the solution, their toxicity increases. Low temperatures (2-5 °C) significantly increase the survival rate of rhizobia.

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SPECIFIC FEATURES OF DEVELOPMENT OF SPIDER MITE *Tetranychus urticae* Koch RESISTANCE TO ACARICIDE FLORAMITE® (BIFENAZATE)

I.N. YAKOVLEVA, Yu.I. MESHKOV, N.N. SALOBUKINA, V.V. MIKHAILOVA, T.A. BERESHCHUK

All-Russian Research Institute of Phytopathology, Federal Agency for Scientific Organizations, 5, ul. Institute, pos. Bol'shie Vyazemy, Odintsovskii Region, Moscow Province, 143050 Russia, e-mail innayakovleva@mail.ru (✉ corresponding author), yimeshkov@rambler.ru, Nsalobukina7liza0904@mail,ru, abramova1984@mail.ru
ORCID:
Yakovleva I.N. orcid.org/0000-0003-4712-2315 Meshkov Yu.I. orcid.org/0000-0001-5034-2533
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Abstract

Currently, chemical method is deemed the most effective for plant protection against two-spotted spider mite *Tetranychus urticae* Koch. However, pest resistance when using chemicals for a long time remains among the main challenges. Rather short list of insectoacaricides approved for greenhouse farming in Russia aggravates the problem, since effectiveness of pest resistance control via rotation of pesticides decreases. One more thing is imported propagating material of ornamental crops infested by spider mites resistant to commonly used pesticides. Bifenazate is being successfully applied in different countries for phytophagous mite control. This work is the first rationale for the use of a novel acaricide Floramite® (bifenazate, 240 g/l) against spider mites in Russia. Our objective was to estimate the rate of Floramite®-resistance formation and cross-resistance to most used insectoacaricides in laboratory spider mite lines which are highly resistant to commonly used insectoacaricides. These lines were S-vniif (no contact to pesticides), S-floramite (derived from S-vniif to study resistance to Floramite® in the course of selection), R-vertimec (resistant to abamectin, R-fitoverm (resistant to aversectin C), R-talstar (resistabt to bifentrin), R-actellik (resistant to pirimiphos methyl), and R-BTB (resistant to bitoxibacilline biologicals based on *Bacillus thuringiensis* var. *thuringiensis*). The resistance ratios (RR_{50} and RR_{95}) were calculated from the ratio of average lethal concentrations of LC_{50}/LC_{50} and $LC9_5/LC_{95}$ for the selected line and susceptible parental line. The ratio of 10-fold (RR_{50} = 10×) and higher values stand for true resistance, those less than 10-fold value stand for tolerant. We studied formation of Floramite® resistance for 3.5 years. As per our findings, the mites show more than 5000-fold resistance to Floramite® after 53 treatments with this acaricide during 120 generations (for the majority of the most known insectoacaricides maximum resistance appears during 17-25 generations). No cross-resistance to floramite is detected in the lab lines of mites resistant to bitoxybacillin (*B. thuringiensis*) and Vertimec® (abamectin), with 1.2-1.8-fold RR_{50}, respectively. The mite lines resistant to fitoverm, Vertimec® and Actellic® show tolerance to Floramite® at RR_{50} of 2.8×, 2.9× and 3.6×, respectively. Thus, due to slow growth of *T. urticae* resistance to Floramite® and its potential in eradication of mite populations resistant to different pesticides, Floramite® should be approved in domestic protocols for greenhouse farming.

Keywords: two-spotted spider mite, *Tetranychus urticae*, Floramite®, bifenazat, resistance, cross-resistance, insectoacaricides, greenhouses

The two-spotted spider mite *Tetranychus urticae* Koch (*Acariformes: Tetranychidae*) is one of the most dangerous pests of crops in fields and greenhousees. For many decades, it has been controlled by pesticides. However, the ability of phytophages to develop resistance to them quite rapidly often creates unconquerable difficulties in the plant protection practice. This challenge concerns not only the agricultural industry but also other human activities. The issues of the resistance of the spider mites have been widely reported. It has been established that they may develop resistance to almost all groups of pesticides, irrespective of their origin [1-6]. The Insecticide Resistance Action Committee (IRAC) has included the two-spotted spider mite into the list of 20 arthropod

species that have reached critical resistance to pesticides in the pest areas [7]. In Russia, the resistance of *Tetranychus* mites is of particular danger in the protected ground, where beneficiary conditions for massive pest irruptions are provided. The situation is additionally worsened by an insufficiently wide arsenal of insectoacaricides approved for use in the Russian territory.

Over the recent years, a significant problem has been the almost country-wide resistance of the two-spotted spider mite to avermectin agents, which have been continuously used for over 20 years in floral and vegetable facilities. Monitoring of the abamectin resistance carried out by the authors in 2011-2013 has shown that in the vegetable crops the resistance ratio (RR_{50}) of the two-spotted spider mite varied from 48- to 1300-fold; in greenhouse roses, it was from 3.7- to extremely high 3300-fold. Together with this, in the greenhouses the transportation of the avermectin-resistant spider mite populations (on the rose planting material) from abroad was frequently registered [8, 9]. During extensive chemical treatments, the resistant pest populations rapidly restore and significantly increase their numbers, which adversely affects the phytosanitary state of the greenhouses and leads to additional economic costs.

Practice demonstrates that the measures for successful prevention and overcoming of the pest resistance should be based on continuous improvement of the pesticide arsenal, taking into the account the modern physiological, biochemical and genetic impressions about its formation [10]. An important role belongs to the investigation of the population biology of developing resistance to novel pesticides. This provides a possibility to predict the emergence of resistance and take operative measures on selection and rational application of pesticides.

The bifenazate-based acaricides were registered at the end of the last century in the USA and United Kingdom. Currently, these are positioned in many countries as effective agents for controlling the herbivore mites of different crops [11-14]. It is important to note that bifenazate, having a selective activity, is more toxic to herbivore mites than to carnivorous *Phytoseiidae*. Therefore, it may be used in combination with biological means for controlling the pests [15]. In China, monitoring of field populations of the two-spotted spider mite resistant to abamectin has shown that the pests have retained sensitivity to bifenazate [16]. The similar results were obtained at studying various crops in greenhouses and in open ground in Cyprus, where the tested mite populations exhibited sensitivity to bifenazate [17].

The literature data about the emergence of bifenazate-resistant populations of spider mites are rare. Development of resistance to bifenazate at the early stage has been found in two-spotted spider mite in cucumber plants in Jordan [18]. In Korea under laboratory conditions, the acaricides sensitivity was determined in eight populations of two-spotted spider mite collected on roses in greenhouses; two of these were resistant to bifenazate [19]. Further laboratory selection of one of such populations for 4 years has led to the formation of 248.8-fold bifenazate resistance. Cross-resistance of female mites to acenoquinocyl and fenpyroximate, and that of eggs to amitraz, emamectin benzoate, fenpyroximate, milbemectin, pyridaben and spirodiclofen was found. At the same time, the absence of cross-resistance to emamectin benzoate and milbemectin in females and that to abamectin in pest eggs was found [20].

In the studies by T. van Leeuwen et al. [21], the artificial selection of 36 generations of two-spotted spider mite has led to more than 164-fold resistance to bifenazate. The bifenazate-resistant line had no cross-resistance to other acaricides [22]. In other studies, there was no cross-resistance to bifenazate in the laboratory lines of two-spotted spider mite with 580-fold resistance to chlorfenapyr [23]. A possibility of inhibiting bifenazate activity by insectoacaricides from the

organophosphate and carbamate classes has been found at using them against spider mites [24].

In the present papre, the authors have substantiated a possibility of using for the first time the Floramite® 240 SC acaricide, novel in Russia, against populations of two-spotted spider mite, resistant to insectoacaricides from various groups used in the protected ground. It was shown that the resistance to this acaricide develops in mites very slowly, and the resistance to other recommended acaricides does not cause cross-resistance to Floramite.

The aim of the study was to identify the features of forming resistance to Floramite® agent in two-spotted spider mite.

Techniques. The following insectoacaricides allowed for application in Russia were used in this study: Vertimec® EC, 18 g/l (abamectin) (Vertimec® 018 EC, abamectin, Syngenta AG, Switzerland); Phytoverm® EC, 2 g/l, active ingredient aversectin C (Farmbiomed LLC, Russia); Talstar® EC, 100 g/l, active ingredient bifenthrin (Talstar 10 WP, bifenthrin, FMC Chemicals, Belgium); Actellic® CE, 500 g/l, active ingredient pirimiphos-methyl (Actellic® 50 EC, pirimiphos-methyl, Syngenta AG, Switzerland); Bitoxibacillin® P, BA 1500 EA/mg, *Bacillus. thuringiensis* var. *thuringiensis* (Sibbiofarm LLC, Russia).

The experiments were carried out using the standard (sensitive) line of the two-spotted spider mite (*Tetranychus urticae* Koch) cultured in laboratory and never exposed to pesticides (S-vniif); a subline isolated therefrom for studying the rate of forming the Floramite resistance (S-floramite); mite lines selected in the laboratory by resistance to Vertimec® (R-vertimec), Fitoverm® (R-fitoverm), Talstar (R-talstar), Actellic® (R-actellic) and Bitoxibacillin® (R-BTB). The lines were maintained by stabilizing treatments with the said pesticides and were further used for studying cross-resistance. The mites were maintained in isolated glass boxes on the young plants of Sax bush bean at the temperature of 22±3 °C, relative air humidity of 55-70% and 18 h light period.

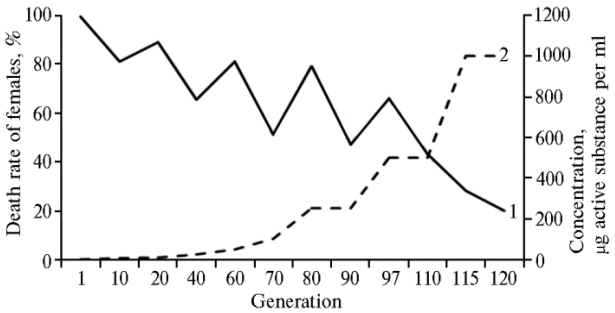
In the experiments on the study of the development of resistance to Floramite® 240 SC (Floramite, 240 g/l, active substance bifenazate, Floramite® 240 SC, bifenazate, Chemtura AgroSolutions, USA), the starting mite subline (S-floramite) was subjected to sequential selective treatment with the pesticide. The bean plants populated by spider mites (about 2000 mobile mites on 100 leaves) were cut and immersed into an aqueous solution of the pesticide for 3 s; the survived mites were allowed to move to the untreated plants. For the starting treatment, the acaricide concentration of 0.0001% AI (or 1.0 μg AI/ml) was selected in such a way so as to cause the death of at least 85% of mites. At the restoration of numbers of the mite to the conditionally starting one, the treatment was repeated. As the mortality of mites decreased, the norm of the pesticide was increased at the next treatment to a value sufficient to maintain its high efficiency. Mites at all stages of development were simultaneously subjected to acaricide, and the results were taken into account with respect to females only. The mortality was assessed after each treatment; the resistance of females to Floramite® was assessed after every 8-10 generations. The criterion of resistance development was the resistance ratios (RR_{50} and RR_{95}), established from the ratio of lethal concentrations LC_{50}/LC_{50} and LC_{95}/LC_{95} in the selected and parental (sensitive) lines. The true resistance was the ten-fold (RR = 10×) and higher, the tolerance was the parameter of less than 10-fold.

In order to determine the lethal concentrations of the pesticides CL_{50} and CL_{95} at determining the rate of resistance development and investigation of cross-resistance, the bean plants populated with female mites (at least 100 mites each) at the phase of 2 true leaves were immersed into aqueous acaricide solutions of 5-7 increased concentrations (from minimal, causing death of 5-10%

sensitive specimens, to maximal, leading to death of 90% specimens and more). Death of females was taken into the account after 24 h (Actellic®), 72 h (Vertimec®, Fitoverm®, Talstar, Floramite®) and 5 days (Bitoxibacillin®) after treatment.

All experiments were carried out in accordance with methodical recommendations for entomological and toxicological studies [25, 26] in 4-fold frequency. The experimental data were processed by the statistical method of probit analysis.

Results. The Floramite® 240 SC (bifenazate) is not yet registered in Russia, but is of interest as a prospective acaricide. It belongs to a new group of chemical compounds with a different mechanism of action and may be used in the pesticide alternation system in order to overcome resistance to avermectins, and it is of low hazard to predatory mites and beneficial insects.



Mortality of female two-spotted spider mites (*Tetranychus urticae* Koch) treated with Floramite® 240 SC (240 g/l, bifenazate) at various concentrations (laboratory experiment).

Formation of resistance to Floramite® in two-spotted spider mite was quite prolonged. In 20 generations, the female mortality was almost constant (81-89%), although the pesticide concentration was twice increased 5- and 10-fold compared to the starting one for this period (Fig.). By the time of development of the 40th generation, the 2.5-fold concentration increase has led to a drastic decrease in female mortality (65%). Further in the selection, the same trend was observed in the mite reaction to the pesticide. In the 60th generation, in response to another 2-fold concentration, an increase (81%) and then a rapid decrease in mortality followed (up to 51%). Alteration of mortality decrease rates indicates a beginning of a new stage of resistant genotype accumulation. However, from the 97th generation, the female mortality began to decrease at a continuous increase in the Floramite® concentration (the pesticide norm was adjusted to 0.1% AI, or 1000 μg AI/ml). This period may be regarded as the beginning of pest resistance stabilization.

The first notable, 5.1-fold, alteration of the average lethal concentration of Floramite® was detected at the 13th generation (Table 1). By the 31st generation, the resistance of the treated line exceeded the control 76.4-fold, by the 45th generation 180.6-fold. Such resistance held for quite a long time, up to the 70th generation; the mites were characterized by an almost identical reaction to the wide range of the Floramite® concentrations. A continuation of selection has led to a rapid increase in resistance (5125-fold to the 113th generation), an extremely high value for the studied pesticide. The experiment took 3.5 years: before the line achieved stable resistance, 53 Floramite® treatments were carried out. The selection was carried out up to the 120th generation. At the final phase of selection, the stabilization of the resistance development process occurred and no reliable alterations in the resistance ratio were observed (RR_{50} 5160). As a rule, at the artificial selection, resistance to most of the known insectoacaricides (organophosphorus, sulfurous and others) emerges in mites quite rapidly, in 17-25 generations on the average [2]. The resistance to Floramite® formed more slowly; however, it should be taken into account that, as is seen from the obtained data (see Table 1), at laboratory selection the use of Floramite® was ef-

fective only up to the 45th generation of the pest (at about one and half years). The subsequent treatments (up to the 70th generation) have led to the development and maintaining of almost 200-fold resistance. It is obvious that at the formation of such resistance in the population, further use of the pesticide is not effective. At approximation under commercial greenhouse conditions, the development of analogous resistance at continuous use of Floramite® may occur approximately in at least 2 to 2.5 years. The similar processes were noted by the authors for the avermectin pesticides in 2-3 years and in 10-15 years in industrial greenhouses [8, 28, 29].

1. Ratios of resistance to the Floramite® 240 SC (240 g/l, bifenazate) pesticide in female two-spotted spider mites (*Tetranychus urticae* Koch) during the selection process (laboratory experiment)

Generation	Number of treatments	LC_{50}, μg AI/ml	LC_{95}, μg AI/ml	Resistance ratio	
				RR_{50}	RR_{95}
P	0	0.07 (0.07÷0.13)	0.57 (0.25÷1.34)	1	1
13	5	0.37 (0.31÷0.76)	2.70 (1.99÷3.33)	5.1	4.7
31	15	5.5 (3.41÷6.00)	32.00 (29.00÷36.00)	76.4	56.1
45	20	13.00 (12.00÷15.30)	440.00 (320.00÷600.00)	180.6	771.9
65	28	12.50 (11.00÷13.10)	260.00 (110.00÷420.00)	173.6	456.1
70	31	13.90 (12.20÷14.40)	490.00 (476.00÷503.00)	193.1	859.6
100	48	130.00 (110.00÷150.90)	2700.00 (1300.00÷5600.00)	1806	4737
113	51	369.00 (300.00÷452.00)	2460.00 (1100.00÷5940.00)	5125	4316
120	53	372.00 (331.00÷491.00)	2808.00 (1900.00÷6900.00)	5167	4926

N o t e. P — parental (sensitive) line, LC_{50} and LC_{95} — lethal concentrations of pesticide causing death of 50% and 95% females, RR_{50} and RR_{95} — the resistance ratios determined from the ratios of LC_{50}/LC_{50} and LC_{95}/LC_{95} in the selected and parental lines. The confidence limits at $p = 0.05$ are recited in parentheses

2. Resistance to Floramite® 240 SC (240 g/l, bifenazate) pesticide in female two-spotted spider mites (*Tetranychus urticae* Koch) from the lines resistant to other insectoacaricides (laboratory experiment)

Line	LC_{50}, μg AI/ml	LC_{95}, μg AI/ml	Resistance ratio	
			RR_{50}	RR_{95}
S-vniif	0.07 (0.07÷0.13)	0.57 (0.25÷1.34)	1	1
R-vertimec	0.13 (0.12÷0.15)	3.30 (1.80÷60.0)	1.8	5.8
R-fitoverm	0.21 (0.17÷0.26)	3.16 (1.30÷7.70)	2.9	5.5
R-talstar	0.26 (0.22÷0.31)	6.10 (3.27÷10.11)	3.6	10.7
R-actellik	0.20 (0.17÷0.24)	3.62 (1.71÷7.69)	2.8	6.4
R-BTB	0.08 (0.07÷0.09)	0.84 (0.42÷1.68)	1.2	1.5

N o t e. See the description of lines in the Techniques section. LC_{50} and LC_{95} — lethal concentrations of pesticide causing the death of 50% and 95% females, RR_{50} and RR_{95} — resistance ratios determined from the ratios of LC_{50}/LC_{50} and LC_{95}/LC_{95} in the selected and parental lines. The confidence limits at $p = 0.05$ are recited in parentheses.

In commercial farming, an extension of the useful life of the pesticide is probable. In the protected ground, at low diversity of pesticides allowed for use against spider mites, it is possible to overcome the resistance at a combination of non-related pesticides. However, the manifestation of cross-resistance significantly decreases the effect of protective measures and sometimes it makes impossible the use of even those pesticides the phytophages never interacted with

[2, 3, 30, 31].

At the present time, the problem of effective control of spider mites resistant to insectoacaricides, firstly, to avermectin pesticides, is of utmost importance. For example, Vertimec® (active ingredient – abamectin) was widely used and exhibited high efficiency for a long time [32, 33], but further, it did not provide the required protective activity [9]. This determines the interest in the assessment of resistance to Floramite® in laboratory lines of two-spotted spider mites resistant to other pesticides. In particular, in the current experiment, the authors used the lines which were highly resistant to Vertimec® (1660-fold), Phytoverm® (1020-fold), Actellic® (2200-fold), Talstar (283-fold) and Bitoxibacillin® (the mortality of resistant females did not exceed 5-7% at treatment with sub-lethal concentration) (Table 2).

The authors hereof have established the absence of cross-resistance to Floramite® in the two-spotted spider mite lines resistant to Bitoxibacillin and Vertimec® (the resistance ratios are correspondingly 1.2× and 1.8×). In the lines resistant to Fitoverm®, Talstar and Actellic®, the 2.8 to 3.6-fold tolerance to Floramite was found. The results concerning Vertimec® and Actellic® obtained in the studies correspond to the literature data. For example, in the studies by Lee *et al.* [19] the populations of two-spotted spider mite resistant to abamectin (3822-fold) and to pirimiphos-methyl (increase to 77-fold) exhibited resistance to bifenazate. There are reports about the absence of cross-resistance to bifenazate in the abamectin-resistant (1294-fold increase) *T. urticae* line [27].

Therefore, at laboratory selection of the sensitive sub-line of two-spotted spider mite (S-floramite) for resistance to the Floramite® 240 SC pesticide, resistance develops quite slowly (RR_{50} over 5,000 for the time of development of 120 generations). In the mite lines that are resistant to Bitoxibacillin® and Vertimec®, no cross-resistance to Floramite® is found. The mites from the lines resistant to Fitoverm®, Talstar 10 WP and Actellic® exhibite tolerance to Floramite®. These results indicate that Floramite® 240 SC (Floramite EC, 240 g/l, bifenazate), if registered in the Russian territory, may be effectively employed against two-spotted spider mite, including its populations resistant to insectoacaricides.

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LIQUID FERMENTATION OF *Stagonospora cirsii* C-163, A POTENTIAL MYCOHERBICIDE FOR *Cirsium arvense* (L.) Scop.

S.V. SOKORNOVA, A.O. BERESTETSKIY

All-Russian Research Institute of Plant Protection, Federal Agency for Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail toxbiotech@vizr.spb.ru (✉ corresponding author), s.sokornova@spbu.ru
ORCID:
Sokornova S.V. orcid.org/0000-0001-6718-4818 Berestetskiy A.O. orcid.org/0000-0002-0612-6996
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Abstract

The bioherbicides should exhibit stable effectiveness in the field, be specific and quick in action, compatible with other preparations and meet market demand. In many ways, the cost and quality of product is determined by the technology of obtaining infectious material. An infectious material is used as a mycelium and its modifications and as conidia as well. The extreme sensitive of the mycelium to drying is often referred as main disadvantage of using it as the basis of a formulation. At the same time, the technological process of obtaining conidia is often more complicated, and the efficiency in the field is less than the mycelium has. The phytopathogenic fungus *Stagonospora cirsii* J.J. Davis, which is causative agent of a leaf spot disease of creeping perennial weeds in the family *Asteraceae*, is considered a potential mycoherbicide of Canadian thistle *Cirsium arvense* (L.) Scop. However, the yield of *C. cirsii* C-163 mycelium on standard nutrient media is significantly lower than that used in biotechnology (3 g/l). Our paper is the first to report that manipulation with liquid nutrient medium allows a significant increase in *S. cirsii* mycelium pathogenicity and tolerance to exsiccation. The study is devoted to the optimization of liquid-phase deep fermentation parameters, as well as the duration of cultivation and composition of a nutrient medium, in order to obtain the *C. cirsii* C-163 mycelium with improved mycoherbicidal properties. This infection material is a good basis for development formulations that can be used both individually and jointly with other protective agents for perennial weed control. The advantage of the approach used in the work is that the resistance to drying, an important technological parameter which largely determines the success of the herbicides, was additionally considered, along with virulence and mycelial yield, during the optimization of fermentation parameters. The strain *C. cirsii* C-163 was used. The 10-day inoculum was obtained in Petri plates on potato dextrose agar medium. The mycelium was incubated in 250-ml Erlenmeyer flasks containing 50 ml of the medium at 130 rpm and 24±2 °C for 2-7 days. The base liquid nutrient media contained carbon source (20 g/l), organic (10 g/l) or inorganic (3.5 g/l) nitrogen source, yeast auto lysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). Dulcite, rhamnose, L-inositol, L-arabinose, D-sorbitol, glucose, trehalose, and sucrose were a source of carbon. Casein, soy peptone, enzyme peptone, soy flour, gelatin, lecithin, ammonium dihydrogen phosphate, ammonium chloride, ammonium sulfate, and sodium nitrate were a source of nitrogen was. The pH of all liquid nutrient media was adjusted to 6.0. To establish the optimum concentrations of sucrose and soy flour in a nutrient medium with yeast auto lysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l), the amount of sucrose was changed from 10 to 70 g/l with a step of 10 g/l and the concentration of soy flour was changed from 5 to 20 g/l with a step of 2.5 g/l. The degree of leaf damage caused by disease was estimated by the necrosis area of leave disks or whole plants (5-6 true leaves). Drying of harvest mycelium, humidity 85-87 %, was carried out in a thin layer (1-2 mm) with flowing air at 30 °C without protectors for 3 hours. The highest yield of mycelium is when the carbon source in the nutrient medium is L-inositol. When inositol is substituted with sucrose or D-sorbitol, the biomass yield reduces by 25 %. At the same time, these nutrient media gave the most aggressive mycelium. Among nitrogen sources, the maximum yield of mycelium is in the case of casein, soy flour and enzymatic peptone. In the process of drying mycelium, loss of viability of propagules turned out to be significant. The mycelium obtained on sucrose-soy nutrient medium is the most resistant to drying. The most viable and aggressive mycelium was formed in the middle of the exponential growth phase which occurred on day 3 when cultivated in flasks on a soya-sucrose nutrient medium. Opti-

mization of the concentration of soybean flour (15 g/l) and sucrose (60 g/l) makes it possible to increase the yield and aggressiveness of mycelium 12 and 4 times, respectively, as compared to Czapek medium. Thus, the present study provides a method for the preparation of a mycelium having a high aggressiveness to the host-plant and a capability to remain viable during drying. The prospects of such a method of obtaining an infectious material are proved.

Keywords: phytopathogenic fungi, *Stagonospora cirsii* J.J. Davis, *Cirsium arvense* (L.) Scop., Canada thistle, submerged liquid cultivation, carbon source, nitrogen source, mycelium, mycoherbicide

Among more than 200 species of fungi and bacteria that were considered as potential bioherbicides of diverse action spectrum, by 2011 only 8.1% had become the basis for production of commercial formulations, 19.4% had been registered but not commercialized, and 72.5% had not confirmed their effectiveness [1]. The reason is that the commercial success of the bioherbicide is determined not only by the virulence for the target object, but also by its effectiveness in the field, the specificity and speed of action (aggressiveness), processability, the cost of the nutrient media used in the manufacturing cycle, the compatibility with other biological and chemical preparations, as well as the market demand [2, 3].

The technology of obtaining biological preparations is determined by the nature of the original infectious material. Most of the registered mycopesticides are developed on the basis of conidia, in some cases mycelium and its modifications are used for this purpose. The sensitivity of the mycelium to drying is often referred to as its main disadvantage. At the same time, the technological process of obtaining conidia is often more complicated, and the efficiency in the field is less stable than the mycelium has [3, 4].

The phytopathogenic fungus *Stagonospora cirsii* J.J. Davis is considered a potential mycoherbicide of Canadian thistle *Cirsium arvense* (L.) Scop. [5]. A leaf spot disease of creeping perennial weeds in the family *Asteraceae* can be caused by conidia as well as *S. cirsii* C-163 mycelium fragments. Strains of this species form conidia only under the influence of ultraviolet [5]. *S. cirsii* C-163 mycelium can cause disease of weeds under more severe temperature and humidity conditions than conidia [6]. The advantages of the use of preparations based on mycelium in the field are also shown for other potential mycoherbicides [6-9]. It is partly explained by the autoinhibition of the conidia development at their high number [10, 11]. The cases are known when phomoid micromycetes conidia obtained in vitro were avirulent [12]. In addition, the emergence of a synergistic effect in the co-use of mycelium and chemical herbicides in low doses provides more stable effectiveness of preparations based on mycelium in the field [13-15]. Therefore, *S. cirsii* mycelium is considered primarily as the infectious material for the development of a bioherbicide against Canadian thistle.

Compared to solid-phase cultivation, liquid-phase deep fermentation is a simpler and faster way to obtain infectious material [16-18]. The development of this technology includes the optimization of the nutrient medium for the viability and aggressiveness of the material [16]. For phytopathogenic micromycetes, it is shown that the aggressiveness of infectious material is determined by the nature of carbon and nitrogen sources, their ratio and absolute concentration [19-21], as well as the physiological state of propagules [6]. It is necessary to note that for phomoid micromycetes, which include *S. cirsii*, the comprehensive study to assess the impact of the cultivation duration, the nature, and concentration of carbon and nitrogen sources on the quality of the mycelium, obtained in the result of deep fermentation, has not been carried out.

This paper is the first to report that manipulation with liquid nutrient medium allows a significant increase in *S. cirsii* mycelium pathogenicity and tolerance to exsiccation.

The work objective was to optimize the composition of the nutrient medium (according to C, N sources) and the duration of deep liquid-phase cultivation to increase the yield of virulent mycelium of *Stagonospora cirsii* C-163.

Techniques. The strain *S. cirsii* C-163, which was stored at 5 °C in test tubes on potato-dextrose agar and at –80 °C in 10% glycerin, was used in the work. The inoculum was obtained on potato-dextrose agar.

The mycelium was incubated in 250-ml Erlenmeyer flasks containing 50 ml of the nutrient medium in the orbital shake-flask propagator (at 180 rpm). The carbon (dulcite, rhamnose, L-inositol, L-arabinose, D-sorbitol, glucose, trehalose, and sucrose) and nitrogen (casein, soy peptone, enzyme peptone, soy flour, gelatin, lecithin, ammonium dihydrogen phosphate, ammonium chloride, ammonium sulfate, and sodium nitrate) sources varied in liquid nutrient media. The following media composition was used: carbon source (20 g/l), organic (10 g/l) or inorganic (3.5 g/l) nitrogen source, yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). The pH of liquid nutrient media was adjusted to 6.0 before autoclaving (taking into account the optimal pH 5-6 for the development of the *S. cirsii* mycelium) [8].

To establish the optimum concentrations of sucrose and soy flour in a nutrient medium with yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l), the amount of sucrose was changed from 10 to 70 g/l with a step of 10 g/l and the concentration of soy flour was changed from 5 to 20 g/l with a step of 2.5 g/l. The optimal cultivation time was determined in the range from 2 to 7 days at 25±2 °C on a nutrient medium (pH 6.0) of the following composition: soy flour (14 g/l), sucrose (60 g/l), yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). The CFU, mycelium yield by dry weight, pH of culture liquid were determined by standard methods [22].

The aggressiveness of the mycelium against Canadian thistle was assessed with the area of damage of leaf disks or entire plants in the rosette phase. Disks with a diameter of 0.8 cm were cut with a Forstner bit from the leaves of the middle layer. They were placed in rows of 12 pcs with the adaxial side up in the leak-proof clear plastic containers on filter paper moistened with sterile water. Leaf disks were inoculated with fragments of *S. cirsii* C-163 mycelium (50 mg/ml) by applying an aqueous suspension (5 μl) to the center of the disc. In experiments on entire plants, they were sprayed with an aqueous suspension of the same concentration at a flow rate of 1.5 ml/plant. The aggressiveness of *S. cirsii* on the leaf disks was assessed 2 days after inoculation on the relative area of necrosis formed at a temperature of 25 °C and intermittent (12 h dark/12 h light) artificial light. The aggressiveness of *S. cirsii* on entire plants was determined by the relative area of leaf necrosis 7 days after inoculation.

Drying of mycelium (humidity 85-87%) was carried out in a thin layer (1-2 mm) with flowing air at 30 °C without protectors for 3 hours.

1. Yield and aggressiveness of 4-day *Stagonospora cirsii* C-163 mycelium at different C sources in the culture medium (*M*±SEM)

Carbon source	Biomass yield, g/l	Necrosis area, %
L-inositol	5.60±0.10	20±4
Sucrose	4.20±0.08	55±4
D-sorbitol	4.13±0.19	50±4
Rhamnose	3.79±0.13	23±2
L-arabinose	3.24±0.23	10±4
Trehalose	3.11±0.22	35±7
Glucose	3.08±0.08	25±4
Dulcite	2.37±0.13	5±2
LSD_{05}	0.29	9

The experiments were carried out in 4 replications. The results were subjected to dispersion analysis. Homogeneity of variances of the samples was checked with the help of Cochran's Q test. Standard deviations (±SEM) are given for the means (*M*). The significance of differences of mean values is determined with the criterion of least significant difference ($LSD_{0.05}$). Calculations were

performed in Microsoft Excel 2007.

Results. The first step in the cultivation conditions optimization for *S. cirsii* C-163 was to select a carbon source for the liquid culture medium. The variant with L-inosite as a carbon source provided the greatest yield. Substitution of L-inosite with sucrose and D-sorbitol resulted in the reduction of biomass yield by 25%. At the same time, the media with sorbitol and sucrose formed mycelium the most aggressive against thistle (Table 1). Due to this reason, and taking into account the commercial availability and stabilizing properties of sucrose [9], it was used as a carbon source when selecting a nitrogen source at the next optimization stage.

2. Yield and aggressiveness of 4-day *Stagonospora cirsii* C-163 mycelium at different N sources in the culture medium (*M*±SEM)

Nitrogen source	Biomass yield, g/l	Necrosis area, %
Casein	55.80±0.11	100±0
Soy flour	25.29±0.09	100±0
Enzyme peptone	21.24±0.13	96±5
Gelatin	18.12±0.14	100±0
Lecithin	12.01±0.08	75±12
Soy peptone	6.11±0.17	100±0
Ammonium dihydrogen phosphate	6.70±0.13	50±4
Ammonium sulfate	5.12±0.08	58±4
Sodium nitrate	4.50±0.08	45±7
Ammonium chloride	4.21±1.30	50±4
LSD_{05}	0.3	9

By varying N sources on the medium with sucrose, it was found that the yield of dry biomass on nutrient media with organic nitrogen (with the exception of soy peptone) was 12-55 g/l, more than by 3 times higher than in the standard Czapek medium with yeast extract (sodium nitrate as nitrogen source) (Table 2). Aqueous suspension based on mycelium fragments, obtained on nutrient media with casein, peptone, soy flour or gelatin, caused the death of disks from the leaves of thistle. The maximum yield of mycelium was found for casein, soy flour, and enzyme peptone (Table 2). Nutrient media of such composition were basic at the third stage of optimization.

3. Vability of 4-day *Stagonospora cirsii* C-163 mycelium at different N sources in the culture medium (*M*±SEM)

Nitrogen source	CFU, $\times 10^6$/g	
	before drying	after drying
Casein	0.4±0.1	0.05±0
Enzyme peptone	1.1±0.1	0.10±0.01
Soy flour	1.2±0.1	0.30±0.01
LSD_{05}	0.20	0.01

The possibility of successful stabilization of infectious material is formed at the very stage of cultivation [23, 24]. Therefore, the main selection criterion at the third stage of optimization of the nutrient medium composition was the stability of the mycelium obtained in different nutrient media to drying. When drying, the loss of propagules viability was substantial. The greatest stability to drying was shown by the mycelium obtained on the sucrose-soy nutrient medium, which was chosen for further optimization (Table 3). The obtained data on the low stability of the *S. cirsii* C-163 mycelium to drying are consistent with those published [3, 8]. In cases when the potential mycoherbicide forms sclerotia, they, taking into account greater thermal tolerance of this life-form, are used as the infectious material [25-27]. Phomoid micromycetes do not have such ability. Softer drying conditions are provided when receiving pesto- and alginate granules. Their use is considered promising in the mycoherbicides development on the basis of the mycelium of phomoid pathogens, since this allows reducing losses during drying, and the composition of such formulations may include additional active ingredients that improve effectiveness in the field [28-30].

At the fourth stage of nutrient medium optimization, the influence of the duration of fungus cultivation on the pathogenic properties of the mycelium and its yield was evaluated (Fig. 1). The maximum yield of mycelium (about 36 g/l)

corresponded to the beginning of the stationary phase of fungus growth and fell on days 4-5 of cultivation. At the same time, the 3-day mycelium showed the greatest activity against the thistle, which corresponds to the middle of the exponential phase of the fungus growth, characterized by the most active metabolic processes.

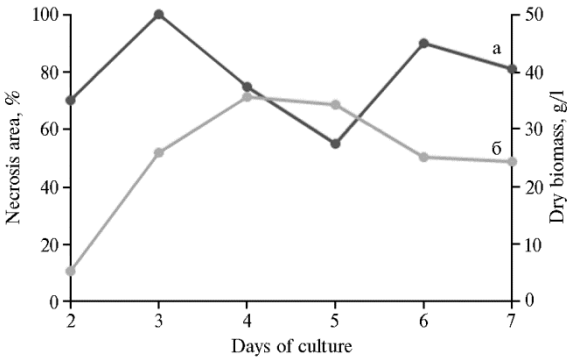

Fig. 1. Aggressiveness (a; LSD_{05} = 8.0) **and the yield of mycelium** (b; LSD_{05} = 0.9) **of *Stagonospora cirsii* C-163 depending on the time of cultivation on the sucrose-soy medium.**

The second peak of biological activity (day 6) was in the stationary phase of growth when the formation and accumulation of secondary metabolites occur usually. It is known that *S. cirsii* at stationary cultivation on the liquid nutrient Czapek medium produces stagonolide-similar toxins exhibiting phytotoxicity [31]. Substrate necrotization may accelerate the development of the disease, so the second peak of biological activity is associated with the beginning of toxin production [32].

Since the reduction of fermentation time becomes an important technological advantage in the deep liquid-phase cultivation, the concentrations of soy flour and sucrose for a 3-day mycelium were optimized in further work. The analysis of variance of the experimental data showed in all cases a statistically significant ($p < 0.001$) joint and individual influence of the concentrations of carbon and nitrogen sources in the liquid nutrient medium on the pathogenicity and mycelium yield. This result is fully consistent with the literature data on the influence of concentration and ratio of these components of the nutrient medium on the yield and pathogenicity of different types of infectious material, such as conidia of *Colletotrichum coccoides* [21], conidia, microsclerotia, and mycelium of *C. truncatum* [24, 27].

The highest yield of dry biomass (36 g/l) was at a concentration of sucrose 60 g/l and soy flour 15 g/l. A further increase in the soy flour amount reduced the yield of mycelium. Evidently, it was due to a decrease in the aeration of the nutrient medium due to an increase in its density. When treating the leaf disks with an aqueous suspension of *S. cirsii* C-163 mycelium (25 mg/ml), the maximum area of the necrosis was observed at sucrose concentration of 30 g/l and above and at a concentration of soy flour 12.5-17.5 g/l. The decrease in pathogenicity at high concentrations of soy flour was also associated with a decrease in aeration, leading to premature aging and degradation of the mycelium. As it can be seen from the graphs (Fig. 2), the range of the maximum values of the mycelium yield of the fungus lies within the range of the maximum values of the necrosis area. Therefore, the optimal concentrations of carbon and nitrogen were chosen according to the maximum yield of the *S. cirsii* biomass. The optimized sucrose-soy medium (pH 6.0) had the following composition: soy flour (15 g/l), sucrose (60 g/l), yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). It is necessary to note that with an increase in the degree of aeration, the concentrations of sucrose and soy flour optimal for the greatest yield of aggressive mycelium may change and require correction [16]. The yield of dry mycelium highly aggressive against Canadian thistle on the optimized sucrose-soy medium for 3 days was 36 g/l. The yield of mycelium did not exceed 3 g/l on the initial Czapek medium with yeast extract.

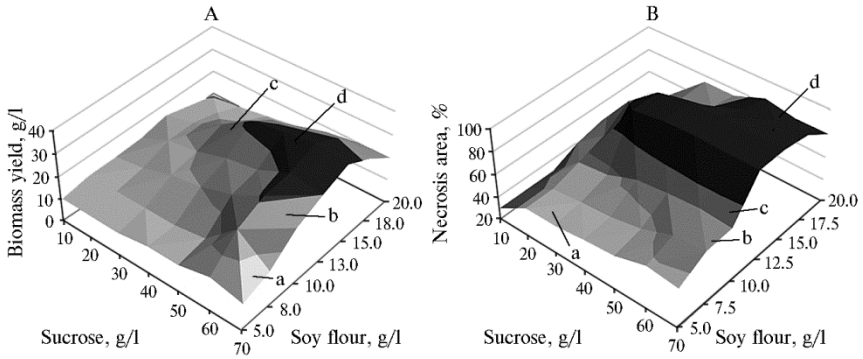

Fig. 2. Yield (A) **and aggressiveness** (B) of ***Stagonospora cirsii*** **mycelium depending on the concentration of sucrose and soy flour in the medium:** a — 0-10, b — 10-20, c — 20-30, d — 30-40 g/l, $LSD_{0.05}$ = 1.5 (A); a — 20-40, b — 40-60, c — 60-80, d — 80-100 g/l, $LSD_{0.05}$ = 7.0 (B).

Thus, optimization of parameters of liquid-phase deep fermentation provides more than 10-fold increase in the yield of virulent mycelium *Stagonospora cirsii* C-163 (to levels comparable with the accepted in the biotechnological practice). The duration of cultivation and the organic nature of nitrogen in the medium significantly affect *S. cirsii* mycelium properties. The proposed high-tech method for obtaining an infectious mycelium that retains viability during drying can be used in the manufacture of a biological preparation against Canadian thistle.

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SEARCH FOR NATURAL ISOLATES OF *Bacillus thuringiensis* FOR DEVELOPMENT OF ECOLOGICALLY FRIENDLY BIOLOGICALS

S.D. GRISHECHKINA, V.P. ERMOLOVA, T.A. ROMANOVA, A.A. NIZHNIKOV

All-Russian Research Institute for Agricultural Microbiology, Federal Agency for Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail: svetagrishechkina@mail.ru (✉ corresponding author), Ermolovavalya1940@mail.ru, tat55176969@yandex.ru, ant.nizhnicov@gmail.com
ORCID:
Grishechkina S.D. orcid.org/0000-0002-4877-705X
Ermolova V.P. orcid.org/0000-0002-9473-8334
Romanova T.A. orcid.org/0000-0003-1773-2956
Nizhnikov A.A. orcid.org/0000-0002-8338-3494
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Abstract

Intensification of agriculture determined the use of biopesticides for plant protection against harmful insects and phytopathogens. Currently, the biological preparations derived from the entomopathogenic bacterium *Bacillus thuringiensis* are of considerable interest since they have high specificity of action, safety for humans, warm-blooded animals, beneficial insects and the environment. High adaptive capacity of *B. thuringiensis* underlies its widespread distribution in nature. This paper represents the results of the screening for novel *B. thuringiensis* isolates from the natural substrates in the Leningrad region. There were 30 samples of substrates collected including soil, potato leaves, sick and dead insects. These samples were colony-purified on the fish agar medium. Based on morphology, 86 candidate colonies were selected from 3 500 colonies analyzed. Aniline Black staining coupled with optical microscopy demonstrated that 12 out of 86 isolates formed crystalline endotoxins of different shapes along with the spores. The isolated microorganisms were selected by their entomocidal activity and identified. As a result of this analysis, isolated bacteria were identified as *B. thuringiensis* and divided onto the two serovars: H_1 (var. *thuringiensis,* isolates 5, 17, 28, 46, 82) and H_{10} (var. *darmstadiensis*, isolates 12, 15, 32, 35, 39, 48, 56). According to their biological characteristics (the formation of acetylmethylcarbinol, lecithinase, pigment, beta-exotoxin, film formation on meat-peptone broth, the utilization of sucrose, mannose, cellobiose, salicin, starch digestion as well as proteolytic activity), the analyzed isolates were similar to the reference strains. The titers of the isolates of the BtH_1 and BtH_{10} serovars varied within limits of 1.3×10^9-2.5×10^9 and 1.5×10^9-2.4×10^9 CFU/ml, respectively. Isolates 17 BtH_1 and 56 BtH_{10} were close to the reference strains by the titer and slightly decreased in the exotoxin content. After selection, the titer and the exotoxin content of the 17 BtH_1 and 56 BtH_{10} isolates increased 1.32- and 1.50-fold, as well as 1.52- and 1.70-fold, respectively. In addition, the isolates referred to the BtH_{10} serovars exhibited polyfunctional activity.

Keywords: *Bacillus thuringiensis*, identification, phytophagous insects, phytopathogens, plant protection, polyfunctional biologicals

In environmentally friendly farming systems, a special role is given to phytoprotective products based on microorganisms. Biopesticides are about 1% of the world market of plant protection products. Russia accounts for 0.25% of the global volume of biotechnological products for plant protection [1].

A special place among the microorganisms that serve as the basis of biopreparations is occupied by entomopathogenic bacteria *Bacillus thuringiensis* (Bt) [2, 3] (the market share of biopesticides is about 90-95%). In practice, Bt is used as an entomocidal [4, 5], antifungal [6-8] and growth-stimulating agent [9, 10]. Bt is characterized by workability, a wide and selective action spectrum, safety for non-target insects, warm-blooded animals, and humans [11, 12]. Bacteria

form spores, parasporal crystalline endotoxin, and, some of them, thermostable exotoxin. Such properties were the reason to use *B. thuringiensis* as a basis for environmentally friendly entomopathogenic preparations and alternative to synthetic chemical pesticides.

B. thuringiensis representatives are widespread in nature due to the ability to effectively adapt to various extreme conditions. These bacteria are isolated from soil samples, sick and dead insects. More than 70 species, effective against phytophagans from the orders of *Lepidoptera*, *Coleoptera*, *Diptera* and *Hymenoptera*, were identified. After the treatment of plants, Bt bacteria remain viable for a long time [13, 14].

High biological effectiveness of Bt is associated with antifeedant, teratogenic, and dereproductive properties. Pathovar A is active mainly against lepidopterans (*Lepidoptera*), B against nymphs of bloodsucking mosquitoes and midges, as well as herbivorous mosquitoes (*Diptera*), and C against coleopterans (*Coleoptera*) [15].

Bacteria of the *Bacillus* genus exhibit poly-enzymatic properties (due to the presence of hydrolases, they are active simultaneously against harmful insects and phytopathogenic fungi), as well as form antibiotics and toxins [16], among which the protein δ-endotoxin is the most important. In the intestine with alkaline pH, δ-endotoxin becomes protoxin, which is hydrolyzed by serine proteases with the formation of a true toxin (according to modern nomenclature Cry-toxin).

Thermostable β-exotoxin of nucleotide nature produced by the bacterium into the external environment and acting through the insect covers also plays an important role in the suppression of insect population, which expands the scope of Bt application. Exotoxin-containing preparations are recommended against the Colorado beetle (*Leptinotarsa decemlineata* Say) and red spider (*Tetranychus urticae* Koch) [5].

Due to the presence of crystals of endotoxin, exotoxin A, phospholipase C, and the endospores, Bt exhibits its entomotoxic, entomopathogenic and metatoxic effects. Bacteria, penetrating into the body of insects, cause diseases that are accompanied by septicemia. The parasite is transferred in large amounts in hemolymph, enters the epithelium of the intestine, where reproduces intensively and causes the death of insects [2].

The antifungal activity of Bt is associated with the production and release of protease and chitinase, which lyse the cell walls of phytopathogenic fungi [17], and the content of the fungus hyphae becomes a source of nutrition and energy for *B. thuringiensis*. Lipopeptide cyclic antibiotics, which have received a lot of attention in recent years, are also responsible for the antagonistic effect. In addition, *B. thuringiensis* can produce antibiotics of polypeptide and aminoglycoside families, capable of suppressing the growth and development of harmful organisms [18].

A combined action of bacteria of the *Bacillus* genus (their strains are able to produce from 50 to 200 biologically active substances) creates the basis for the effective reduction of the number of harmful organisms. However, the range of biological pesticides is much smaller than that of the chemical ones.

For the development and production of biological products, active initial strains are required, which are searched in natural sources according to the criteria of workability, activity, and action spectrum. An important role here belongs to specialized banks of biological agents (collections of microorganisms).

In this investigation, the isolates BtH_1 No. 17 and ByH_{10} No. 56, which can be used in the creation of multifunctional biological pesticides, were obtained for the first time.

The work objective was to isolate from natural substrates, identify, and

select bacteria of *Bacillus thuringiensis* – promising producers of biopreparations against pests and plant diseases.

Techniques. Collection of 30 samples from various substrates (soil, potato vine, nymphs and imagoes of Colorado beetle) was carried out in the Leningrad Region in potato fields.

To isolate microorganisms, 1 g of soil, leaf blade, or insects (sick and dead ones), flamed over an alcoholic lamp flame and mashed in a mortar with a sterile pestle, were placed in a tube with 5 ml of sterile physiological solution and shaken for 1 min. One drop of the received suspension was added in Petri dishes on fish agar (FA) by the dwindling smear method. The dishes were incubated in the thermostat at 28 °C. After 7 days, the morphological type of colonies was investigated, and the formation of δ-endotoxin crystals and endospores was registered under the microscope (Laboval 4 microscope, "Carl Zeiss Jena", Germany, magnification ×900). Colonies selected by morphological features typical for Bt were reinoculated in lines on FA in Petri dishes, divided into 8 sectors for convenience.

At the same stage, the smears stained according to Smirnov [19] with the use of black aniline dye were prepared and examined.

Preliminary screening of isolates was carried out on the basis of entomocidal properties, identification – according to schemes for *B. thuringiensis* by De Barjac and Bonnefoi [20] and Lysenko [21].

During the study of the biochemical properties of microorganisms, instead of liquid differential diagnostic media, the systems of indicating paper (RSI) disks (SPA Microgen MHRF, Russia) were used with a certain amount of substrate in combination with the corresponding indicator, stabilized with the film-forming coating (polyvinyl alcohol). When determining the ability to use carbohydrates in 0.3 ml of sterile 0.85% NaCl solution (pH 7.3±0.1), the content of one microbiological loop of the daily agar culture grown at the temperature of 29±1 °C were suspended and a disk with the corresponding carbohydrate was immersed in it (control – disks immersed in a sterile 0.85% NaCl solution). The accounting was carried out in 5-18 hours. In the same way, with the use of RSI, the indole formation and urease activity, the absorption of hydrogen sulfide, the formation of acetylaminophenol (AMC) were evaluated.

The productivity of isolates was determined on yeast-polysaccharide media when grown in depth in Erlenmeyer flasks in the shake-flask propagator with aeration (220 rpm) for 72 h at 29 °C up to the formation of endospores and crystalline endotoxin. The titer of cells was taken into account by the conventional method of serial dilutions with inoculation on FA.

Entomocidal activity expressed as LC_{50} for nymphs of the Colorado beetle (*Leptinotarsa decemlineata* Say) of the natural population, as well as the content of the exotoxin as LC_{50} for nymphs of the housefly (*Musca domestica* Linnaeus), was determined according to the description [22].

Antifungal activity was assessed by the agar block method [23]. To do this, the testing cultures of fungi (*Botrytis cinerea, Fusarium oxysporum*) were grown on potato agar in Petri dishes with entire lawn for 7 days. The culture of Bt isolates with the appropriate titer in an amount of 10% of the volume of the medium was introduced in the melted and cooled up to 40 °C potato agar, stirred and poured into Petri dishes. The blocks (1 cm diameter) cut from a 7-day lawn test culture of the fungus were placed on the surface of the solidified agar with Bt. The control was in the medium without Bt. The dishes were placed in a thermostat at 28 °C. After 7 days, the diameter of the fungus colonies was measured. The percentage of growth inhibition of the fungus colonies was calculated with the formula by Abbot [24] as $(D_c - D_e) \cdot D_c^{-1} \cdot 100$, where D_c and D_e are the diameters of the fungus colonies in the control and experiment, respectively, cm.

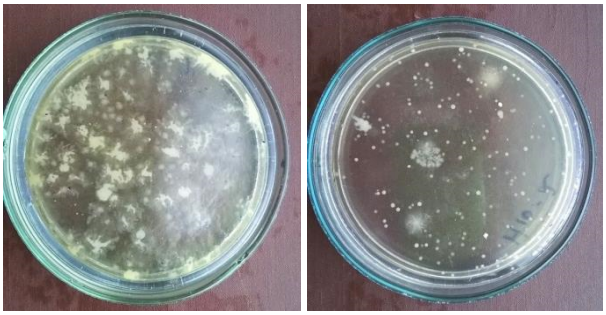
Bt selection was carried out in several stages: inoculation of culture on FA by the dwindling smear method, selection of typical Bt colonies (grayish-white color, rounded or odd-shaped, with a little rough surface), microscopic analysis of culture according to δ-endotoxin formation and endospores in the ratio 1:1, assessment of workability in the cultivation with the in-depth method and on the content of exotoxin in LC_{50}, μl/g of feed for nymphs of *Musca domestica*.

The obtained data were processed with the use of the conventional method of variance analysis [25]. The mean values (*M*) and standard errors of means (±SEM) were calculated. The statistical significance of differences was assessed according to the Student *t*-criterion at 95% confidence interval ($p < 0.05$).

Results. Thirty samples were obtained from different substrates and examined for microflora. Inoculations were carried out on fish agar (FA). Among more than 3,500 colonies analyzed according to their morphology, color, shape, and texture, 86 isolates of the *Bacillus* genus were selected. After 7 days of cultures inoculation at 26-28 °C, the sporangia lysis and release of endospores and endotoxin crystals occurs. When inoculating, grey or cream flat matte colonies with irregular contours were formed, soft, fine-grained in consistency. According to morphological and cultural characters (Table 1), 12 isolates related to BtH_1 and BtH_{10} were selected.

1. Morphological and cultural characters of the *Bacillus thuringiensis* ssp. isolates, separated from natural substrates in the Leningrad Province

Character	BtH_1	BtH_{10}
Gram stainong	Gram-positive	Gram-positive
Shape and size of vegetative cells	Straight or slightly tortuous rods of 2.5-3.0×0.8-0.9 μm	Rod cells of 1.2-1.5×2.5-4.5 μm
Mobility	Peritrichous flagellation	Peritrichous flagellation
Cell connection	Short chains	Single or short chains
Endospores formation	Subterminal	Subterminal
Shape and size of endospores	Oval 0.8-0.9×1.1-1.3 μm	Oval 0.8-1.4 μm
Shape and size of crystalline protein endotoxin	Regular rhombic with clear lines of 1.2-4.5×0.5-2.3 μm	Tetragonal bipyramid or ovoid of 0.2-0.6×0.3-0.8 μm
Diameter	0.8-1.5 cm	0.4-0.8 cm
Surface	Little rough	Rough
Profile	Flat	Flat
Optical properties	Mat	Opalescent, mat
Colonies color	Gray-white	Off-white
Substrate color	Not change	Not change
Line	Irregular	Irregular
Structure	Fine-grained	Fine-grained
Consistency	Soft	Soft



Colonies of isolates *B. thuringiensis* H_1 (on the left) and *B. thuringiensis* H_{10} (on the right) on fish agar.

Colonies appearance on fish agar in isolates differed slightly (Fig.). Microscopy showed that they form endotoxin crystals of different shapes along with endospores. In the study of the main physiological and biochemical features, it was found (Table 2) that among the 12 selected isolates 5 belong to *Bacillus thuringiensis* var. *thuringiensis* (BtH_1) and 7 were *Bacillus thuringiensis* var. *darmstadiensis* (BtH_{10}). As nitrogen sources pepton, meat and fish broth, protein feed yeast, cleaved glucose, mannose, levulose, maltose, cellobiose were used; galactose, arabinose, xylose, rhamnose, lactose, raffinose, mannitol, dulcite,

sorbitol, inulin, inosite were not used as nitrogen sources. These isolates liquefied gelatin, peptonized milk, hydrolyzed starch, used citrates, formed acetylmethylcarbinol. Pigment and urease formation has not been observed. They did not use indole and hydrogen sulfide, reduced the nitrate to nitrite. BtH_1 isolates, unlike isolates BtH_{10}, formed lecithinase, cleaned sucrose, salicin, glycerol. In other aspects, they were similar in biochemical features (see Table 2).

2. The main physiological and biochemical features of the BtH_1 and BtH_{10} isolates, separated from natural substrates in the Leningrad Province

Isolate No.	1	2	3	4	5	6	7	8	9	10	11
5	+	+	–	+	+	+	+	+	+	+	+
17	+	+	–	+	+	+	+	+	+	+	+
28	+	+	–	+	+	+	+	+	+	+	+
46	+	+	–	+	+	+	+	+	+	+	+
82	+	+	–	+	+	+	+	+	+	+	+
BtH_1 (standard)	+	+	–	+	+	+	+	+	+	+	+
12	+	–	–	+	+	–	+	–	–	+	+
15	+	–	–	+	+	–	+	–	–	+	+
32	+	–	–	+	+	–	+	–	–	+	+
35	+	–	–	+	+	–	+	–	–	+	+
39	+	–	–	+	+	–	+	–	–	+	+
48	+	–	–	+	+	–	+	–	–	+	+
56	+	–	–	+	+	–	+	+	–	+	+
BtH_{10} (standard)	+	–	–	+	+	–	+	+	–	+	+

N o t e. "+" and "–" — manifestation or absence of a trait; 1, 2, 3, 4, 5 — formation of acetylmethylcarbinol, lecithinase, pigment, exotoxin, film on meat-peptone broth (MPB), respectively; 6, 7, 8, 9 — use of sucrose, mannose, glycerin, salicin, respectively; 10 — starch cleavage; 11 — meat-peptone gelatin proteolysis, respectively.

The selected isolates were compared by workability, exotoxin formation, and antifungal activity (Table 3).

3. Biological characteristic of the BtH_1 and BtH_{10} isolates, separated from natural substrates in the Leningrad Province ($M \pm$SEM)

Isolate No.	The titer of endospores in culture fluid, $\times 10^9$/ml	The content of the exotoxin, LC_{50}, μl/g of feed for *Musca domestica*	Colony growth inhibition, %	
			Botrytis cinerea	*Fusarium oxysporum*
5	2,0±0,3	6,7±0,2	13,9±1,2	11,2±0,9
17	2,5±0,2	5,0±0,2	18,2±1,9	13,4±1,1
28	1,7±0,2	7.6±0,3	15,3±1,8	12,4±1,2
46	1,3±0,4	8,4±0,2	11,8±0,9	9,7±1,5
82	1,7±0,3	8,1±0,1	12,7±1,1	8,2±0,8
BtH_1 (standard)	2,5±0,2	4,1±0,2	18,8±1,4	12,1±1,2
12	1.4±0,1	6,9±0,2	50,5±1,9	37,4±1,4
15	2,3±0,3	6,1±0,1	61,9±1,8	57,4±1,7
32	1,7±0,2	7,5±0,3	52,1±2,0	40,9±1,6
35	1,9±0,1	7,8±0,2	48,4±1,6	38,1±1,3
39	1,5±0,1	8,1±0,2	58,4±1,8	42,3±0,9
48	1,9±0,2	6,3±0,1	60,3±2,1	52,1±1,6
56	2,4±0,2	4,9±0,2	72,3±1,7	65,3±1,2
BtH_{10} (standard)	2,6±0,1	3,9±0,2	73.3±1,9	66,1±2,0

N o t e. The data were processed by the method of variance analysis at 95% confidence interval.

Isolated strains of BT in addition to insecticidal activity had antifungal action, but in the strains of BtH_1, it was significantly lower than in BtH_{10}. According to the authors' data [15, 17], the mechanism of antifungal action of Bt is associated with several factors. Bacteria produce and release lytic enzymes into the environment, in particular, protease and chitinase, which lyse the cell walls of phytopathogenic fungi. When lysing, the content of the fungus hyphae becomes a source of nutrition and energy for bacilli [26].

The analysis of the parameters important for production made it possible to select strains BtH_1 No. 17 and BtH_{10} No. 56 that demonstrated the best results. For example, according to workability and the content of exotoxin, the strain BtH_1 No. 17 was superior to the other by 1.25-1.90 and 1.34-1.68 times, respectively, the strain BtH_{10} No. 56 – by 1.23-1.70 and 1.28-1.65 times. To im-

prove these characteristics, the selection of isolates BtH_1 No. 17 and BtH_{10} No. 56 was held. After 7 days of growth and inoculation with the dwindling smear method, from 50 colonies of each isolate, 25 variants with the formation of crystalline endotoxin and endospores in a ratio of 1:1 were selected by microscopy. Then, during in-depth cultivation, 10 variants with a titer of not less than 3×10^9 CFU/ml have been selected and evaluated on the exotoxin content and insecticidal activity [22]. The isolate BtH_1 No. 17 titer of endospores ($\times10^9$/ml of culture liquid) as a result was 3.3±0.2 against the initial 2.5±0.2 (by 32.0% more workable), the content of exotoxin (LC_{50}, μl/g of feed for *Musca domestica*) was 3.8±0.2 against 5.0±0.2 (by 24.0% more); for BtH_{10} No. 56, these indicators were 3.8±0.2 against 2.4±0.2 (by 58.3% more workable) and 2.9±0.2 against 4.9±0.2 (by 40.8% more) respectively (differences are statistically significant at $p < 0.05$).

The entomocidal activity of isolates BtH_1 No. 17 and BtH_{10} No. 56, expressed in LC_{50} for nymphs of Colorado beetle of the second age, was high – 0.26 and 0.24%, respectively (in determining the culture Bt liquid in concentrations 5; 1; 0.25% was used; control – water).

In conclusion, it is necessary to note that the study of the potential use and mechanisms of action of Bt strains, the search and description of the properties of newly separated isolates as possible agents in the biocontrol of pests and plant diseases remain extremely relevant [27-29]. The obtained results indicate the prospects of strains BtH_1 No. 17 and BtH_{10} No. 56 as producers of biopreparations, and BtH_{10} No. 56 has both entomocidal and antifungal activity, that is, it is polyfunctional (the claim for an invention of this strain has been applied). These data are consistent with the results of previously performed studies of the polyfunctional activity of the bacteria *B. thuringiensis*, which, along with bioinsecticide properties, has antifungal and growth stimulating effects [26].

Thus, the investigation confirms once again that entomopathogenic crystal-forming bacilli of *Bacillus thuringiensis* can be found in soil, leaves, infected and dead insects, and in insect habitats. Identification and biotesting showed that the separated isolates related to the serovars *Bacillus thuringiensis* var. *thuringiensis* and *B. thuringiensis* var. *darmstadiensis* by their biological properties and practical significance are close to typical strains. It is obvious that with the help of analytical selection, selection of nutrient media and cultivation regimes, it is possible to enhance the valuable properties of these isolates practically and successfully use them as producers of biopreparations with entomocidal and antifungal properties to control the number of harmful insects and phytopathogens. Isolates with multiple activity related to serovar BtH_{10} are of special interest. Microbial preparations of multifunctional action can compete with chemical pesticides not only in terms of environmental safety but also in economic terms, taking into account the protective effect against pests and diseases.

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NATURAL REPRODUCTION OF ENTOMOPHAGES TO RESTORE BIOCENOTIC REGULATION IN CEREAL CROPS

Zh.A. SHIRINYAN, M.V. PUSHNYA, E.Yu. RODIONOVA, E.G. SNESAREVA, V.Ya. ISMAILOV

All-Russian Research Institute of Biological Plant Protection, Federal Agency of Scientific Organizations, 14, ul. Vavilova, Krasnodar, 350039 Russia, e-mail mar.pushnya2014@yandex.ru (✉ corresponding author), rigaey@gmail.com, greas23@yandex.ru, vlyaism@yandex.ru
ORCID:
Shirinyan Zh.A. orcid.org/0000-0002-7198-2350
Pushnya M.V. orcid.org/0000-0002-7133-9533
Rodionova E.Yu. orcid.org/0000-0001-5631-2204
Snesareva E.G. orcid.org/0000-0003-4617-3604
Ismailov V.Ya. orcid.org/0000-0002-6713-0059
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Abstract

Recently, plant protection methods based on pest regulation by entomophages, naturally inhabiting the crops or attracted thereto, become more preferable. This relevant approach to agro landscape construction necessitates seeking for ways to activate natural cenotic mechanisms, e.g. via optimized crop rotation, created micro reservoirs of desirable species, botanical diversity of protective forest belts, roadsides and other natural plant communities etc. Telenomines (*Hymenoptera: Platygastroidae: Scelionida, Telenominae* subfamily), the egg parasites of *E. integriceps*, are a powerful regulator of sunn pest *Eurygaster integriceps* Put. abundance. These entomophages are of particular interest worldwide as they are the most numerous not only on bread wheats but also on tilled crops. For a long time, researchers from Russia and other wheat producing countries, where *E. integriceps* pests pose a threat to crops, were concentrated on the laboratory reproduction of telenomines for field use. However, exploitation of naturally reproduced telenomines has not yet been studied. This paper is the first to report on use of entomophilic fennel strip plots with arranged kairomone-based feeding places as baits for *Graphosoma lineatum* L., the alternative hosts of telenomines, to further attract and massively reproduce these egg parasites of *E. integriceps* in field conditions. This technology is based on a long time study (2006-2018) of factors affecting reproduction of telenomines in cereal cenoses. Our data indicate the key role of winter generation of these egg parasites, which reproduce successfully during the second half of the season last year, in the harmful bug control. Additional hosts, the bugs of *Pentatomidae* family among which the Italian striped bug *G. lineatum* is the most frequent (7.20±1.20 insects per square meter), provide a significant increase in count of wintering telenomines. Sunflower and soybean agrocenoses with bait strip plots of entomophilic fennel (coriander) are optimal to provide reproduction of telenomines. In the kairomone-based feeding places, telenomines laid their eggs into 52.1±2.2 to 84.9±3.2 % eggs of *G. lineatum*. Due to use of this technology during 2008-2010, farther use of kairomone-based feeding places for accumulation of telenomines was not needed. Currently, the entomophages actively reproduce due to crop rotations with up to 40 % cultivated plants favorable for *Scelionidae* species. In experimental wheat crops of All-Russian Research Institute of Biological Plant Protection, in 2011-2017 from 53.1±2.7 до 88.2±2.0 % eggs of *E. integriceps* contained eggs of telenomines. This allowed for cancellation of other protective measures against the bug's larvae.

Keywords: phytophagous insects, *Eurygaster integriceps* Put., sunn pest, *Graphosoma lineatum* L., shield bugs, entomophages, egg parasites, telenomines, reproduction, natural habitats, spatial patterns, grain crops, tilled crops, kairomone-based feeding places, biocenotic regulation, efficiency

Ecologically oriented agriculture involves optimization of phytosanitary measures based on the predominant substitution of synthetic plant protection products [1]. Avoiding chemical pesticides provides the maximum use of self-regulation mechanisms in cenoses to reduce the number of pests [2-4]. Natural aboriginal entomophages are one of the biotic factors determining the stability of biocenotic regulation of phytophage populations in the biological protection system [5-9].

Wheat is the leading cereal in many countries, including Russia. Winter

wheat crops occupy more than 30% of arable land (up to 1.6 million hectares) annually in the south-west of the North Caucasus in the conditions of the Krasnodar Territory. There are up to 30-40 species of phytophages in the agrocenosis of the wheat field in the Kuban region, including 18 specialized ones, to 7-8 species of which chemical treatments are applied annually [3, 7-9]. The most dangerous threat is the corn bug (*Eurygaster integriceps* Put.) that reduces the grain quality; the greatest amount of pesticides accounts for the treatments against this pest [10-13]. The corn bug has a wide geographical range: it is common in the steppe zone in the south of the forest-steppe of Russia and Ukraine, in the Caucasus, in Albania, Greece, Bulgaria, Romania, Turkey, Syria, Lebanon, Iran, Iraq, Afghanistan, and Pakistan [14].

The most effective entomophages of *E. integriceps* Put. include egg-eating parasites from the *Scelionidae* family [3, 4, 7, 8, 15]. In the general geographical range of the bug, including the areas close to the Russian Caucasus in the south, e.g. in Turkey, Iran, Iraq [16-20], as well as in the region of wheat cultivation located to the north, in the Volga Region [21], about 20 species of telenomines are described, mainly from the *Trissolcus* and *Telenomus* genera. In some years, they are able to infect up to 80% of the pest egg-laying, but the mechanisms regulating the increase in activity and the natural reproduction of this group of parasites are not well studied [7, 8, 22]. In Russia and other countries, technologies for mass cultivation of telenomines under laboratory conditions have been developed for many decades, both using host insects, mainly on the striped bug *Graphosoma lineatum* L., and using artificial nutrient media (ANM) [23-25]. However, this method is labor-consuming and expensive, since entomophages develop on ANM for 3-5 days longer than on the insect host [23].

In the USSR, as early as 1940-1975, on the basis of a long-term analysis of seasonal colonization of local and introduced species of the egg-eaters of the *Trissolcus* genus (*Tr. grandis* Thoms., *Tr. simoni* Mayr and others), attempts were undertaken to introduce large-scale practical use of these entomophages for controlling phytophages, which, however, did not give a positive result [25]. The authors' long-term studies [7, 26] showed that the cause was the death of laboratory populations of *Trissolcus* in early spring due to their inherent increased xero-and thermophilicity.

An essential role in maintaining the potential of the telenomines of the *Scelionidae* family is played by their additional hosts, the stink bugs of the *Pentatomidae* family, which ensure the survival of telenomines in natural conditions before and after the eggs are laid by the corn bug [6, 7, 19]. According to different sources, these are mainly representatives of *Hemiptera* family, the *Pentatomidae* (20-30 species). The known domestic and foreign publications indicate only the species composition of these *Hemiptera* groups whereas their role in ensuring the maximum effectiveness of telenomines remains insufficiently studied [26-29].

This study for the first time determines that the key role in ensuring the reproduction of overwintering stock of egg-eating telenomines, which are the main regulators of the corn bug abundance in the North Caucasus, is played by their additional hosts, bugs of the *Pentatomidae* family, which are constantly present in the agrocenosis of agricultural crops.

The purpose of the paper was to identify the factors that determine the increase in the activity and reproduction of egg-eating telenomines in the cenosis of grain crops, taking into account the features of the stationary distribution and the reproduction rate of their additional hosts, the *Pentatomidae* bugs.

Techniques. Observations (2006-2018) were carried out with respect to the most common species of stink bugs, the sloe bug (*Dolycoris baccarum* L.), the striped bug (*G. lineatum* L.), the pointed head bug *Aelia acuminata* L., *Car-*

pocoris fuscispinus Boh. and rape bug (*Eurydema oleracea* L.), which ensure the preservation and accumulation of the aboriginal populations of the corn bug (*E. integriceps* Put.) egg parasites, as well as the dominant species of the corn bug egg-eating telenomines, the *Telenomus chloropus* Thoms., *Tr. grandis* and *Tr. simoni* regulating the number of pests. Surveyed plots were located in the Central zone of the Krasnodar Territory (experimental crop rotation of the All-Russian Research Institute of Biological Plant Protection, RIBPP, a total area of 247 hectares, 2006-2017); OAO Chistaya yeda (Krymsky District, Krasnodar Territory, 200 hectares, 2011-2013); farms of the south of the Rostov Province (300 hectares, 2014-2015). The soil type of the Central zone of the Krasnodar Territory is southern leached chernozem, the weather conditions during the years of observation were mostly typical for the region: the average monthly temperature was 21.7 °C, the air humidity was 66.9% with the warm humid weather in July (the hydrothermal coefficient (HTC) was 0.9-2.2), dry hot weather in August (HTC 0.01-0.10) with severe drought in July-August (HTC 0.04-0.20) in 2007 and 2010. The zone is characterized by the presence of a network of field-protective belts. The southern part of the Rostov region belongs to the steppe zone, the soil is ordinary black chernozem, weather conditions typical for the region: the average monthly temperature is 19.6 °C, the air humidity is 69.9%.

In field experiments, cages-insulators (aluminum frame 20-25 cm in diameter and 13-15 cm in height with stretched mesh material, which allows free access of telenomines) were used as kairomone feeding pads (KFP). Insulators were secured at the top with rubber rings for limiting the movement of the bugs. One drinking trough with a moistened cotton swab and feeders with dill seeds or wheat germ were placed at the bottom of each KFP. The number, species composition and reservation sites of the corn bug egg parasites were determined using KFP. Males and females of *G. lineatum* striped bug and *E. integriceps* corn bug from natural populations were used as a live source of kairomone and ovipositors. *G. lineatum* specimens were selected on special small (up to 50 m^2) plots of dill (*Anethum graveolens* L.) or coriander (*Coriandrum sativum* L.), and the corn bug was selected on wheat crops.

To account the field number of adults and bugs larvae, their species composition and reservation sites, mowing was carried out using a standard entomological net with a diameter of 30 cm [30]. The infestation of bug eggs with telenomines and the species composition of the latter were studied during individual and mass laboratory breeding of parasites using phytophage ovipositors collected in the field [30]. The concentration sites, the species composition and the number of egg parasites of the corn bug were determined using KFP, which ensured the attraction of telenomines.

The taxonomic identity of telenomines was identified by the determinant of the Zoological Institute of the Russian Academy of Sciences (St. Petersburg) [31] by viewing under an MBS-10 binocular microscope (magnification ×8, OAO LZOS, Russia). The insects were kept in MLR 35 OH artificial climate chambers (Panasonic, Japan).

The number of stink bugs of the *Pentatomidae* family was counted monthly. The obtained results were processed according to generally accepted methods of statistical analysis using the Statistica v.12.6 software (StatSoft, Inc., USA) [32]. The tables show the mean (M) and standard error of the mean (±SEM).

Results. To attract entomophages of the corn bug, kairomones were used, which are considered as effective agents against this pest [33]. In field experiments to assess the natural distribution, as well as for attraction and reproduction of telenomines, specially designed kairomone feeding pads (KFP) were used.

Kairomone feeding pad used in the experiment for recording, attracting and reproducing egg-eating telenomines of the corn bug: 1 — cage-insulator, 2 — drinking trough with a moistened cotton swab, 3 — feeder with dill seeds.

Long-term landscape biotopical monitoring of the stink bugs on winter wheat crops (2006-2017) showed that bioecologies of the *Pentatomidae* family representatives are generally similar. Insects overwinter in the imago stage under the canopy of trees and shrubs, mainly oak (*Quercus robur* L.), as well as on the fields and roadsides under the remains of grassy plants. At an average daily temperature above 11 °C, the bugs came out of the overwintering areas almost simultaneously with the telenomines (1 month before egg-laying by the corn bug). An exception was the striped shield bug (*G. lineatum*) which left the overwintering at an average daily temperature above 20 °C simultaneously with *E. integriceps*. Development cycles of the corn bug and the striped shield bug coincide, but shield bugs do not live on wheat. After completing feeding on young leaves of oak, acacia (*Caragana arborescens* L.), ash (*Fraxinus excelsior* L.), and blackthorn (*Prunus spinosa* L.), shield bugs mated.

1. Number (ind/m^2) **of *Pentatomidae* family shield bugs in the studied agrobiocenoses** (*M*±SEM, RIBPP, Krasnodar, 2006-2017)

Month	*Carpocoris fuscispinus* Boh.	*Dolycoris baccarum* L.	*Aelia acuminata* L.	*Graphosoma lineatum* L.	*Eurydema oleracea* L
Flowering herbs					
April	1.60±0.30	4.04±1.10	0.09±0.09	0.20±0.08	0.70±0.09
May	1.10±0.50	1.50±0.80	0.90±0.10	0.40±0.03	0.20±0.07
June	0.70±0.40	3.03±1.10	0.40±0.25	0.50±0.07	0.02±0.10
July	–	–	–	0.70±0.16	0.70±0.09
Winter rape					
April	0.02±0.10	0.70±0.15	0.20±0.09	–	1.07±0.70
May	0.10±0.07	0.20±0.10	0.10±0.09	–	1.03±0.65
Winter wheat					
April	0.10±0.08	0.20±0.10	0.30±0.10	–	0.10±0.07
May	0.30±0.11	1.00±0.15	1.20±0.20	–	0.10±0.07
June	0.20±0.09	0.90±0.17	1.50±0.20	–	–
Corn					
July	0.10±0.06	0.80±0.10	–	–	–
Sunflower					
July	0.10±0.08	0.50±0.09	–	–	–
August	–	0.10±0.08	–	–	–
Soy					
July	0.10±0.07	0.06±0.23	–	–	–
Август	0.01±0.08	0.30±0.11	–	–	–
Entomophilous cultures (dill, coriander)					
May	–	–	–	0.60±0.11	–
June	–	0.30±0.11	–	3.30±0.75	–
July	0.10±0.06	0.30±0.12	–	7.20±1.20	–

Note. A dash means that no shield bugs were found on the plants.

During the formation of reproductive products (usually the 2nd decade of April), the bugs in the forest shelter belts were inhabiting wild plants from different botanical families (*Asteraceae*, *Lamiaceae*, *Caryophyllaceae*, *Brassicaceae* and *Poaceae*) eating their generative organs. In these areas, the number of shield bugs was many times greater than that of the cultural station, i.e. in crops of early flowering winter rape (Table 1). Under favorable weather conditions, at the end of April, most of the shield bugs began egg-laying, which passed into the

mass egg-laying in the winter wheat cenosis. Striped shield bugs developed on flowering umbrella plants, rape bug developed on winter rape (the number of *E. oleracea* during all the years of observation was small, therefore infestation of its egg-laying was not determined).

Due to the earlier egg-laying of the shield bugs, which begins 7-10 days earlier than that of the corn bug, there was an increase in the number of egg parasites (5 times or more) and in occupation by their first generation of the wheat crops adjacent to the field-protective plantations, which allowed overwintering telenomines populations to wait for the *E. integriceps* eggs. Thus, asynchrony in the development of parasites and their host was overcome to some extent, which increased the efficiency of *Scelionidae* [7, 26].

Colonization of the winter wheat by the shield bugs began during the stem elongation phase (Z 31-35 by Zadoks scale). Biocenosis of wheat in the Krasnodar Territory is characterized by the 6 species of pentatomids, among which the most common are the pointed head bug (*A. acuminata*), the sloe bug (*D. baccarum*) and *C. fuscispinus*, while the pointed head bug occupied the wheat before the other shield bugs (7 days earlier) and finished the egg-laying later (14 days later) [26]. The development cycles and the nature of harmfulness of the *D. baccarum* and *C. fuscispinus* almost coincide. Shield bugs cause white-spikes and underdeveloped ears, and later the change in physiological properties of the grain [34, 35].

2. Infestation of corn bug (*Eurydema integriceps* Put.) eggs and shield bugs of the *Pentatomidae* family with telenomines of the *Trissolcus* genus on winter wheat crops (*M*±SEM, RIBPP, Krasnodar, 2007-2013)

Wheat development phase (according to Zadoks)	Analyzed eggs, pcs	Infested by parasites	
		total	%
Corn bug			
Z 31-35	140.0±6.7	99.0±2.5	70.7±4.4
Z 50-59	610.0±5.5	521.0±7.5	85.4±3.2
Z 70-75	590.0±4.8	557.0±3.3	94.4±3.6
Z 75-80	260.0±3.2	244.0±5.0	93.8±5.1
Shield bugs (during egg laying by corn bug)			
Z 31-35	70.0±2.6	25.0±1.7	35.7±4.4
Z 50-59	70.0±4.1	28.0±2.2	40.2±2.4
Z 70-75	90.0±3.9	41.0±1.5	45.6±3.7
Z 75-80	250.0±3.1	201.0±7.2	80.4±3.6
Shield bugs (after completing egg laying by corn bug)			
Z 75-80	510.0±6.7	470.0±5.9	92.1±4.8
Z 80-90	710.0±7.8	610.0±6.3	85.9±4.1

Note. Infestation rate is an indicator of EEL (entomophage efficiency level).

The number of *Pentatomidae* bugs in the cenosis of winter wheat during observations did not reach the economic threshold of harmfulness (3.0-3.5 ind/m^2) due to the activity of their natural enemies, the egg-eating *Scelionidae* wasps. Parasites infested up to 80.4±3.6% of shield bugs eggs and 93.8±5.1% of *E. Integriceps* eggs (Table 2). The first egg-laying of both the shield bugs and the corn bug were 80-100% affected by hygrophilic species *T. chloropus* Thorns. After completing of the egg-laying by the corn bug, development of the third generation of egg-eaters (phases of wax and full ripeness of grain, Z 75-90) continued on the eggs of additional hosts, the shield bugs of *Dolycoris*, *Aelia*, *Carpocoris* genera. The infestation rate of their eggs by telenomines in the absence of egg-laying of the main host reached 92.1±4.8%. Such values were recorded during almost the entire observation period (see Table 2).

An important condition for the development of telenomines is the presence of eggs of their additional hosts in other stations [24, 28, 29]. When noting this fact, the authors, however, did not determine which conditions contributed to the preservation of entomophages, in essence, limiting themselves to analyzing

the taxonomic structure of the described groups of insects. Only a few papers report on the infestation of up to 30% of eggs in *Dolycoris* genus [18, 28]. The authors of this paper showed that at the end of the grain crops harvesting, the shield bugs migrated to row crops and plantings with a variety of flowering grassy vegetation in search of food. Sunflower and soybeans during budding—flowering were the most attractive for pentatomids in the indicated timeframes, crops of entomophilous dill (coriander) and, to a lesser extent, melliferous umbrella plants for the shield bugs. The formation of the telenomines overwintering stock is most intense in the biocenoses of these cultures. On average, the number of striped shield bug eggs per KFP ranged from 1170.0±6.5 to 2460.0±8.3, among them 610.0±4.4 to 2090±7.3 were attacked by egg parasites. Greater infestation of egg-laying by telenomines was noted near the crops of entomophilous dill, 2090±7.3 of 2460±8.3 individuals (84.9±3.2%).

3. Infestation of shield bug eggs (*Graphosoma lineatum* L.) by telenomines of the *Trissolcus* genus on the kairomone feeding pads, providing optimal conditions for attracting and reproducing the telenomines (*M*±SEM, RIBPP, Krasnodar, 2006-2010)

Crop	Laid eggs	Infected by telenomines	
		total	%
Sunflower	2440.0±7.2	140.0±3.0	57.4±3.0
Corn	1170.0±6.5	610.0±4.4	52.1±2.2
Soy	1790.0±7.9	1080.0±6.9	60.3±4.2
Dill, coriander	2460.0±8.3	2090.0±7.3	85.0±3.2

N o t e. Infestation rate is an indicator of EEL (entomophage efficiency level).

Reactivation of egg-eaters and their spread to the fields in early spring occurred at a lower temperature (11-12 °C) than the beginning of the egg-laying of the corn bug (20 °C), which led to the death of a significant part of overwintered egg parasites [6, 26]. According to this study, telenomines survival is facilitated by the presence of flowering plants in their habitats during this period (insects eat their nectar), so the highest infection rate of the shield bugs eggs by telenomines on KFP was noted in wheat crops near the flowering wild-growing grass [3, 7, 26]. Therefore, it is very important to keep intact the borders of the forest belts with grassy vegetation as places for wintering, feeding and breeding entomophages and those phytophages that may be used as additional hosts of entomophages [3, 6, 7, 36].

According to the authors' data, the number of *Scelionidae* on cereal crops can be increased by creating their additional reserves when grown in crop rotations of entomophilous dill for the accumulation of the striped shield bugs. To this end, in 2008-2010 the method of mass reproduction of the egg-parasites of the corn bug at the entomophilous dill sites in the field was tested. The originality of the approach used is that synchronous reproduction of egg-eaters and their additional host, *G. lineatum*, occurred on dill. The proposed technology was as follows. The strips of planted entomophilous dill, on which, during the development of the striped shield bug summer generation (July—August) the KFP was installed in a row with 10 m spacing (a KFP per 4 hectares of protected wheat crops), were placed near areas of overwintering and constant reproduction of telenomines and their additional host, namely, near the trees and shrubs, orchards, row-crops plantings (corn, soybean, sunflower). Mass accumulation of telenomines in natural cenoses included i) the production of the insect host, a polyvoltine striped shield bug, by growing entomophilic dill (or coriander), which ensures attraction and accumulation of the bug; ii) mass reproduction of entomophage parasites in KFPs located in the agrocenoses of tilled crops (maize, soybean, sunflower) and in bait plots of dill (coriander) (to preserve and increase the overwintering stock of entomophages); iii) cultivation of nectarifer-

ous crops (phacelia, coriander, fennel) for additional feeding of egg-eaters; iv) testing the ability of breeding parasites to control the pest (the corn bug). It should be noted that that the previous studies [5, 8, 22, 27, 28] do not show the possibility of breeding *Scelionidae* by providing field conditions conducive to reproduction and preservation of their additional hosts. A number of papers propose mass production of entomophages grown in the laboratory, but their efficiency often did not exceed 50% [6, 24].

The intensive use of the developed technology during 2008-2010 allowed abandoning the KFPs for additional accumulation of telenomines. The reproduction of entomophages is carried out by maintaining 7-40% of tilled, entomophilous and nectar-bearing crops in the structure of crop rotation, due to which the populations of aboriginal species of the *Scelionidae* family are activated. During the last 9 years, telenomines infested from 53.1±2.7 to 88.2±2.0% of the corn bug eggs in the experimental crop rotation (All-Russian Research Institute of Biological Plant Protection), which allows excluding all treatments with preparations against the bug larvae in the grain milky phase (such treatments are recommended in case of egg-laying infection below 40-50%) (Table 4). The number of bug larvae born of uninfected eggs did not exceed 2.1 ind/m^2 on average.

4. Prolonged effect of crop biodiversity in crop rotation while maintaining the natural reproduction of *Scelionidae* family telenomines populations on winter wheat crops (*M*±SEM, kairomone feeding pads, RIBPP, Krasnodar, 2010-2018)

Year	Ovipositor of corn bug (*Eurygaster integriceps* Put.)		
	eggs laid in KFP	eggs infested by telenomines	
		total	%
2010	280.0±4.1	210.0±3.2	75.0±1.8
2011	380.0±4.2	240.0±2.6	63.1±3.3
2012	340.0±5.1	210.0±3.8	61.7±4.0
2013	360.0±5.0	290.0±6.1	80.5±2.2
2014	510.0±3.1	450.0±4.4	88.2±2.0
2015	490.0±4.6	260.0±5.1	53.1±2.7
2016	560.0±4.1	360.0±4.3	64.2±1.9
2017	550.0±2.9	420.0±3.8	76.3±3.1
2018	520.0±4.5	405.0±1.9	77.8±2.6

N o t e. Infestation rate is an indicator of EEL (entomophage efficiency level).

Elements of the technology (crop rotations with sunflower and soybean, strip crops of entomophilous dill) in 2011-2013 passed production testing in the Krasnodar Territory (OAO Chistaya yeda, Krymsky District) and in 2014-2015 in the Rostov region (KFH Biokhutor, Taganrogsky District, SPK AF Novobatayskaya, Kagalnitsky District). After 2-3 years of using the proposed techniques, the infection rate of *E. integriceps* egg-laying with telenomines was 45.5-69.0% regardless of weather conditions. In each farm, the area of chemical treatments against the pest was reduced by 500 hectares on average. At the same time, the number of its imago and larvae remained below the economic threshold of harmfulness (no more than 1.8 ind/m^2).

Thus, this paper has shown that the preservation and reproduction of the main egg-eating telenomines with the participation of their additional hosts, the shield bugs, under natural conditions is provided by the inclusion of tilled and entomophilous crops in the crop rotation. This for the first time made it possible to form, and subsequently, by constantly sowing tilled, entomophilous and nectariferous crops in crop rotation, to maintain the “natural biolaboratory” in agroecosystems for restoring the natural biocentric regulation of phytophage numbers by entomophages in wheat crops. In such natural biolaboratory, the wintering stock of telenomines is activated and reproduced during the second half of the summer season. The proposed technology for 9 years provides 88.2% infestation of the corn bug laid eggs by egg-eaters of *Scelionidae* family, which

made it possible to completely abandon chemical treatments against the pest.

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THE BIO-ECOLOGY OF NORTHERN POPULATIONS OF THE PLUM MOTH *Grapholitha funebrana* Tr. (*Lepidoptera*: *Tortricidae*) IN THE CONTEXT OF CLIMATE CHANGE IN THE CENTRAL NECHERNOZEM ZONE OF RUSSIA

A.S. ZEYNALOV

All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery, Federal Agency for Scientific Organizations, 4, ul. Zagor'evskaya, Moscow, 115598 Russia, e-mail adzejnalov@yandex.ru (✉ corresponding author)
ORCID:
Zeynalov A.S. orcid.org/0000-0001-5519-2837
The author declares no conflict of interests
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Abstract

Climate change over the last decades and rising global temperatures, especially in the Northern latitudes, affect ecosystems, including agricultural sector. The crops naturally grown in southern and central Russia are now expanding to northern areas along with dangerous pests that are not characteristic of these areas. Plum moth *Grapholitha funebrana* Tr. causes over 80 % yield losses. At least 3-4 treatments are required during the growing season to ensure the profitability. Chemical and environmentally friendly methods (pheromone traps) are of practical use. Anyway, successful protection requires detailed biological and ecological characterization of the pest in a specific area. In the Central Non Chernozem Zone of Russia such studies have not yet been conducted. This paper is the first evidence of *G. funebrana* wide spread in the Central Non Chernozem Zone where the pest can produce two generations. The study was carried out in the fruit-bearing plum plantations (All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery, Moscow Region, Leninskii district) in 2015-2017. Summer dynamics of *G. funebrana* population was monitored using pheromone traps with Denazil-P («Schelkovo Agrokhim», Russia) to attract moth males. Two days after the first plum moth butterflies flied, 400 leaves and the same number of ovaries (after their formation) were collected from 10 trees standing evenly along diagonals of the plantation to determine the start date of egg laying. From each tree the leaves and ovaries were collected daily from four sides (10 samples per side). The beginning of caterpillar hatching was determined by daily viewing 400 ovaries, starting from day 4 after the detection of the first eggs laid. Our survey shows that northern populations of *G. funebrana* are presently widely spread and successfully adapted outside 52° north latitude previously deemed the northern border of plum moth area. In Central Non-Chernozem Zone, particularly in the Moscow region (55° north latitude), the pest is capable to produce not one but two generations if sum of effective temperatures (SET) above 10 °C is 854-1124 °C. This is much lower than the value for the forest-steppe zone in Ukraine (1231-1353 °C). In some years the second *G. funebrana* generation exceeds the first generation in abundance. The start date of the overwintered generation flight varies greatly depending on the weather conditions during spring. The favorable sum of effective temperatures is from 59.4 to 159.8 °C which coincides with flowering and ovary formation in plum trees. So SET values and phenophases of plant development are not reliable reference points to forecast the beginning of the butterfly flight. Unlike geographical populations of *G. funebrana* from the southern and central zones of horticulture, butterflies of northern *G. funebrana* population remain active at daily air average temperature of 11-14 °C. This study indicates that in the northern gardening protective measures should be planned against both the first and second generation of plum fruit moth to prevent mass damage to fruits and to reduce the number of overwintering *G. funebrana* population

Keywords: *Grapholitha funebrana* Tr., northern populations, pheromone traps, the sum of effective temperatures, the dynamics of the flight

Climate changes over the last decades, as well as the lengthening of the vegetation period, lead to an imbalance in the habitat of living organisms, disrupting the order of the relationship between them [1-4]. Weather anomalies, temperature rise, shifts in precipitation, causing fluctuations in other environ-

mental factors, to which living organisms react depending on their inherent ecological plasticity and adaptive capabilities, cause qualitatively new transformations in ecosystems [(5-8]. Similar dynamics can be observed in agroecosystems, including horticultural plantations. The impact of climate changes on these systems has been evident since the early 1980s [9-11].

Climate warming, on the one hand, promotes a number of crops grown in the southern and middle zone of horticulture, in the Northern regions, on the other hand – leads to the penetration of harmful organisms to new territories. In particular, in the Central Non-Chernozem Zone, noticeable changes occur in the bio-ecology of phytophages and pathogens, the period of their harmfulness is lengthened, new, unusual to the region, pests and pathogens occur, those pests which previously had secondary importance are activated. The emergence of new varieties and modifications of cultivation technologies favor this process [11-13].

The species composition of plum pests, the degree of their danger and economic significance is exposed to significant metamorphoses. Thus, the damage of the fruit by plum moth (*Grapholitha funebrana* Tr.) and crop losses from this phytophage in different regions [14, 15] are up to 80% or more, and to ensure the profitability of crop production, it is necessary to apply at least 3-4 chemical treatments for the growing season [16-19]. To control numbers and prevent crop losses from moth, the environmentally friendly methods of struggle, including breaking chemical communications, pheromone confusion and mass trapping of males [20-22], the use of oophages [23] based on monitoring of pest population are offered [24]. However, the features of bioecology and related harmfulness of the phytophage in the Central Non-Chernozem Zone are still not investigated.

In this paper, it was first established that the plum moth is widely distributed in the Central Non-Chernozem Zone and develops here in two generations. The limits of the sum of effective temperatures for the beginning of flying of butterflies of the overwintered generation, the formation of two generations, the beginning of egg laying, and the pest caterpillar hatching are determined. The minimum air temperature for the active swarming of the Northern population butterflies and the dynamics of swarming during the vegetation period, which differ significantly from the indicators for the southern zone, were established.

The work objective was to study the bioecology of plum moth in the Central Non-Chernozem Zone and assess the dynamics of this phytophage during the growing season.

Techniques. The study was carried out in the fruit-bearing plum plantations (the laboratory lot and the demonstration garden) of All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery (ARHIBAN, Moscow Region, Leninsky District) in 2015-2017. The swarming dynamics of *G. funebrana* population was monitored using the pheromone Atrakon-A traps of the triangular form (AO Shchelkovo Agrokhim, Russia) with the size of the adhesive liner 10×17 cm. To attract males, the pheromone Denazil-P (AO Shchelkovo Agrokhim, Russia) was used with a dispenser, the rubber capsule impregnated with the active substance (sex pheromone) Z8 (dodecyl acetate, 1 mg/dispenser). Pheromone traps were hung at a height of 2 m directly in front of flowering plum varieties of early maturing (Utro, Opal, Skoroplodnaya). Dispensers were placed in the center of the adhesive liner with a tweezer, changed every 6 weeks (adhesive liners were replaced as contaminated). Inspection of pheromone traps and the counting of the caught males before the first butterflies flew were carried out daily, at the end of swarming of the 2nd generation (since the beginning of the 2nd ten-day period of September) in 1 day, and then 2-3 times a week. Dispensers were

stored in the refrigerator before use.

To establish the start date of egg laying by females in 2 days after the flight of the first butterflies from 10 trees evenly distributed over two diagonals of planting, 400 leaves and the same number of ovaries (after their formation) were selected. The selection was carried out daily, on 10 pieces from four parties of each accounting tree. The samples were viewed (microscope MBS-10, OAO LZOS, Russia). The beginning of caterpillar hatching was determined on a daily basis (starting 4 days after the detection of the first eggs of the phytophage) scanning for 400 ovaries.

The mean by dates of observations (sample mean $\bar{x}$) and the standard deviation for each observation site (σ) were calculated. The correlation between the number of males of the 1st and 2nd generations of plum moth and the sums of effective temperatures in the period of intensive swarming (SET 1 and SET 2) was analyzed according to B.A. Dospekhov [25] using the Microsoft Excel software package. Correlations were considered statistically significant with confidence probability P = 99%.

Results. In the period from the late 1980s to the early 1990s, 52° of Northern longitude was considered the Northern boundary of the *G. funebrana* distribution and within the Orel, Kursk, Voronezh Regions and the Northern part of the Belgorod Region, the pest developed in I generation [26-28]. However, nowadays, the plum moth has penetrated and intensively develops in the Central Non-Chernozem Zone, including in the Moscow Region (55° of Northern longitude). Even in the abnormally cold growing season 2017 (Table 1), it gave not one, but two generations.

1. Indicators of the average temperature and precipitation for the vegetation periods of 2015-2017 in the Leninsky District of the Moscow region (according to the weather station of the Domodedovo Airport, Moscow Province

Parameter	Year	Month					
		April	May	June	July	August	September
Average temperature, °C							
Long-term		6,7	13,2	17,0	19,2	17,0	11,3
Actual	2015	6.1	14.3	18.0	18.1	17.6	13.8
	2016	8.1	15.0	18.2	20.9	19.5	11.4
	2017	5.3	10.9	14.5	17.9	18.8	13.0
Deviation	2015	–0.6	+1.1	+1.0	-1.1	+0.6	+2.5
	2016	+1.4	+1.8	+1.2	+1.7	+2.5	+0.1
	2017	–1.4	–2.3	–2.5	–1.3	+1.8	+1.7
Precipitation, mm							
Long-term		37	50	80	85	82	68
Actual	2015	44	119	94	121	14	88
	2016	34	63	61	122	167	59
	2017	79	84	140	105	68	38
From long-term, %	2015	119	238	117	142	17	129
	2016	92	126	76	144	204	87
	2017	214	168	175	124	83	56

Certainly, the temperature regime, in particular, the sum of effective temperatures (SET) has a dominant effect on the rate of phytophage development in different phases, but precipitation, humidity, and a combination of these factors also have a significant impact. The development dynamics of plum moth in stable warm weather during the growing season is smoothed. That is, for each generation, the beginning of swarming, one peak, and the decline in numbers is clearly distinguished, as it was noted in Northern Italy, the southern part of Bulgaria [29, 30], a number of regions of the Czech Republic and Slovakia [31]. The models of development dynamics of moth developed in these conditions concerning the sum of effective temperatures well correlate with the actual situation in a concrete zone [29], but cannot be universal for regions where climatic and geographical conditions differ essentially [31].

In the Northern regions, depending on the weather conditions of the year, more complex development dynamics of *G. funebrana* is observed, often with several periods of increase and decrease in population during the swarming period of one generation, which is noted from the forest-steppe zone of Ukraine [30, 32]. In contrast to the southern and middle horticulture zones, where the sum of effective temperatures by the beginning of swarming of the overwintered generation of butterflies has more or less similar indicators over the years, in the Central Non-Chernozem Zone, they vary greatly. In the 1980s, swarming of the moths of the overwintered generation *G. funebrana* began with the accumulation of SET above 10 °C in the range of 105-120 °C [28]. According to I.V. Shevchuk et al. [30], in the forest-steppe zone of Ukraine, butterflies of the overwintering generation fly when SET is 45.5-47.0 °C, and in Bulgaria, this is 32.6-67.6 °C. In comparison with the beginning of the 1960s, in the Northern forest-steppe zone of Ukraine, butterflies fly 33-36 days earlier, and swarming ends much later, in early or mid-October. The authors explained it with the global climate changes.

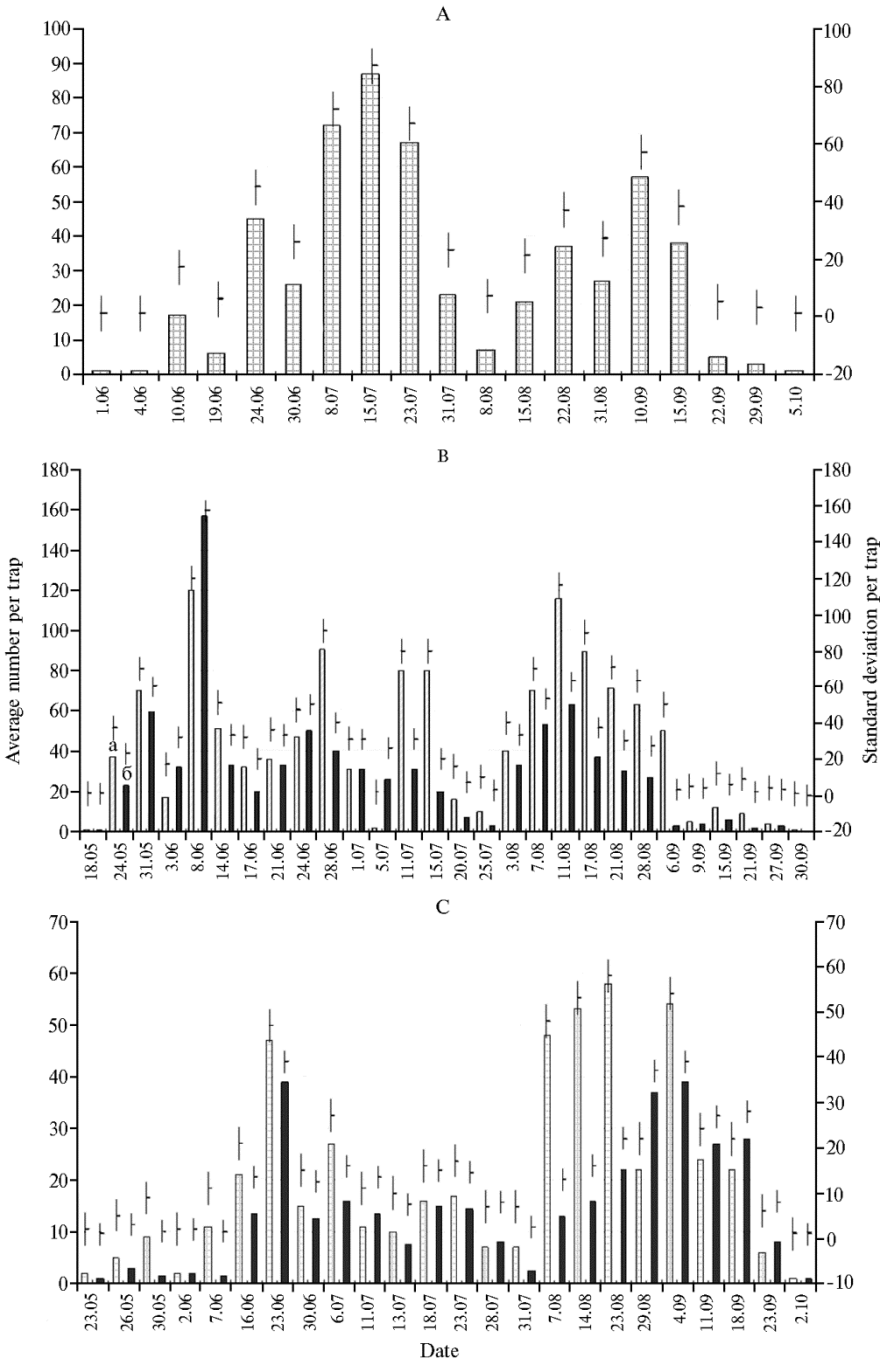
According to the authors' data, in 2015, SET higher than 10 °C at the beginning of swarming of butterflies *G. funebrana* was 159.8 °C (the 1st of June), in 2016 – 86.9 °C (the 18th of May), in 2017 – 59.4 °C (the 23rd of May). The beginning of butterflies swarming also varied significantly over the years and accounted for the phenophase of the flowering beginning, the end of flowering or the ovaries formation of later varieties of plums Pamyat Timiryazeva, Renklod Tambov, Aleksii. The maximum and minimum SET indicators for 3 years differed 2.7-fold. The time of egg laying by females of pests also varied year by year (on 2015 June 4 at SET of 185.6 °C, on 2016 May 24 at SET of 105.4 °C, on 2017 May 30 at SET of 102.6 °C) and the beginning of hatching of the first caterpillars (on 2015 June 12 at SET of 240.1 °C, on 2016 June 3 at SET of 191.2 °C, 0n 2017 June 16 at SET 147.7 °C).

This indicates high ecological plasticity of the phytophage, which promptly reacts to the actual changes in the environment and optimally harmonizes its development, on the other hand this fact points to a continued adaptation within the new area. In the conditions of sharp climatic changes, typical for the Non-Chernozem Zone of Russia, the determination of the beginning of plum moth swarming only by SET does not guarantee high enough accuracy and can serve as a guide for planning, but not carrying out protective measures, as well as the phenophase of plant development. Other climatic factors, including the difference between day and night temperatures, their value and distribution by day, intensity and duration of precipitation, the presence and strength of the wind, etc., have a significant impact on the dynamics of the phytophage development. More reliable information on the dynamics of butterflies swarming can be obtained by pheromonitoring [20, 21], and measures to protect the crop from the pest should be carried out on the totality of the data obtained [24], including the results of visual observation.

Plum moth swarming in 2015 lasted for 127 days, in 2016 for 136 days, in 2017 for 132 days, which is more than 4 months (Fig.). The period of flight of the overwintering generation of moths took about 1.5-2 months. In 3-5 days after the flight, females began to lay eggs. The duration of the embryonic development period and development of caterpillars, depending on weather conditions, was from 1 week to 12 days and from 17-20 days to 1 month, respectively. It is known that the development at the pupal stage needs 1.5-2 weeks, and the life expectancy of butterflies in different conditions varies from 4-5 to 15 days [26-28].

In case of only one generation, the number of butterflies could not sig-

nificantly increase after mid-August—early September and swarming could not continue until the beginning of October. Thus, in the conditions of the Central Non-Chernozem Zone, the plum moth gave not one, but two generations in a year. The second generation could be complete or optional depending on weather and fodder supply.



Plum moth (*Grapholitha funebrana* Tr.) swarming in 2015 (A)**, 2016** (B)**, and 2017** (C) (number on average per 1 trap) **at a laboratory plot** (a) **and in the demonstration garden** (b) ($x \pm \sigma$, the Leninsky District, Moscow Province).

In 2015-2016, the weather conditions favored the development of *G. funebrana* of the 1st and 2nd generations, but the period of maturation and

harvest came much earlier than in 2017. In these conditions, the 1st generation was more numerous. It is particularly explained by the fact that even in the southern regions from 25 to 55% of the caterpillars of each generation go to the winter diapause (certainly, except for the last generation, which is completely diapaused) [28].

In 2017, despite the abnormal for this period cold that lasted till mid-summer, combined with prolonged heavy rains (see Table 1), butterflies swarming began on May, 23, lasted continually. Butterflies of the 1st (overwintered) generation reached the maximum number in pheromone traps in late June—early July (see Fig.). The relatively high number fluctuated depending on weather conditions (with two peaks), but remained until the middle of the third ten-day period of July. Then, it decreased to the middle of the 1st ten-day period of August, reaching its minimum. However, the process was not interrupted and intensified with the beginning of butterflies swarming of the 2nd (summer) generation. During this period, the weather changed quickly: precipitation was less; the air temperature was much higher than the average annual data. In these conditions, the majority of caterpillars of the 1st generation did not diapause, and gave rise to the second generation of the plum moth. As a result, it was more numerous than the first generation. Along with the change in weather, this was facilitated by the abundant fodder supply, since the ripening and harvesting period was delayed by about 3 weeks, and in late varieties, the fruits remained until the beginning of October. The high number of butterflies of the 2nd generation (also with two peaks) was noted until the second half of the 2nd ten-day period of September with changes depending on weather conditions. Further on, the number of pests constantly and sharply decreased, and the last butterflies were found in late September—early October.

In the abnormal weather conditions of spring and the first half of summer 2017, some important features in the pest bioecology were found. Butterflies maintain swarming activity at an average summer air temperature of 11-14 °C. In early June, when average daily air temperature fell below 10 °C and did not rise above 12.9 °C, they did not interrupt swarming unlike the traditional pest in this area, the apple worm *Cydia* (*Laspeyresia*) *pomonella* L. (its swarming was not observed).

It was found that in the Central Non-Chernozem Zone with the accumulation of SET above 10 °C to 854-1124 °C developed two generations of plum moth − overwintered and summer. Similar indicators for the forest-steppe zone of Ukraine are 1231-1353 °C [30], which emphasizes the high ecological plasticity of *G. funebrana*. The value of SET 854 °C was recorded in 2017, when the 2nd generation was superior to the overwintered generation in number. However, it should be emphasized that in 2017, the SET during the intensive swarming of the summer generation by 148.7 °C exceeded the value of the SET in the period of the intensive swarming of the overwintered generation (in 2015 and 2016 by 10 and 7.3 °C, respectively). The analysis of the multiple correlations between the number of the 1st and 2nd generations in the period of intense swarming and the corresponding values of SET also showed a closer relationship between the number of the summer generation and SET 2 (Table 2). This research found average strength positive correlations between the numbers of the 1st and 2nd generations, as well as SET 1 and the number of the 2nd generation. At high SET 1 (within the optimal temperature for the phytophage 23-29 °C), the development accelerated and caterpillars that have completed the nutrition earlier could pupate and give rise to the second generation. The decrease of SET 1 shifted the formation and maturation of the crop to a later date, that is, it influenced the fodder base (taking into

account the varietal factor, the activity of entomophages and entomopathogens) and in combination with a higher SET 2 stimulated pupation of caterpillars and the flight of butterflies of the second generation.

2. Correlation between SET 1, SET 2 and the number of male plum moth (*Grapholitha funebrana* Tr.) of the 1st and 2nd generations (Moscow Province, 2015-2017)

Indicator	1st generation	2nd generation	SET 1	SET 2
1st generation	1			
2nd generation	0.4361*	1		
SET 1	0.3740*	0.5741*	1	
SET 2	0.2492	0.5923*	0.4701*	1

Note. SET 1 and SET 2 — sums of effective temperatures above 10 °C in the periods of intense swarming of butterflies of the 1st and 2nd generations.
* Correlations are statistically significant with confidence probability P = 99%.

Certainly, bioecological development of any organism in a particular environment are determined by the interaction of a set of factors, each of which deserves special attention, but a more significant role is played by the dominant. By using data on the initial number of the phytophage and its relationship with the SET indicators, it is possible to predict and pre-plan the necessary protective measures, but their multiplicity and specific timing should be established taking into account the results of pheromonitoring.

Thus, the plum moth *Grapholitha funebrana* Tr. in the Central Non-Chernozem Zone of Russia develops not in one, but in two generations. The beginning of swarming of butterflies in the overwintered generation depends on spring weather conditions, strongly fluctuates on years and occurs at SET above 10 °C from 59.4 to 159.8 °C (from the beginning of flowering to plum ovaries formation). Two-generation development of *G. funebrana* in the Central Non-Chernozem Zone requires SET above 10 °C within 854-1124 °C, and butterflies swarming can continue at 11-14 °C. The second generation of *G. funebrana* in some years may exceed the first one in number, which requires appropriate measures to combat the pest. This will ensure crop preservation and the high fruit quality, also significantly reducing number of overwintering population.

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