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

AGRICULTURAL BIOLOGY

Since January, 1966

PLANT BIOLOGY

Vol. 52, Issue 5
September-October

2017 Moscow

Covered in Scopus, Web of Science (BIOSIS Previews, Biological Abstracts, Russian Science Citation Index), Agris

Science editors: E.V. Karaseva, L.M. Fedorova

Publisher: Agricultural Biology Editorial Office NPO

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For citation: Agricultural Biology,
Сельскохозяйственная биология, Sel'skokhozyaistvennaya biologiya

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

SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2017, Vol. 52, № 5

CONTENTS

FUTURE AGRICULTURE SYSTEMS

GENETIC RESOURCES, INTROGRESSION, IMMUNITY
(toward 130th Anniversary of N.I. Vavilov)

SYMBIOGENETICS

BIOFORTIFICATION

GENETIC AND PHYSIOLOGICAL BASIS OF CROP BREEDING

AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 843-855
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Future agriculture systems

UDC 631.522/.524:581.132.1:57.05

doi: 10.15389/agrobiology.2017.5.843rus
doi: 10.15389/agrobiology.2017.5.843eng

CHLOROPHYLL b AS A SOURCE OF SIGNALS STEERING PLANT DEVELOPMENT

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported by Russian Science Foundation (grant № 14-16-00120)
Received December 12, 2016

Abstract

Crop yield strongly depends on time of the onset of flowering as well as of the initiation of senescence. These processes are under tight control of multiple gene complexes. Suboptimal environmental conditions, as well as mutations, may cause changes in the expression levels of these genes, which, in turn, can result in a delay of flowering and/or early senescence, and, ultimately, in a decrease of yield. Recently, crucial role in the regulation of plant development via retrograde signaling pathways has been revealed for chlorophyll b. Chlorophyll b is an obligate component of the photosynthetic apparatus of land plants, and the main regulator of the biosynthesis and degradation of photosynthetic antennae. It is becoming clear that the size and stability of photosynthetic antennae are not only important for photosynthesis but also represents a source of signaling beyond chloroplasts. The absence of chlorophyll b in mutants of *Arabidopsis thaliana* (*ch1*) and *Hordeum vulgare* (*chlorina f2 3613*) leads to a decrease in the growth rate, leaf size and biomass production. In addition, and independently of the downregulation of photosynthesis, the lack of chlorophyll b results in the delay of flowering and early onset of ontogenetic as well as induced senescence. This review addresses the role of chlorophyll b in energy balance, and discusses new data on the role of chlorophyll *b* in regulation of ontogenesis not related to photosynthesis. Mutants of economically important crops impaired in chlorophyll b biosynthesis represent promising models for physiological, biochemical and molecular studies of regulation of flowering and senescence, as the results can be directly applied to agricultural practice. Also, we review the novel data on the potential importance of plants with truncated photosynthetic antenna for increase in vegetative and grain biomass production. A decrease in chlorophyll b contents and the following down-regulation of antenna proteins were shown to influence the rate of electron transport within the photosystem II, as well as the rate of CO_2 assimilation relative to chlorophyll unit. Strikingly, these parameters in *chlorina* mutants are higher than in wild type plants by 15-20 %. Using plants with this type of photosynthetic apparatus can potentially bring about a considerable increase in yield. This suggestion has been recently supported by data on transgenic tobacco plants with truncated photosynthetic antenna (H. Kirst et al. 2017). At the same time, the consequences of the decrease in chlorophyll b levels for ontogenetic regulation and photoprotection typically negate the potential benefit of the acceleration of the limiting factor of photosynthesis, the photosystem II. This review discuss the possible ways to search for optimization of plant functions regulated by chlorophyll b, to provide new mechanisms of the increase in photosynthesis and crop production in agriculture.

Keywords: yield, chlorophyll b, flowering, ontogenesis, senescence, photosynthetic antenna

The yield of cereals depends on many factors. In addition to environmental conditions (availability of necessary nutrients, light, soil moisture, etc.), it is affected by endogenous processes. For example, violation of the expression of several regulatory genes can result in delayed flowering and accelerated senescence [1-2]. It is obvious that such changes in ontogenesis adversely affect yield.

Even a slight delay in the initiation of floral transformation of the vegetative apical meristems of shoots can lead to a substantial reduction in harvest, and at the shift of ontogenesis phases in time, the negative impact of environmental factors on the plant growth and yield dramatically increases. Blocking of transition to flowering leads to a complete loss of the grain harvest, as the spikes (with accelerated senescence they cannot be formed) are of agricultural value for these crops. At the same time, the acceleration of start of floral transformation of vegetative meristems even by 2-3 days (for example, early flowering barley and wheat mutants) [3] has a positive impact on productivity. Searching for such mutants and their introduction into the culture are actively carried out in the largest center for the study of barley – the Carlsberg Research Centre (Denmark).

Currently, there are a large number of mechanisms governing the change of ontogenesis phases [1, 4-6]. It is well known that for the timely passage of plants from one ontogenesis phase to another one, the availability of nitrogen and carbon is important [5]. For many cultures, it is established that the transition to flowering is initiated at a high ratio of far-red light to the red in the spectrum [7]. The most important factors regulating flowering are the length of daylight and temperature [8]: the cold pathway is stimulated at specific exposure to low temperatures (vernalization), and the photoperiodic one is triggered by a certain length of daylight [9]. Gibberellic signaling and the so-called autonomous induction of flowering also refer to the major signaling pathways that initiate flowering [4]. The structural and molecular genetic mechanisms of flower formation in response to floral transformation of the vegetative apical meristems of a stem have been studied [10-13].

The process of plant senescence is of key importance in the formation of crop yield [14, 15]. Senescence is the terminal stage of the development of tissue, organ or a whole plant [16], a genetically determined and an integral part of ontogenesis [17-19]. In early induction caused by adverse environmental conditions, senescence can result in a decrease of yield of vegetable and fodder crops [20, 21]. In most cases, the decay of leaves is consistent with the development of seeds, apical leaves and storage organs [22, 23], since the primary purpose of senescence is the remobilization of nutrients to plant young parts and seeds [24, 25]. For annuals, which include most cereal crops, the transport of nutrients from vegetative tissues into seeds during the senile stage is crucial: over 70 % of nitrogen is supplied to seeds from senescent leaves [14, 26-28]. Like flowering, senescence depends on the expression of regulator genes [27-29], which act both as activators and transcriptional repressors of target genes [30-33].

Previously, the biosynthesis of photosynthetic pigments, in particular, chlorophylls, was not considered a factor activating signaling pathways that lead to the initiation or a delay of flowering and senescence. However, recent works show the importance of the auxiliary photosynthetic pigment chlorophyll b (Chlb) in the regulation of plant ontogenesis. Chlb overproduction in *Arabidopsis thaliana* transgenic lines with overexpression of the chlorophyllide-a-oxygenase (CAO) gene from prochlorophyte cyanobacteria slowed down the initiation or stopped the senescence progress. These changes were marked when growing plants in low light and in the dark [34, 35]. With a high probability, we can expect that a shortage of Chlb leads to accelerated senescence. Besides, the data testifying that the absence of Chlb negatively affects the change of ontogenesis periods of barley has been published [36].

Chlb impact on the development of plants is convenient to be studied on model objects, the mutants unable to synthesize this pigment. *Chlorina* mutants lacking Chlb are well-known for many species, i.e. barley, corn, peas, rice, soybeans, sweet clover, wheat, rape and *A. thaliana*. Chlb biosynthesis in photosyn-

thetic tissues of higher plants is carried out by the chlorophyllide-a-oxygenase [37, 38]. The gene encoding this enzyme is presented in the genome by a single copy. Chlorophyll a (Chla) serves as the CAO substrate. Mutants by the *CAO* gene are not able to convert Chla to Chlb [39]. By the example of the most studied barley mutants of allelic series *chlorina-f2* and *Arabidopsis ch1* mutants, it was shown that a mutation in the *CAO* gene has a pleiotropic effect and is accompanied by numerous violations of the functions at the molecular, cellular and organismal levels. This indicates the importance of the pigment in the key life processes of plants.

This review discusses the Chlb functions in chloroplasts and energy metabolism of plants, as well as the role of Chlb in the regulation of the ontogenesis phases, which does not depend on the pigment photosynthetic function. Mutants of cultivated plants with the impaired Chlb biosynthesis are described as the models to identify and study mechanisms for the increase in photosynthesis and productivity. Prospects for the use of plants with the truncated size of the light-harvesting antenna complex for the increase in yield of vegetative and seed biomass are discussed.

Localization of chlorophyll b in the photosynthetic apparatus of higher plants. In plants, chlorophylls are found only in the pigment-protein complexes, as in free form, being the strongest photosensitizers, they can cause destruction of the thylakoid membranes and chloroplast stroma through the photodynamic effect. Chlb serves as an accessory light-harvesting pigment absorbing and transferring light energy to the reaction centers of photosystems. It accounts for approximately 15-25 % of the total chlorophyll content [40, 41]. Unlike Chla, which is part of the core complexes of photosystems, Chlb is found only in the light-harvesting complexes (LHC) of photosystems (LHC I and LHC II) and in the so-called minor antenna of photosystem (PS) II [42, 43]. In LHC I, Chlb accounts for about 22 % of the total amount of chlorophylls, in LHC II reaches about 43 %, and in the pigment-protein complex of the minor antenna is 31-46 % [44]. In transgenic plants with the increased Chlb biosynthesis, the antenna size can be greatly increased due to superstabilization of antenna proteins [34, 35, 45].

The role of chlorophyll b in plant energy metabolism. Chlb has a unique physico-chemical property to absorb light in the short-wave region (425-475 nm), in which Chla absorbs weakly. Chlb significantly increases light collection when the light levels are low, under mutual shading of plants in thick plantings. In the process of light collection, the energy of the excited by absorbing light quanta singlet states of pigment molecules, associated to antenna proteins, is transferred from carotenoids to chlorophylls, then from Chlb to Chla and in molecule chain order Chla reaches the reaction center of photosystems. The first two stages of energy transfer are highly efficient and occur in less than a picosecond [46, 47], while the energy transfer between Chla molecules within the same protein and between adjacent monomers takes several picoseconds [48, 49]. Chlb is responsible for transfer to Chla of about 50 % of the energy absorbed by carotenoids [50]. The efficiency of absorption of light energy and its transfer to the photosystems of mutants unable to synthesize Chlb is significantly reduced.

We should also mention the so-called red forms of chlorophylls, which are represented by Chla and Chlb located on the antenna proteins of PS I. The absorption and fluorescence spectra of these forms are shifted into the far red region [51], which makes them able to capture light energy in this range and transfer it to the reaction centers of PS I against the energy gradient in low light conditions. Such transfer is possible at physiological temperatures due to thermal energy, through which the energy gap between the donor and the acceptor is

overlapped. Such spectrum range extension due to the chlorophyll red forms provides absorption of almost 40 % of light energy in shading [52].

At high light output intensity, the chlorophyll molecules are involved in the process opposite to light collection — the diffusion of the absorbed light energy excess in the form of heat (non-photochemical quenching) potentially dangerous for plants. The functions of individual Chlb molecules are different in photoprotection. Thus, the efficient quenching of the excited chlorophyll singlet states is carried out by clusters of the paired Chla-Chlb dimer and zeaxanthin [53]. These quenching centers are localized on the minor antenna proteins of PS II [53]. For quenching of triplet chlorophyll states, in the vicinity of the neoxanthin binding site on the antenna proteins Chlb has a maximum value: in its absence, the production of singlet oxygen increases sharply [54]. It should be noted that mutants lacking Chlb, as a rule, have a strong oxidative stress, primarily, due to high production of singlet oxygen [55-56]. Since the main source of singlet oxygen in chloroplasts is PS II, these mutants may have obstruction of outflow of electrons from the reaction center of PS II into the electron transport chain (ETC). Recently, experimental evidence for this hypothesis has been obtained [57].

Chlorophyll b as a regulator of the antenna size. According to modern concepts, Chlb functions in photosynthesis are not limited to light collection and light scattering. It is known that the ratio of Chla:Chlb in high light is higher than in low one. The regulation of Chlb synthesis is essential for adaptation of plants to light of varying intensity [58]. Chlb is a major regulator of the antenna size of the photosynthetic apparatus: Chlb binding with the antenna proteins of LHC stabilizes it, and the initial reaction of Chlb catabolism activates a cascade of proteins that carry out disassembly of the antenna. Because Chlb is concentrated only in the antenna, the reduced antenna protein content leads to the change of the Chla:Chlb ratio. The stabilization mechanism of the antenna proteins with Chlb participation was described for LHCB1 [59]. In Chlb, 7-formyl group pulls electron density of magnesium central atom to the periphery of the molecule; therefore, the positive charge of magnesium is shielded by the electron cloud less than in the Chla molecule. In this regard, Chlb is easier than Chla forms electrostatic bonds with Lewis bases, namely with the carbonyl groups of the peptide chain. In addition, between the protein and the 7-formyl group, the formation of hydrogen bonds is possible. Binding Chlb to the protein leads to the fact that the conformation of the latter becomes more stable and allows it to gain a foothold in the membrane [59]. It is important to note that in the case where the molecule of LHCB1 protein is not bound to Chlb, its degradation occurs and LHC II cannot develop. It is known that in Chlb absence in chloroplasts, the contents of some other antenna proteins are reduced; there may be a similar mechanism, although experimental evidence has not yet been received. However, it can be assumed that the work of the CAO enzyme is coordinated with the import systems of synthesized in the cytosol apoproteins of the antenna complexes in chloroplasts.

Recent studies have allowed establishing that Chlb has an impact not only on the assembly of antennas, but also on the degradation of antenna proteins [60, 61]. The synthesis of apoproteins of LHC II decreases at the beginning of senescence, but due to the relatively high stability these proteins are detected in the leaves, even at its later stages [62]. While the chlorophylls are bound to proteins, the latter are not accessible to proteases [18, 62-64]. Chlb catabolism is impossible without its turning into Chla [61]. The reaction is catalyzed by two enzyme isoforms of chlorophyll-b-reductase: non-yellow coloring 1 (NYC1) and NYC1-like [65]. From this stage, the disassembly of LHC II starts [18]. NYC1

does not accumulate in Chlb absence; this protein is undetectable in *Arabidopsis* mutants lacking Chlb [66]. On the whole, Chlb synthesis and catabolism are regulated by the principles of negative and positive feedback respectively: in Chlb excess, the CAO enzyme is subjected to degradation [42], which allows the cell to maintain a low pigment content, and for the accumulation of NYC1 protein, Chlb, on the contrary, is necessary [66]. This data confirms the role of Chlb as the main regulator of the size and light-harvesting capacity of the photosynthetic antenna [44].

Participation of chlorophyll b in the maintenance of the supramolecular organization of thylakoid membranes. In chloroplasts of the mutants with impaired Chlb biosynthesis, an ability to form grains is reduced [67, 70], and also the nature of packing the pigment-protein complexes in the granule membrane is changed. The ability to form grains is reduced, because in the granule membranes, the content of integral proteins of LHC II, which play a major role in the stacking, decreases [70]. Besides, in connection with the reduced antenna protein content in grains of such plants, the pigment-protein complexes form supercomplexes with the changed composition and size [67-70]. A smaller particle size promotes a more dense packing in the granule membrane. However, this limits the lateral diffusion of membrane components, i.e. the photosynthetic proteins and the low molecular weight hydrophobic molecules, including the carrier of electrons in the photosynthetic electron transport chain of plastoquinone [71]. Recent studies have revealed that diffusion limitations hamper the timely repair of the damaged photosynthetic complexes, which prevents normal work of the latter [71]. Thus, part of the pleiotropic effects caused by *CAO* mutation in *chlorine* plants with a high probability is due to the lateral mobility limitations of membrane components of thylakoid membranes in connection with the changed stoichiometry of photosynthetic complexes because of Chlb lack. This assumption is supported by recent studies of *chlorina* mutants lacking Chlb, in barley and *A. thaliana* [57].

Participation of chlorophyll b in the regulation of the ontogenesis. Chloroplasts are the most important sources of signaling for other organelles and the cell in general. The retrograde signaling pathway from chloroplasts and mitochondria to the nucleus modulates the anterograde control in accordance with the needs of the cell [72-74]. In the absence of signaling from chloroplasts, the expression of several nuclear genes encoding proteins of these organelles, including the antenna proteins of LHC, is inhibited [74]. For chloroplasts, signaling is associated primarily with the photosynthetic function, and since the photosynthesis intensity is influenced by various factors, signaling from chloroplasts can serve as sensors of environmental conditions [75]. Among the major sources of retrograde regulation signaling there are the formation of tetrapyrroles, the expression of chloroplast genes, the change in the redox state of ETC components and the formation of reactive oxygen intermediates (ROI) [76, 77]. In addition, an important source of plastid signaling is the stability of pigment-protein complexes. This signaling carries information on environmental conditions and the age of the cell [35, 38, 65, 77].

Stay-green mutants preserving steady green coloring at later ontogenesis stages, when senescence begins in the wild type, which is accompanied by yellowing of leaves, can serve as an example of plants with a very high degree of stabilization containing chlorophyll of pigment-protein complexes. *Stay-green* mutants are known in many plant species. In particular, they include Mendelian pea mutants with green color of the seeds. There are functional and cosmetic phenotypes in *stay-green* mutants. In the functional one, the expression of senescence-associated genes (SAG) decreases and the intense photosynthesis remains

longer than in wild-type plants. In the cosmetic phenotype, senescence of mutants is induced in the same way as in wild-type plants. Moreover, they have the reduced intensity of photosynthesis, but the coloring remains green. Such phenotype is observed in mutants by *SGR* (*Stay-GReen*) genes encoding components of a complex involved in the degradation of proteins and chlorophylls of LHC II, including Chlb [34, 78]. The components of this complex are the chlorophyll catabolism enzymes (CCE), including NYC1 [62], and the very LHC II [34]. At the knockout of genes encoding proteins of CCE, stay-green phenotype is also observed [35, 65].

In the study of regulation of ontogenesis, mutants with the functional stay-green phenotype, for example, autophagy gene mutants, are of particular interest. As it is known, autophagy plays an important role in recycling of chloroplast proteins, primarily ribulose bisphosphate carboxylase/oxygenase (rubisco), especially during senescence [79]. Unable to autophagy *atg*5 mutants under mild abiotic stress, when in the wild type early senescence was induced and *SAG* genes activated, demonstrated the functional stay-green phenotype, i.e. the delayed onset of senescence [80]. It still remains unclear why in some cases the functional stay-green phenotype is implemented, and in others, when the LHC II degradation complex formation is affected directly, the "cosmetic" one is involved.

Even more intriguing is the fact that the functional stay-green phenotype appears in plants due to the accumulation of Chlb above normal. In transgenic *Arabidopsis* plants, the overexpression of the *CAO* gene from prochlorophyte cyanobacterium led to overproduction of Chlb, since, in contrast to the endogenous enzyme in plants, this enzyme in plant cell is not exposed to the regulation by the feedback principle. The Chlb content in such plants was so great that it replaced Chla in the core antenna of PS I and PS II, and the size of the light-harvesting antenna and its stability were very high [34, 35]. These transformants showed the functional stay-green phenotype and differed from wild-type plants in the delayed onset of leaves senescence both at a shortage of light and in the dark [34, 35]. Perhaps, superstabilization by Chlb of pigment-protein complexes in the light-harvesting systems and prolongation of their active functioning change the number of still unidentified signaling molecules necessary for switching the ontogenesis programs, which leads to the modulation of genome expression, including the reduced expression of *SAG* genes. The role of such signaling molecules could be performed by chlorophyll catabolites, apoproteins of LHC II lacking Chlb or products of their proteolysis. We can also assume that the long-term maintenance of the antenna of LHC II in the functional condition enhances photoprotection and, therefore, provides the reduced ROI level by the beginning of cell senescence. Probably, this slows down the initiation of subsequent stages of the senescence process. In the cosmetic stay-green mutants, the pigment-protein complex of LHC II remains, but loses the ability to interact with PS II. Thus, it is quite possible that the reason why plants obtained a negative feedback regulation of CAO enzyme was the influence of excessive amounts of Chlb on ontogenetic signaling mediated by superstabilization of the antenna of LHC II.

In the works done by us on *chlorina* mutants with a complete block of Chlb bio-synthesis (*ch1 A. thaliana* and *chlorina f2*^{3613} *Hordeum vulgare* mutants), the preliminary data on the influence of the antenna, destabilized by Chlb absence, on the time of the beginning of flowering has been obtained [36, 81]. *Chlorina* mutants of both species differed from plants of the parental lines in the later onset of floral transformation. Additionally, in 30-40 % of the barley mutants, the growth and differentiation of the ear structural elements stopped [36, 81]. In the initiation of flowering, in *chlorina* mutants the expression of *FT* gene (florigen), the main regulator of floral transformation, was disrupted, and also

the expression of genes-markers of senescence and catabolism of *SAG* and *NYC1* chlorophyll increased [81]. Violations of these processes are likely associated with changes in the retrograde signaling cascades activated by the antenna complexes of chloroplasts.

Prospects for the use of plants with the truncated light-harvesting antenna size to improve photosynthesis and productivity. In recent studies, in transgenic tobacco plants through directed modification of the light-harvesting antenna relaxation of the absorbed excessive light energy dissipating in the form of heat has been accelerated [82]. Production of vegetative biomass in such plants was higher by 15% than in the wild type. Based on these results in another study [83], the authors purposefully have examined the transgenic lines of tobacco with the truncated light-harvesting antenna size (TLA-plants) and have found a significant increase in photosynthetic productivity and growth of vegetative biomass, especially in thick plantings, noting the prospects of using TLA-plants in applications. However, manipulation through transgenesis is undesirable for crops. At the same time, *chlorina* mutants have features that bring them closer to TLA-plants, including their reduced size of the photosynthetic antenna. The data on the high intensity of photosynthesis and productivity of some chlorina wheat [84-85], soybean [86] and barley [87] mutants has been published [87], although in most cases, such mutants are characterized by the reduced photosynthesis and the growth retardation. Therefore, chlorina mutants could be promising replacement for TLA-plants, but the diversity of Chlb physiological functions described above suggests that the negative effects of *chlorina* mutations most often will level out the possible increase in photosynthesis. Such effects include the negative impact of shortage or complete absence of Chlb on ontogenetic regulation.

The nature of signaling molecules and signal transmission pathways from (de)stabilization of the antenna beyond the limits of chloroplast, which are involved in the initiation and passage of ontogenesis phases, remains unclear. However, recently we have proposed a mechanism, by which the absence of Chlb can affect the regulation of flowering [88]. In chlorina barley and arabidopsis mutants, violation of redox balance of chloroplasts changes the number and permeability of plasmodesmata, changing the conductivity of the symplastic channel that carries the macromolecule signals inducing flowering, which could be the reason for a delay of floral transformation [88-90]. This is the first work discussing the nature of the relationship between the stability of the light-harvesting antenna of the photosynthetic apparatus and regulation of flowering in plants.

Summarizing the data on Chlb functions, we can conclude that in *chlorina* mutants suppression of the photosynthetic function and productivity decrease (and, potentially, violation of the ontogenetic regulation) are due to changes in the redox balance in chloroplasts and increased production of ROI in the leaves [44, 57, 89]. Therefore, it is necessary to search for mechanisms improving the redox status of such plants. If to consider that Chlb is primarily needed at a shortage of light and for rapid rearrangements of the photosynthetic complexes caused by light flecks under the forest canopy, for agricultural crops, when grown in open spaces, many other effects of reducing chlorophyll b contents will be negligible. Furthermore, the reduction of the costs for synthesis of unnecessary for photosynthesis antenna proteins, which constitute a considerable portion of proteins in chloroplasts, will allow plants to save some resources, and the reduced light absorption by the leaves will increase the amount supplied to the leaves of lower layers in thick plantings. Our studies have shown the possibility of formation of highly productive phenotype of *chlorina f2 3613* barley mutant, when grown in open ground [36].

So, the recently discovered Chlb function associated with the regulation of the ontogenesis phases in plants deserves a detailed study. A convenient model may be mutants of *CAO* gene for Chlb biosynthesis, i.e. *chlorina-f2* (barley), *ch1* (*Arabidopsis*), as well as mutants of the *NYC* genes encoding isoforms of enzymes of Chlb catabolism. The results of such studies will be used in breeding, and also in the improvement of agrotechnical methods to ensure timely flowering and prevent early plant senescence. In general, the study of mutants with the changed Chlb biosynthesis is of practical interest to identify new mechanisms for increasing photosynthesis and crop yield.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 856-868
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 631.522/.524:581.1:581.144.2

doi: 10.15389/agrobiology.2017.5.856rus
doi: 10.15389/agrobiology.2017.5.856eng

MOLECULAR, GENETIC AND HORMONAL OUTLOOK IN ROOT BRANCHING

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (grant № 16-16-00089). Study of the role of auxin in lateral root initiation in *Cucurbitaceae* was supported by Russian Foundation for Basic Research (grant № 14-04-01413-a)
Received November 15, 2016

Abstract

The most important function of any plant root system is the supply of mineral nutrients. The soil is a heterogeneous environment characterized by irregular distribution of nutrients. The branching of the main root which leads to the formation of the root system is regulated by the necessity of compensation for this unpredictable environment. Different types of root systems may reflect different strategies of adaptation of vascular plants to land (L. Kutschera et al., 1997). In recent years, a vast array of experimental data on this subject has been collected. Investigations were carried out on the model plant *Arabidopsis thaliana* (J.G. Dubrovsky et al., 2001; B. Parizot et al., 2012; J.G. Dubrovsky et al., 2017) as well as on a wide range of crops (cereals, crucifers, gourds, buckwheat etc.). The accumulated data allow the identification of economically important traits of root systems that can be exploited to design breeding strategies to optimize root system function. This review contains an analysis of the current data on cellular, molecular genetic and physiological mechanisms of lateral root initiation and development. The phytohormone auxin performs multiple functions during lateral root initiation (Y. Du et al., 2017). It participates in the earliest stages by determining of competence for the first division by pericycle cells that leads to primordium formation. Furthermore, auxin facilitates the emergence of the primordium from the parental root cortex. Recent studies have shown that the formation of the lateral root begins with the oscillation of auxin concentrations in the basal part of the parental root meristem and the formation of an auxin response maximum in some cells of central cylinder (I. De Smet et al., 2007; K.H. ten Tusscher et al., 2017). The next stage is the specification of founder cells in the pericycle and the subsequent formation of the prebranch site (M.A. Moreno-Risueno et al., 2010). Questions ranging from the mechanisms that determine which pericycle cells can become founder cells for lateral root primordia, the mechanisms of regulation of cell proliferation, the positioning of lateral roots along the axis of the parental root, and hormonal factors and their targets, all leading to the successive development of lateral roots, are discussed in this review. Data on the role of auxin in this process and on the mechanisms of auxin signal transduction in the course of lateral root initiation are provided. The key factors involved in the determination of the competence of pericycle cells to initiate lateral root primordia are the transcription factor GATA23 (B. De Rybel et al., 2010) and the membrane-associated kinase regulator MAKR4 (W. Xuan et al., 2015). Special attention is paid to the role of neighboring cell layers in the control of the initial stages of cell proliferation in the pericycle that result in the formation of a new organ. However, there are a number of families among flowering plants in which the initiation and development of lateral root primordia occurs directly in the parental root meristem (J.G. Dubrovsky, 1986, 1987; K.N. Demchenko et al., 2001; E.L. Ilina et al., 2012). For the first time, data on the key role of auxin in lateral root primordia initiation in these species, in particular in *Cucurbitaceae*, are presented in this review, and the mechanisms that open the opportunity for early and rapid branching of the main root are discussed. Special attention is paid to evolutionary mechanisms of branching site determination in flowering plants.

Keywords: auxin, cell proliferation, lateral root initiation, meristem, root branching, root development, transcriptional factors

Numerous studies have shown the relationship between the genetically determined root traits and the productivity of crops [1-3], including that under drought conditions [4]. For selection for improving the properties of root systems, it is necessary to identify those features of the root that allow the plant to use water and nutrients most effectively under different conditions. It is important to identify the genetically determined features of the root, which lead to increased yield and resistance to stress. The success of the selective change in the architecture of the root system in crops depends on the specific feature and nature of its inheritance, as well as on the use of a particular system of farming and soil characteristics [5].

The root system of the plant ensures the absorption of water and nutrients necessary for growth and development, fixing plants in the soil, storage of reserve constituents. In addition, it interacts with the roots of other plants, soil microorganisms and fungi. The root system is a dynamic formation that is affected by environmental factors [6-8]. The ability of the root to adapt in response to a change in the moisture level and the amount of nutrients in the soil makes it possible to study the natural plasticity of the root in order to identify its features, which can increase the yield [9-11]. The study of molecular mechanisms that control the architecture of the root system in crops [5] is also of interest. Strategies of study of the root system development include methods for direct and reverse genetics, the use of *Arabidopsis thaliana, Medicago truncatula* and *Brachypodium distachyon* mutants, and identification of loci of quantitative characters that determine the phenotypic variability of the root in populations [12-13].

This review presents current understanding of the molecular genetic mechanisms and key genes involved in the earliest stages of initiation of the primordium of the lateral root. For the first time, we present comparative data on the hormonal mechanisms of initiation of the lateral root in various root zones. Particular attention is paid to the evolutionary mechanisms for determining the location of initiation of the lateral root.

Cell-based mechanism of initiation of the lateral root. The root system consists of the main root and lateral roots of different orders. In most species of flowering plants (monocotyledons and dicotyledons), lateral roots are formed endogenously in the pericycle and extend to the surface much higher than the zone of extension of the maternal root [14, 15]. In the apical meristem of the root, the initial cells proliferate, separating the sister cells that are constantly moving away from the root tip (the age structure of the cells along the root axis), pass from the apical meristem to the stretch zone, reaching a finite size, and acquire functional characteristics of their type in the differentiation zone [16-18]. The study of *lhw*, *wol* and *ivad* mutants in *Arabidopsis* showed that the heterogeneity of the pericycle and the organization of conducting tissues are regulated by the same cascade of genes and are determined in the meristem at the early stages of development [19]. The first cellular events in the initiation of the lateral root, which include the migration of the nucleus of two neighboring cells of the pericycle and subsequent unequal division of these cells, in *Arabidopsis* are detected at a distance of several millimeters from the root tip [20, 21]. Despite this, the group of pericycle cells opposite to the xylem pole, which will take part in the initiation, is also determined in the basal part of the apical meristem of the root [22-24].

The role of auxin in the regulation of the initial stages of initiation of the lateral root. Auxin plays a leading role in regulation of the development of the lateral root [25-28]. The family of proteins-repressors of

Aux/IAA auxin signaling suppresses the work of the ARF (Auxin Response Factor) transcriptional factors group. At physiologically low auxin concentrations, Aux/IAA proteins form dimers with ARF transcriptional factors, preventing their binding to DNA and transcription of auxin-sensitive genes. Auxin regulates morphogenetic processes through rapid ubiquitin-mediated degradation of Aux/IAA proteins. At physiologically high concentrations, it binds to the receptor F-box protein TIR1 (Transport Inhibitor Response 1), which is included in the oligomeric complex SCFTIR1 with ubiquitin-ligase activity [30], which leads to proteolytic degradation of Aux/IAA in the 26S proteasome and release of the transcriptional factors ARF [31]. In *Arabidopsis*, Aux/IAA and ARF proteins are encoded by extensive gene families. Growth processes are regulated through a specific interaction between conjugate synthesized ARF and Aux/IAA proteins.

The most important question in the study of the morphogenesis of the root system is which genetic factor or group determines the program for the development of the founder cells of the lateral root and regulates the spatial distribution of primordia along its longitudinal axis. I. De Smet et al. [23] showed that the cellular response to auxin oscillates in the basal part of the root meristem at intervals of 15 hours, which reflect peaks in the activity of the auxin-sensitive DR5 promoter. It is believed that this particular oscillation serves as a mechanism that determines the marking of the initial primordium cells of the lateral root [33].

Genetic targets for auxins. The transcriptional factor *GATA23* is one of the targets of the mediated ARF action of auxin in the founder cells of the lateral root in *Arabidopsis* [34-36]. *GATA23* relates to the B-class of GATA proteins and is characterized by the degenerated domain of LLM (leucine-leucine-methionine). The *GATA23* gene is specific for *Brassicaceae*; its orthologs have not yet been identified in other families [37, 38]. *GATA23*, identified in connection with initiation of the lateral root in the meta-analysis of transcriptomic databases in *Arabidopsis*, is the earliest indicator of lateral root development [36, 39]. *GATA23* is expressed in all pericycle cells at the end of the stretch zone, and in pericyclic founder cells expression occurs before their first asymmetric division initiating the lateral root. In RNAi plants with suppression of *GATA23* expression, the number of primordia of lateral roots decreases (both left from the maternal root and stopped in development at earlier stages). Increased expression of *GATA23* leads to an increase in the frequency of ectopic primordia formation and previous increase in the number of founder cells of the lateral root.

As a result of the study of consecutive time points of expression of the auxin-sensitive structures *pDR5::GUS* and *pGATA23::GUS* in the roots of *Arabidopsis*, a relationship between the expression of *GATA23* and the cellular response to exogenous auxin processing was established [36]. In the basal part of the meristem, the oscillating peaks of *pDR5::GUS* activity in the protoxylem cells and the related local expression of *pGATA23::GUS* in the cells of the pericycle of the xylem pole begin 10 hours after the peak of the cellular response to auxin, which is approximately equal to the duration of the mitotic cycle. Therefore, the expression of *pGATA23::GUS* depends on the TIR1-mediated pathway of the auxin signal transmission in the basal part of the meristem.

A study of the expression of *GATA23* in *aux/iaa Arabidopsis* mutants showed that both the relative expression and the promoter activity of the *GATA23* gene were reduced only in the mutant with overexpression of the *iaa28-1* gene. In *iaa28-1* mutants, the number of primordia of lateral roots decreased; hence, the expression of the *IAA28* gene is related to the mechanism for forming the competence of pericycle cells to the formation of lateral roots. The ectopic expression of

GATA23 in the pericycle cells of the xylem pole in *iaa28-1* leads to a phenocopy of the wild type [34]. That is, the GATA23 protein as a component of the TIR1-IAA28 signaling system works after IAA28. A number of ARF factors (ARF5, ARF6, ARF7, ARF8 and ARF19), interacting with the IAA28 protein and synthesized in the basal part of the meristem, have also been detected. The expression of *GATA23* was completely absent in the double mutants *arf7arf19*, which indicates the involvement of ARF7 and ARF19 in activation of *GATA23* and initiation of the lateral root. Thus, the first molecular component of the specification of the pericycle cell was identified, which establishes the competence of the pericycle cells in the basal part of the meristem to participation in the initiation of the lateral root. The expression of the *GATA23* gene is considered to be the earliest event associated with the initiation of the primordium of the lateral root. *GATA23* controls the initial stage of the specification of the founder cells of the root, although its expression is not localized only in them. A mechanism by which the oscillation of maxima of auxin concentration and expression of *GATA23* are precisely established and correlated in the meristem of the stretch zone is of interest [15]. It is necessary to identify the positional signals, due to which the specification of the pericycle cells occurs and the competence to the formation of lateral root is formed.

It was also shown that indole acetic acid (IAA) formed in the root cap of indole-3-butyric acid (IBA) modulates the amplitude of the oscillation of IAA concentration in the root meristem [40, 41]. This oscillation, in turn, determines whether a competence zone will be created for the formation of the lateral root (the so-called prebranch site) [42]. Studies of the *Arabidopsis* transcriptome have made it possible to identify a new, regulated IBA root marking component – MEMBRANE-ASSOCIATED KINASE REGULATOR4 (MAKR4) [41]. It transforms competent cells into the initial cells of the future primordium of the lateral root. In the authors' opinion, the space-time root marking is determined by the transformation of IBA into IAA in a cap and the subsequent launch of *MAKR4* expression [41]. In addition, AtMYB93 from the subfamily R2R3 MYB (MYELOBLASTOSIS), the expression of which is induced by exogenous auxin in the basal meristem, can be potentially involved in the formation of oscillations of the endogenous auxin and specification of the initial cells of the primordium [43]. These studies allowed suggesting the concept of prebranch sites, the appearance of which is regulated by cyclic apoptosis of the root cap cells [44].

Control of the resumption of the cell cycle. Asymmetric divisions of the pericycle cells, which in the future will give rise to the primordium of the lateral root, are controlled by the activity of the cell cycle [45-50]. In *Arabidopsis* and other flowering plants, in which primordia are formed above the stretch zone, in order to implement the program of the lateral root initiation, it is necessary for pericycle cells to leave the cell cycle in the G1 phase [46, 51-52] at the end of the meristem. However, before asymmetric division, they must be ready to resume proliferation [51, 53]. It is assumed that the *ABERRANT LATERAL ROOT FORMATION 4 (ALF4)* gene, encoding a poorly studied protein with nuclear localization [47], is important in order to determine the ability of pericycle cells to continue proliferation. In *alf4* mutants, the initiation of the lateral root was disrupted [27, 45, 47]. The functional role of ALF4 is still poorly understood. In *alf4* mutants, the formation of calluses is also disrupted [54]. ALF4 is probably needed to ensure the competence of pericycle cells to resume proliferation when forming a lateral root above the stretch zone. The presence of the product of this gene allows the cells to be in a state of temporary rest before the first division, which initiates primordium of the lateral root.

The transition of a portion of the pericycle cells at the xylem pole above

the stretch zone from the stage G_1 to the stage S (resumption of proliferation) and their subsequent division are stimulated by auxin. These cells resume the mitotic cycle after reaching the initiation zone of the lateral roots [51, 55]. Most likely, switch genes activating the mitotic cycle are not able to start the process of forming the lateral root without additional stimulation with auxin [48].

SKP2A (S-Phase Kinase-Associated Protein 2A) is an *Arabidopsis* F-box protein that regulates the proteolysis of transcription factors affecting the mitotic cycle. Auxin activates ubiquitin-dependent degradation of SKP2A protein by directly binding to it. SKP2A stimulates the degradation of E2FC/DPB and induces the proliferation of root meristem cells. Also, auxin enhances the interaction between SKP2A and DPB. That is, SKP2A is an auxin-binding protein that coordinates the transmission of the auxin signal with cell proliferation [56].

The transcriptional factor E2F stimulates the transition to asymmetric cell divisions during the initiation of the lateral root [49, 57]. The expression of *E2Fa* is regulated by the dimer of the transcriptional factors LBD18/LBD33, which in turn is associated with the transmission of the auxin signal [57]. LBD18/LBD33 serves as a link for the formation of the lateral root by activating the transcription of *E2Fa*. The initiation of transcription of *E2Fa* by LBD factors is a common mechanism of auxin-dependent activation of the mitotic cycle [57].

The formation of the competence to initiate the lateral root (prebranch sites) in the pericycle cells in the basal part of the meristem occurs, in the opinion of some authors, due to the formation of a local maximum of auxin in the adjacent protoxylem cells [23]. This blocks the transition to the S phase in the cells of the pericycle. Such cells leave the meristem at the end of the G_1 phase and at the end of the stretch zone, and are able to resume cyclic motion, resulting in two synchronous divisions initiating the lateral root [51]. Subsequently, the ability of the pericycle to resume proliferation is determined with the participation of D-type cyclin *CYCD4;1* [58]. A decrease in the expression of *CYCD4;1* in the pericycle under the influence of the local maximum of auxin in the basal part of the meristem leads to a stop in the proliferation of some pericycle cells in G_1 (before DNA synthesis) [59]. In the formation of primordia of the lateral root above the stretch zone, the resumption of the pericycle cells motion in the S phase is accompanied by the formation of a new local maximum of auxin due to its transport from the endoderm [60]. This allows continuing the proliferation of cells and forming an axis of primordium. Thus, the synchronous priming of two adjacent pericycle cells determines the point of initiation of the lateral root. In our opinion, it is the simultaneous resumption of proliferation through the transition from G_1 to S in these cells and the cells of two adjacent rows of the pericycle determines the exact location of initiation of the formation of the lateral root.

Role of the cell environment in the initiation of the lateral root. In the recent time, the regulatory role of the cell environment of the pericycle in the initiation zone of the lateral root has been widely discussed in the literature [60-64]. The function of mechanical interactions between the pericycle and endoderm cells in the process of initiation and development of the lateral root was studied [64]. It was shown that even before the first division leading to its initiation, the growth and increase in the volume (protrusion) of two adjacent pericyclic cells and the simultaneous decrease in the adjacent endoderm cells occur. Further, the expression of the *GATA23* gene is activated and the first unequal anticlinal division occurs. Auxin signal from pericycle cells should be perceived in endoderm cells. In order to study this relationship, the *Arabidopsis CASP1pro::shy2-2* line was created, which has a specific suppression of the response to auxin in endoderm cells [64]. *SHORT HYPOCOTYL 2*

(*SHY2*), the gene of repressor of the auxin response, was controlled by the promoter of the *CASP1* gene, the protein of which is associated with Casparian strips. In plants expressing the *CASP1pro::shy2-2*, the development of the lateral roots was blocked until the first asymmetric division. Processing such plants with exogenous auxin (naphthylacetic acid) induced the development of a small number of lateral roots, but primordia did not reach the root surface and were flat, which indicates the need of the response to the auxin in the endoderm cells in order to release the lateral root. Indeed, endodermal cells in *CASP1pro::shy2-2* plants remained bulky, although they normally decrease and allow the lateral root to grow.

With the disruption of the endoderm by the laser, proliferation in the pericycle resumed; however, the division plan changed from anticlinal to periclinal, and the development program of the lateral root primordium did not start [63]. The resumption of proliferation in the cells of the pericycle occurred regardless of their position on the longitudinal axis of the root. The disruption of xylem, bark and rhizodermis cells did not affect the resumption of divisions in the pericycle. In the authors' opinion, all the pericycle cells in *Arabidopsis* are potentially capable of resuming proliferation, but adjacent endoderm cells block this transition [63]. Indirectly, this is confirmed by the ability of the roots of *Arabidopsis*, as well as other representatives of *Brassicaceae*, to form multiple lateral roots after treatment with auxins in high concentrations (up to 90 μM) [65, 66].

In *Arabidopsis* yucca mutants with increased auxin biosynthesis [67, 68], endoderm disruption did not lead to a change in the plan of pericycle cells division from anticlinal to periclinal [63]. In mutants for the transmission of the auxin signal *tir1/afb2/afb3* (*transport inhibitor1/auxin signaling f-box2/afb3*) and *slr/iaa14* (*solitary root/indole-3-acetic acid14*) with multiple root development disruptions in the destruction of the endoderm, the pericycle cells changed the division plan into periclinal, as well as in wild-type roots. However, when processing the roots of the *tir1/afb2/afb3* mutant with exogenous auxin, the number of reorientations of divisions decreased. The treatment of wild-type roots with a destroyed endoderm with naphthylphthalamic acid (auxin transport blocker) did not affect the change in the plan of pericycle cells division. When the endoderm was destroyed, they became periclinal, as in control plants. Perhaps the change in the plan of the pericycle cells division depends on the preservation of the pathway for the transmission of the auxin signal, but not on the auxin transport [63].

A local increase in the auxin content in the group of pericycle cells not only forms their competence for the lateral root initiation, but also starts response to auxin in adjacent endoderm cells [60]. In the latter, a short-term expression of the auxin gene of the *PIN3* transporter occurs, and the synthesized protein is localized on the membrane of the endodermal cell in contact with the pericycle cell. This ensures the outflow of auxin from the endoderm cells to the pericycle cells. The level of expression of *PIN3* in the endoderm begins to decrease 15 hours after the first initiating division, and after 2 hours the protein completely disappears. In the mutant *pin3*, an increase in the number of pericycle cells with a maximum of auxin and a decrease in the number of the first asymmetric divisions were observed. The *PIN3* gene mutation disrupts the transition of the founder cells to the division, initiating primordium. Therefore, due to PIN3-mediated outflow of auxin from the endoderm, the amount of this hormone in the pericycle cells increases again, which stimulates their transition to the first asymmetric division.

Mechanism for determining the size and shape of the primordium of the lateral root. The number of cells participating in the divisions that specify the diameter of the primordium is limited by the receptor-like ACR4 kinase [69]. The volume of primordium is obtained due to the peri-

clinal divisions, increasing the number of layers. At this stage, the formation of a correct dome-shaped primordium is regulated by several mechanisms, including through a directed auxin flow [63]. The *MYB36* gene is expressed in the pericycle at the base of the primordium, which begins at the stage V of development [70]. It was shown that *MYB36* directly participates in the control of the borders of the primordium, since the *myb36-5* mutant has increased number of cells along the width of the primordium. *MYB36* is required for the transition from flat to domed shaped primordium. At the same time, cell divisions stop at the periphery and the final width of the primordium is determined. The expression of *MYB36* at the level of mRNA and protein occurs in some pericycle cells without transmitting signals to the surrounding endodermal cells, as shown for the SHY2-mediated response to auxin [60, 64].The expression of the *PER9* and *PER64* peroxidase genes belonging to secondary targets of *MYB36* is greatly reduced in the *myb36-5* mutant. Probably, as a result of the above, it has a higher content of hydrogen peroxide, since the treatment with potassium iodide (peroxide absorber) resumed the development of primordium in mutant roots. Thus, the final size and shape of primordium depend on the sum of the signals that stimulate or inhibit the proliferation in its cells. These signals include active forms of oxygen, the amount of which is indirectly regulated by the *MYB36* gene.

The data presented show that the main genetic processes of determining the competence of pericycle cells and initiation of primordium of the lateral root in *Arabidopsis* have been sufficiently studied. However, there is a group of plants in which initiation and development of primordium of the lateral roots occur directly in the apical meristem of the main root. This type of the lateral roots formation is characteristic for species from the *Cucurbitaceae* [71-75], *Polygonaceae* [76], and *Convolvulaceae* families, and some aquatic plants from the *Pontederiaceae* [75, 78] and *Araceae* [79] families. In addition, due to the formation of groups of primordium of the lateral roots in embryogenesis, these species undergo an early branching of the main root during germination [72, 80]. Rapid development of a powerful root system makes it possible to successfully compete with representatives of other species for soil resources and to gain significant biomass. The regulation of root formation in these plants has not been studied.

The results obtained by us in studying the cellular and hormonal mechanisms of the initiation of the lateral root of courgette (*Cucurbita pepo*) clearly indicate that the initial stages of the determination of the pericycle and endoderm cells, as well as their transition to the first anticlinal division in the species of the *Cucurbitaceae* family, are identical to the processes in *Arabidopsis* and other plants initiating the lateral root above the stretch zone [81]. Thus, the first stage of determination is the appearance of a local maximum of the cellular response to auxin in pairs of sister cells of three internal rows of the pericycle, two rows of the outer pericycle, and a row of endoderm. For *Arabidopsis*, simultaneous activation of cell pairs of three rows of the pericycle on the xylem pole was shown [55]. In courgette, the first anticlinal division is preceded by the formation of a local maximum of the cellular response to auxin in two adjacent cells in the row. In *Arabidopsis* and cereals, in addition, there is a directed movement of the nucleus of these cells towards each other [36, 73].

Consequently, the first divisions initiating the lateral root, regardless of the place of its initiation, are the anticlinal divisions of a pair of sister cells. Some difference between the representatives of *Cucurbitaceae* is the absence of unequal anticlinal divisions and migration of nucleus, since all the initiation processes take place in the root meristem and the cells do not stretch. According to

our data, in the courgette when initiating the lateral root in the meristem, there is no resumption of the mitotic cycle from the G_1 phase, as in the formation of the lateral root above the stretch zone [51, 53]. As a result of one of the anticlinal divisions in the pericycle row (as for the endoderm), two sister cells of origin are formed. Their further advancement along the mitotic cycle G_1-S-G_2 will be accompanied by the occurrence of a local maximum of auxin and will be finished with the first anticlinal division. It will be the first division in the initiation of the primordia of the lateral root.

Thus, we assume that the physiological and molecular genetic mechanisms of initiation of the lateral root in different groups of plants have a single origin. Ancestral forms of all flowering plants had endogenously formed rudiments of lateral roots, and the site of initiation of the lateral root was located in the immediate vicinity of the initial cells of the apical meristem. In the course of evolution, the site of initiation of the lateral root gradually shifted from the apex cells to the basal part from the meristem of the parent root beyond the stretch zone. However, in some families of flowering plants, the archaic type of initiation of the lateral root preserved, i.e. directly in the apical part of the meristem of the parent root. When the site of initiation changed, there was also a decrease in the role of tissues surrounding the pericycle in the formation of temporary primordium structures.

So, in the studies of cellular, molecular genetic and physiological mechanisms of initiation and formation of lateral roots, extensive factual material has been accumulated, but many processes accompanying the formation of a new organ on the maternal root still need to be studied. We still do not know how the cells of origin of the lateral root receive a signal and what leads to the determination of competence of the cell to further formation of the lateral root in this place. Studies of regulatory gene networks have only begun. The prompt resolution of these issues will allow us to proceed to the selection based on the characteristics that determine the ability of root systems to better adapt to changing environmental conditions, and, ultimately, to increase yields.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 869-877
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.31/.37:631.461.52:577.21

doi: 10.15389/agrobiology.2017.5.869rus
doi: 10.15389/agrobiology.2017.5.869eng

NCR PEPTIDES — PLANT EFFECTORS GOVERNING TERMINAL DIFFERENTIATION OF NODULE BACTERIA INTO THE SYMBIOTIC FORM

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported by Russian Science Foundation (grant № 14-24-00135); V.A. Zhukov is supported by grant № 14-04-01442-a form Russian Foundation for Basic Research
Received December 12, 2016

Abstract

Uptake of mineral nutrients from the soil is the challenge of plant survival. In particular, the availability of such macro-elements as nitrogen and phosphorus is the limiting factor for plant growth and development. Some plant genera overcome this limitation by establishing symbiotic relationships with microorganisms. A remarkable example of such symbiosis is one between legumes and rhizobia — a group of nitrogen fixing soil bacteria. Rhizobial penetration into roots of a specific host plant causes initiation of a specialized organ, symbiotic nodule. Within cells of symbiotic nodule free-living bacteria differentiate into a symbiotic form called «bacteroids». Such organelle-like structures provide plants with fixed nitrogen in exchange for nutrients (B.J. Ferguson et al., 2010). A number of legumes form nodules, in which bacteria terminally (irreversibly) differentiate into bacteroids, thus losing the opportunity to return to the free-living state. Terminal differentiation of bacteroids begins soon after release of the rhizobia into plant cells and leads to morphological, physiological and genetic changes in bacterial cells. It has been shown that a large family of antimicrobial peptides of plants called Nodule-specific Cysteine-Rich peptides (NCR peptides) plays a key regulatory role in this process (P. Mergaert et al., 2003). Its representatives are similar in structure and mode of action to defensins — plant innate immunity factors; however, NCR genes are expressed only in nodules, which fact is reflected in their name. At the moment, about 700 genes encoding NCR peptides that are highly variable in their amino acid sequence but possess a distinct conservative cysteine motif required for the adoption of correct conformation were identified in the genome of the model legume *Medicago truncatula* Gaertn. NCR peptides are delivered to their intracellular target symbiosome (cell compartments containing bacteroides) triggering the process of differentiation by interacting with the components of membranes and various intracellular targets of bacteria (D. Wang et al., 2010). The most studied member of this family in *M. truncatula* is MtNCR247 a cationic peptide with four cysteines forming two disulfide bonds in oxidized form. MtNCR247 affects transcription, translation and cell division processes in *M. truncatula* microsymbiont *Sinorhizobium meliloti* at low concentrations, and also exhibits antimicrobial activity at higher concentrations (A. Farkas et al., 2014). To date, NCR peptides are identified only in plants belonging to IRLC (Inverted Repeat-lacking Clade) legumes which are characterized by terminal differentiation of bacteria into bacteroids. Probably, evolutionary acquisition of the variable gene family encoding NCR peptides has been the selective advantage of this group of plants.

Keywords: rhizobium-legume symbiosis, nitrogen-fixing nodules, differentiation of bacteroides, NCR-peptides, regulation of symbiosis development

The legume family (*Fabaceae*) includes primary food and feed species of cultivated plants such as peas, soybeans, clover, chickpeas and lucerne. It is the third largest group of angiosperms and the second one of food and feed crops grown worldwide [1]. The important environmental advantage of legumes is the

opportunity to grow with virtually no fixed nitrogen in the soil through fixation of atmospheric nitrogen by symbiotic nodule bacteria [2, 3].

When establishing nitrogen-fixing symbiosis, rhizobia selectively penetrate into roots of legumes, which leads to the development of special root structures called nodules [4]. The ontogeny of nodules is a well-organized process based on the coordinated expression of specialized plant and bacterial genes [5[. A large part of a complex system of genes in plants that control the nodule development is needed to control microsymbiont by a host plant.

Inside the nodule, the bacteria differentiate into bacteroids and carry out symbiotic nitrogen fixation, i.e. reduction of molecular nitrogen to ammonium ion using the enzymic complex of nitrogenase [6, 7]. In the legume nodules belonging to the IRLC (Inverted Repeat-lacking Clade) group, the transformation of rhizobia into bacteroids is irreversible (terminal differentiation); in other plants, it is a reversible process [8-10]. If the rhizobia strain is capable of nodulation on the roots of a wide range of plants, the degree of bacteria differentiation into bacteroids and its reversibility strictly corresponds to species of a host plant, from which it follows that the differentiation process is induced through plant signals [11-13].

In *Medicago truncatula* model legume, as such factors there may be at least 800 nodule-specific symbiotic peptides, the majority of which (over 700) belong to the group of nodule-specific cysteine-rich (NCR) peptides [14-17]. NCR peptides have been also described for other legumes belonging to the same IRLC clade, i.e. beans (*Vicia faba*) [18], white clover (*Trifolium repens*) [19], Eastern galega (*Galega orientalis*) [20], and English peas (*Pisum sativum* L.) [21-23]. NCR peptides have been identified in experiments on identification of nodule-specific protein molecules (nodulins) [21], but not all nodulins refer to NCR peptides.

The aim of the present article is generalization and structuring of information accumulated over the last decade, in relation to the regulation of symbiotic bacteria differentiation in establishing nitrogen-fixing symbiosis. For the first time, a complete characterization of the numerous protein NCR peptides family has been given. The issues of their structure, functions, targets, modes of action and its intended path of evolution have been also discussed in detail.

The genes of NCR peptides belong to the extensive group of defensin-like genes, various representatives of which are found in the genomes of vertebrates (encode proteins involved in the acquired immunity), invertebrates (e.g., encode a component of the scorpion toxin) and plants [14, 24, 25]. In turn, defensins are a group of antimicrobial peptides (AMP), which are produced by almost all living organisms and play a key role in the innate immunity [26-28]. The general mode of action of antimicrobial peptides is the disruption of microbial membranes and (or) inducing the formation of pores, which leads to lysis of bacterial cells and also makes possible the interaction of peptides with intracellular targets (DNA, RNA and various proteins) [29-31].

NCR peptides, along with plant defensins, by amino acid composition belong to a vast family of cysteine cluster proteins (CCPs) containing a conservative cysteine cluster of 4, 6, 8 or 10 cysteine residues in conservative positions [32-34]. Nodule-specific CCPs, that is, NCR peptides, represent one of 10 CCP subgroups [34, 35]. Like the genes of defensins, the genes of NCR peptides encode short (30-60 amino acid residues) secretable polypeptides with a high variability in amino-acid sequence, which determines their specificity and different mechanisms of action [14, 36, 37]. Unlike defensins, the key function of which is involvement in protective processes and a negative impact on bacteria, NCR peptides play a positive regulatory role in the nodules of legumes controlling the rhizobia differentiation into nitrogen-fixing bacteroids [11, 38, 39]. The antimicrobial activity

of NCR peptides is reflected in the fact that the differentiation into bacteroids under their action is irreversible. Furthermore, the bacteria lose their ability to reproduce.

The expression of the genes encoding NCR peptides is specific to the nodules. However, certain groups of genes are activated together, resulting in several successive "waves" of their expression at different stages of nodule development [40]. Just synthesized NCR peptides contain an N-terminal signal sequence that defines their transport to the endoplasmic reticulum [11, 14]. In the transport of NCR peptides, the key role is played by signal peptidase cutting off the signal sequence from the mature peptide in sorting proteins on the endoplasmic reticulum [41]. In *M. truncatula* mutants of *MtDNF1* gene encoding a nodule-specific signal peptidase subunit, NCR peptides retain the signal sequences and accumulate in the endoplasmic reticulum, without getting into symbiosomes resulting in the absence of bacteroids differentiation [11, 16, 41]. In the case of the normal development of symbiosis, with the gene expression different sets of NCR peptides are delivered to the endosymbiont and mediate the subsequent events of its differentiation [16].

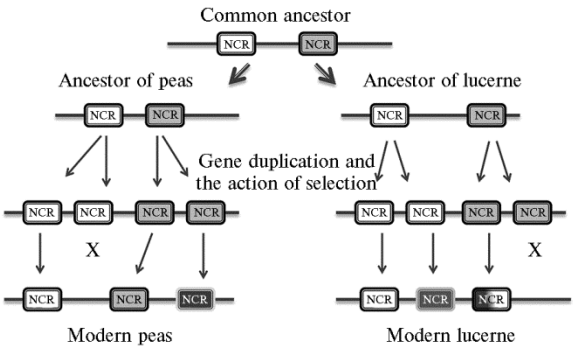
For some NCR peptides (e.g., MtNCR247 and MtNCR335), an ability to interact with the membrane of bacterial cells has been shown, which in vitro leads to the loss of both rhizobia and human pathogens and plants [11, 42]. However, in vivo (in the nodule cells) symbiotic peptides do not violate the permeability of the bacterial membrane so much that it should lead to cell lysis [31]. Probably, the increase in the permeability of the membrane promotes the penetration of NCR peptides into the bacterial cells.

Of bacteroids present in the cells of *M. truncatula* nodules, about 140 different NCR peptides can be identified, which indicates the possibility of their penetration into the bacterial cell, as well as their high stability and potential interaction with intracellular targets [43]. One of MtNCR247 targets is bacterial protein FtsZ, which plays an important role in the formation of the cell wall in the cell division [44-46]. Another partner of MtNCR247 is chaperone GroEL, which is required for full activation of nodulation genes and assembly of nitrogenase complex [47]. Also, MtNCR247 exposure changes the expression of some regulatory genes critical to the cell cycle progression (*ctrA, gcrA, dnaA*), which might be related to stopping the proliferation of bacteria in the plant cells [45].

Rhizobia have protective mechanisms to withstand the effects of vegetable NCR peptides [48]. In particular, some protection against NCR peptides is provided by the *BacA* gene encoding the protein of the family of ABC transporters [49-51]. Thus, *S. meliloti* mutant of the *bacA* gene shows hypersensitivity toward MtNCR247 in vitro and in vivo (bacteria are degraded soon after release of the lucerne nodule into cells, while bacterial strains of the wild type remain viable and are differentiated into bacteroids) [52-54]. In addition, the metallopeptidase gene *HrrP* (host range restriction peptidase), encoding enzyme capable to cut NCR peptides, has been described [55, 56]. Some *Bradyrhizobium* strains for a successful nitrogen-fixing symbiosis require the presence of styrole-like lipids (hopanoids) in the membrane, probably, enhancing its strength and providing protection against NCR peptides [57, 58].

An important feature of the gene family of NCR peptides is their clustered organization within the genome. The Clusters of genes, which encode NCR peptides, are evenly distributed on eight chromosomes in *M. truncatula* and, apparently, originated from the repeated duplication and subsequent diversification of sequences [9]. Since the promoter regions of genes encoding NCR peptides are also similar, their expression is highly consistent. Sequencing RNA samples from the nodule zones, carved out of the preparation by laser micro-dissection,

showed that the genes of NCR peptides can be divided into several groups based on their spatial-temporal expression profile [16]. Obviously, the expression of certain groups of NCR peptides genes is needed only at the specific stage of nodule development [40].



Scheme of the proposed evolution of NCR peptides genes of *Medicago truncatula* and *Pisum sativum*. Genes of NCR peptides originated about 25 million years ago in the common ancestor of the IRLC (Inverted Repeat-lacking Clade) group. Subsequently, the duplication, diversification and selection in favor of the highest functionality led to the emergence of a large number of genes encoding antimicrobial peptides with low sequence conservation, which became the basis of the diversity of their activity and physiological functions. Some of NCR peptides gained unique functions distinguishing them from the main group and essential for successful symbiosis; part of the sequences was becoming pseudogenes and was losing a biological function. The color indicates the degree of differences in the variability of the nucleotide sequences of NCR peptides genes and their changes in the evolution (mutation and recombination). X means that the gene, which encoded NCR-peptide, due to mutation evolved in pseudogene and lost its original function.

Despite the previously described interchangeability of antimicrobial molecules [14], there is data on the unique properties and functions of NCR peptides, the absence of which interrupts establishment of nitrogen-fixing symbiosis. Thus, MtNCR211, toxic to *S. meliloti* in vitro, is necessary for the survival of bacteroids in planta, because mutation in its gene leads to the death of bacteroids in the nodule cells [60]. Similarly, to maintain bacteroids, MtNCR169 is necessary, in the absence of which their differentiation practically does not take place and, as a result, nitrogen is not fixed [61]. These two NCR peptides are characterized by similar localization in planta [60, 61]. *M. truncatula* mutants of the *MtDNF4* (=*MtNCR211*) and *MtDNF7* (=*MtNCR169*) genes have similar transcriptomic profiles [62]. Despite this, the role of each of these peptides is unique, because in the mutant by the *MtNCR211* gene, lysis of bacteroids occurs after differentiation, and in the mutant by *MtNCR169* gene, undifferentiated bacteria are exposed to lysis [63]. The sequences of NCR peptides genes are extremely variable even within the same species of legumes. The found homologous sequences in different representatives of the IRLC group have a very low percentage of similarity, so the definition of orthologous pairs becomes impossible [64]. Apparently, these plants diverged from a common ancestor, which already had the genes of NCR peptides, a long time ago (Fig.). Subsequently, in the genome of each of them there was duplication of the genes of NCR peptides, which then evolved independently under the influence of a positive (driving) selection [59]. Some genes of different types due to the accumulation of mutations could turn into pseudogenes and be eliminated, so non-orthologic genes began to perform the same biological function. Finally, the similarity of individual sections of sequences of NCR peptides genes indicates the possibility of their variability increase due to illegitimate recombination. The result of the evolution of this gene family was a wide variety of NCR peptides observed in modern representatives of the IRLC group.

According to research of the efficiency of symbiotic nitrogen fixation, bringing bacteroids differentiation to the terminal phase provides the plant (and the symbiotic system as a whole) a significant benefit [65]. Probably, for this

reason the acquisition in the evolution of the family of NCR peptides genes was a selective advantage of IRLC group plants. In addition, NCR peptides are involved in the background immune response of nodules, the activity of which does not depend on whether there are undesirable (pathogenic) microorganisms in them [63]. The activity in the symbiotic plant organs of the genes of NCR peptides related to the plant immune system and quickly evolving towards diversity, together with the presence of rhizobia systems, which resist the lethal effects of NCR peptides, is a good example of co-evolution of partners in the case of nitrogen-fixing symbiosis.

Thus, to date, in the limited group of legumes, a large gene family has been discovered, which has an organo-specific expression. Its representatives perform a function of a terminal, that is, irreversible differentiation of the symbionts inside the nodule. It is assumed that the genes of NCR peptides arose from related genes of plant defensins in the rapid duplication and diversification of sequences. Many of NCR peptides are interchangeable due to the similarity of the structures, but some acquired new, more specialized functions. The roles, the targets and the modes of action of NCR peptides have been described only for several representatives of this family, and therefore, further research may lead to unexpected and valuable discoveries.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 878-894
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.31/.37:631.461.52:577.1

doi: 10.15389/agrobiology.2017.5.878rus
doi: 10.15389/agrobiology.2017.5.878eng

ANTIOXIDANT DEFENSE SYSTEM IN SYMBIOTIC NODULES OF LEGUMES

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (№ 14-04-00383) and by grant from President of the Russian Federation for leading scientific schools (НШ-6759.2016.4)
Received December 22, 2015

Abstract

Nitrogen-fixing nodules are formed on the roots of leguminous plants as a result of their interaction with soil bacteria, called rhizobia. Nodule development is based on the exchange of signaling molecules that leads to coordinated gene expression in both partners. This process is accompanied by differentiation of both plant and bacterial cells leading to formation of infected plant cells, filled with nitrogen-fixing forms of rhizobia, called bacteroids. The bacteroid is separated from the plant cell cytoplasm by the peribacteroid membrane and forms an organelle-like structure called the symbiosome (A.V. Tsyganova et al., 2017). The main function of the symbiotic nodule is to maintain the microaerophilic conditions required for working of the rhizobial nitrogen fixation enzyme — nitrogenase, which is extremely sensitive to oxygen. Nitrogen-fixing nodules produce an abundance of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These are formed due to auto-oxidation of leghemoglobin in the cytoplasm, oxidation of nitrogenase and ferredoxin in symbiosomes, and functioning of electron transport chains in mitochondria, symbiosomes, and peroxisomes (C. Chang et al., 2009). ROS and RNS molecules are involved in different signal transduction pathways; therefore, the nodule antioxidant system cannot simply eliminate ROS and RNS, but must maintain their concentration in the cell at the certain level (C.W. Ribeiro et al., 2015). Most antioxidants presented in plant organs are also found in the nodule, however, at a higher concentration, which is probably due to the high intensity of the processes associated with biological nitrogen fixation. These are enzymes superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, and peroxiredoxins, as well as millimolar concentrations of non-enzymatic elements (primarily ascorbic acid and glutathione) (M. Becana et al., 2010). It has been discovered that Legumes harbor a unique homologue of glutathione, homoglutathione, both of which exhibit similar functions and specificity. However, it is still not clear why some Legumes evolved the ability to synthesize two different thiol compounds and require a double regulatory mechanism of the cell cycle including activation by glutathione and inhibition of cytokinesis by homoglutathione (T. Pasternak et al., 2014). It has now been shown that an increase in the level of glutathione leads to an increase in the efficiency of nitrogen fixation, while there is no similar data for homoglutathione. Considering that for the functioning of the nodule a balance in the ratio of glutathione and homoglutathione is necessary, it is evident that increasing the level of nitrogen fixation by modifying the levels of these thiols is a non-trivial task. Moreover, it is necessary to account for the influence of other components of the antioxidant system. It should be noted that the rhizobial antioxidants play an important role in the functioning of the nitrogen fixing nodule (C.W. Ribeiro et al., 2015). In this review, we will consider the main components of the plant antioxidant system in the nodule. A deeper understanding of its functioning is necessary to develop conditions for increasing the efficiency of biological nitrogen fixation.

Keywords: symbiotic nodule, antioxidants, redox potential, glutathione, homoglutathione, ascorbate, ascorbate-glutathione cycle, thiol peroxidases, redoxins, superoxide dismutase

Formation of a nitrogen-fixing nodule requires implementation of two specific developmental programs, one of which is responsible for its morphogenesis, and the other for formation of infection threads (tubular structures via which the rhizobia penetrate the root) [1, 2]. In response to flavonoids secreted

by legume plants, the rhizobia produce Nod factors (lipochitooligosaccharides from the N-acetylglucosamine residues and fatty acid with different length and degree of unsaturation), which are recognized by plant LysM-receptor-like kinases [3]. Nod factors trigger both development programs; however, for their successful realization additional components are required [4].

Simultaneously with the development of infection threads, the nodule primordium is formed that is associated with mitotic reactivation, dedifferentiation and proliferation of cells [5]. In indeterminate nodules characterized by the presence of a stable apical meristem, the divisions occur in the inner cortex, root endodermis and pericycle [5]. The prolonged functioning of the meristem providing growth and constant renovation of nitrogen-fixing tissue leads to zone formation. Meristem, infection, nitrogen fixation and senescence zones can be distinguished in indeterminate nodule [6]. Only a limited number of evolutionally advanced legume plants from the sub-family of *Papilionoideae*, the tribes *Trifolieae* (clover, alfalfa) and *Vicieae* (pea, vetch) form indeterminate nodules. At the same time, in many papilionaceous plants, such as soybean, beans (*Phaseoleae* tribe) and deervetch (*Loteae* tribe), determinate nodules are formed from the cells of outer cortex with a meristem functioning for the limited period of time [6]. After its disappearance, the growth and renovation of nitrogen-fixing tissues cease, and zones are not identified in the central part of the nodule. The plant cells infected with rhizobia in indeterminate and determinate nodules pass through several endoreduplication cycles, which is accompanied by a significant increase in their sizes and resulted in cell susceptibility to filling with multiple bacteroids [7]. The differentiation of bacteroids in indeterminate nodules is a sequential process [8]; it is accompanied by the amplification of the entire genome and an increase in their sizes. Bacteria lose the ability to reproduce, i.e. their differentiation is irreversible. The bacteroids in the determinate nodules are comparable with free-living bacteria in the amount of genomic DNA, cell size and ability to reproduction [9].

Thus, nitrogen-fixing nodules are formed as a result of multistep differentiation of both symbiotic partners. At each of these steps, the redox cell balance plays a crucial role. Reactive oxygen species (ROS), reactive nitrogen species (RNS) and also the components of plant and bacterial antioxidant defense system are involved in maintaining of that balance in the nodule.

The principal value of the antioxidant defense system is determined, on the one hand, by the sensitivity of the nitrogenase, the main enzyme of atmospheric nitrogen fixation, to oxygen; and on the other hand, by multiple processes facilitating the formation of ROS and RNS in nitrogen-fixing systems. The synthesis of superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) is associated with high respiratory activity required for maintaining effective nitrogen fixation, autooxidation of oxygen-containing forms of leghemoglobin and oxidation of some proteins with high reductive potential (nitrogenase, ferredoxin, hydrogenase). Nitrogen monoxide (NO) is produced in infected cells of functioning nodules [10] by nitrate reductases of bacterial and plant origin [11, 12], and also due to plant NO-synthase activity [13]. Peroxynitrite ($ONOO^-$) can be formed via reaction of O_2^- and NO. The antioxidants prevent development of oxidative and nitrosative stresses in the nodule, modulating the ROS and RNS concentrations and thus allowing them perform various functions in metabolism, including signal interactions during nodule formation [14-16].

Prevention of oxidative stress in plant cells is provided by a complex mechanism. Multiple enzymes such as ascorbate peroxidase, glutathione peroxidase (Gpx), catalase, peroxiredoxin (Prx), and superoxide dismutase (SOD) protect from prooxidants. The redox control of protein activity is carried out by thi-

oredoxin (Trx) and glutaredoxin (Grx) [17, 18]. The non-enzymatic molecules, capable of acting as direct antioxidants, control the redox potential of the cell, thus affecting signal cascades, the cell cycle and the synthesis of various metabolites. Contrary to animal cells where the main non-enzymatic antioxidant is glutathione (GSH), in plants ascorbate (ASC) executes the function of reducing hydrogen peroxide. However, GSH performs a number of unique functions in plant cells, which do not allow replacing it with another thiol or antioxidant [19-21].

During formation of symbiosis the microsymbionts should also cope with the constantly oscillating contents of ROS and RNS. Rhizobia use different strategies in order to modulate the amount of these molecules, including inhibition of their synthesis and detoxication, and also controlling the activity of enzymes. During colonization and bacterial differentiation, a well-coordinated work of antioxidant systems of both partners is required to trigger the signal cascade activated by ROS and RNS. Not only successful development but also further functioning of the nodule depends on this balance. It has been shown that ROS also participate in the senescence of the micro- and macrosymbionts.

The aim of the present review was to generalize the modern views on the plant antioxidant system at different steps of rhizobium-legume symbiosis development and to highlight new components of this system. Special attention was paid to differences in the functions of glutathione and homoglutathione identified till now.

Non-enzymatic antioxidants. The positive correlation between the contents of GSH and ASC, the activity of enzymes participating in the ascorbate-glutathione cycle and the efficiency of nitrogen fixation in nodules suggests the importance of these antioxidants for nitrogen-fixing symbiosis [14, 23, 26-29].

(*Homo*)*glutathione*. The thiol tripeptide GSH is a water soluble antioxidant and redox buffer of plants involved in cell cycle and processes of development, in sulfur transport and accumulation, in response to stress and detoxication of heavy metals [30]. GSH exists in cells in two main stable forms, as reduced and oxidized, the latter is glutathione disulfide (GSSG). Under optimal conditions, the ratio GSH:GSSG in most cellular compartments of the plant is fairly high. The sub-optimal external conditions lead to a shift of this ratio due to accumulation of GSSG which can cause alterations in acceptance and transduction of stress signals. In contrast to many other redox pairs (e.g. $NADP^+$/NADPH), in case of GSH and GSSG the redox potential of GSH depends not only on ratio of these forms, but also on absolute GSH concentration. Thus, even if the value of GSH:GSSG remains unchanged, a reduction in the concentration of GSH form leads to an increase in the redox potential [31].

The GSH synthesis comprises two ATP-dependent steps. The first limiting step of γ-Glu-Cys formation from glutamate and cysteine is catalyzed by γ-glutamylcysteine synthase (γ-ECS), which is encoded by the *GSH1* gene. The second step of GSH synthesis from γ-Glu-Cys and glycine is catalyzed by glutathione synthetase GSHS (*GSHS* gene). Homoglutathione (hGSH), GSH homologue wherein glycine is replaced with β-alanine, was found in the members of the *Fabaceae* family [27, 32, 33]. The synthesis of hGSH is carried out by homoglutathione synthetase (hGSHS) encoded by the *hGSHS* gene. The *Arabidopsis GSH1* knockout mutant is lethal at the embryo stage [34], whereas knockout of the *GSHS* gene leads to seedling-lethal phenotype [35]. The subcellular fractionation and immunolocalization have shown that in nodules γ-ECS is present in plastids, whereas GSHS and hGSHS predominate in cytosol [36, 37]. However, no mutants knocked out only in one of the genes – *hGSHS* or *GSHS* among leg-

ume plants have been obtained so far. This would allow determining the degree of interchangeability of these thiols and possible differences in their functions.

The substantial role of GSH and (or) hGSH in nodule formation was demonstrated for alfalfa (*Medicago truncatula*) using antisense constructs of *GSHS* and *hGSHS*, and also via transcriptomic analysis of plants with a reduced content of (h)GSH as a result of treatment with L-buthionine-[S-R]-sulfoximine, a specific inhibitor of (h)GSH biosynthesis. At the early stages of nodule formation in the plants with reduced synthesis of both thiols, there was an increase in the expression of genes controlled by salicylic acid [38]. The activation of these genes is, apparently, determined by redox-sensitive NPR1 protein that suppresses deformation of root hairs and expression of nodulins. The inactive NPR1, located in cytoplasm, is an oligomeric form resulted from reaction with S-nitrosoglutathione. The monomerization of NPR1, catalyzed by Trx, unmasks the signal motive of nuclear localization allowing for this protein to be transported in the nucleus wherein it interacts with redox-sensitive transcription factors [39]. Therefore, at the early stages of interaction, presence of a certain pool of (h)GSH in plant cells is the required for inhibiting the salicylate-induced defense mechanisms and colonization of plant host by rhizobia [40]. The reduction of the (h)GSH synthesis significantly decreased the number of nodules and suppressed the expression of the early nodulin genes (*MtENOD12* and *MtENOD40*), which are gene markers of nodule formation. At the same time, both in control plants and in plants with reduced thiol level, no alterations were observed in the number of infection sites in the roots, which was confirmed by the similar expression of the *Rip1* gene, marker of infection process.

The inhibition of nodule formation was accompanied by a reduction in the number of the lateral roots [41]. It was shown that GSH is necessary for cell division in the root apex [42], and its amount controls the transition of cells from G_1 to S phase of the cell cycle. The relocation of GSH to the nucleus at the G_1 phase strongly affects the redox state of the cytoplasm and expression of redox-sensitive genes. A further increase in the total cellular pool of GSH above the level observed in G_1 is required for transition of cell to the S phase of the cycle [43, 44]. Apparently, exactly GSH stimulates the meristematic activity in nodules as well that correlates with maximum of GSH concentration in the meristem and in the infection zone of the nodule [45]. It is possible that the fluctuations of GSH pool in cytoplasm and in the nucleus may control the repetitive rounds of endoreduplication of infected cells and also the irreversible differentiation of bacteroids.

Using promoter of a gene, encoding cysteine-rich peptide (NCR001) specific for nitrogen fixation zone of the nodule, the role of the (h)GSH in mature nodules of *M. truncatula* was studied [46]. It was shown that the overexpression of the *GSH1* in the nitrogen fixation zone led to an increase in the GSH but not the hGSH content that correlated with increase in the efficiency of nitrogen fixation. A decrease in the *GSH1* gene expression by RNA interference reduce nitrogen fixation efficiency, (h)GSH content, nodule size, and expression of *TrxS1* and *LEG* genes encoding Trx and leghemoglobin, respectively. In such nodules, the amount of the *GSHS* transcripts drastically increased compared to the control, whereas the expression of the *hGSHS* gene did not change. Recently it was shown that *TrxS1* controls differentiation of bacteroids via the redox state of cysteine-rich peptide NCR335 [47]. Analysis of the spatial localization of transcripts revealed the expression of the *GSH1* gene in the meristem, the infection zone and in the beginning of the nitrogen fixation zone, while expression of *hGSHS* observed in the cortex and vascular bundles, and that of *GSHS* was in

the cortex and in the nitrogen fixation zone. This further confirms the important role of GSH for functioning of meristematic and nitrogen fixing cells and differentiation of cells in the infection zone. Also it is likely that in the cortical cells both thiols play an important role in maintaining the oxygen barrier of the nodule [46].

In 73 species of 3 subfamilies, the distribution of (h)GSH in the *Fabaceae* family was studied [48]. It was shown that hGSH was absent in the members of *Caesalpinioideae* subfamily, but it was found in two species of *Mimosoideae* and in species of the Old World clade in *Papilionoideae* subfamily. Distribution of (h)GSH was tissue-specific. The hGSH content was often higher in leaves and roots, the GSH in seeds, which can reflect a difference in the functions of both thiols. At the same time, in the species forming and not forming nitrogen-fixing nodules, the distribution of hGSH did not change; therefore, the accumulation of hGSH in the roots probably is not related to nodule formation [48].

Previously in *M. sativa* it was shown that GSH is associated with meristematic cells, activation of the cell cycle and induction of somatic embryogenesis, whereas hGSH is associated with differentiated cells and embryo proliferation. Thus, the ratio of hGSH:GSH was the lowest in the root meristem and the highest in the fully differentiated organs (mature leaves and root elongation zone). It supposed that alterations in hGSH/GSH occur during dedifferentiation and (or) activation of the cell cycle that leads to transition from differentiated to dividing cells [49]. As already noted, these processes take place during formation of the nodule primordium. In *M. sativa*, in the formed nodules, as compared to leaves and roots, GSH becomes the main thiol compound. Alterations in the thiol ratio are reversible: in the cell culture after development of somatic embryos and cell differentiation, the ratio of hGSH:GSH increased again, and in the nodules the hGSH content grew as the tissues differentiated [45]. Probably, local alterations in amounts of phytohormones, associated with developmental programs and (or) environmental influence, could regulate *(h)GSHS* expression or (h)GSHS activity and therefore lead to the observed patterns of hGSH and GSH distribution.

Indeed, the expression of the *GSHS* and *hGSHS* genes not only strongly varies depending on the species and tissue of legume plant, but is also regulated differently in response to signal molecules or stress conditions. E.g. in *M. truncatula* the *hGSHS* expression can be detected in roots and nodules, and that of *GSHS* is found in all plant tissues [50]. Moreover, in the roots of *M. truncatula* the expression of the *GSH1* and *GSHS*, but not *hGSHS* is induced by nitrogen oxide [51]. In deervetch (*Lotus japonicus*) GSHS is found in nodules only, and hGSHS is present in leaves and roots as well [33]. In the roots of *L. japonicus GSHS* is activated by auxins, cytokinins and polyamines, whereas the expression of *hGSHS* remains unchanged [14]. The addition of exogenous hydrogen peroxide induced increase the expression of the *GSH1* and *hGSHS* genes in beans nodules, whereas $CdCl_2$, NaCl or jasmonic acid did not cause such an effect [52]. Long-term treatment with cadmium chloride of pea (*Pisum sativum*) mutant $SGECd^t$ [53] resistant to cadmium a slight decrease in the expression of *GSH1* and *GSHS* genes was observed, whereas the expression of *hGSHS* increased in the roots of the mutant and wild type plants [54]. The analysis of the obtained data suggests the presence of specific *cis*-regulatory elements in the promoter region of *GSHS* and *hGSHS* genes and (or) different regulatory mechanisms for *GSHS* and *hGSHS* [14].

The synergism of phytohormones and oxidative stress plays an important role in the control of plant growth and development [55], and GSH and hGSG may mediate such control in legume plants. Nodule formation is an energy con-

suming process. Integration of oxidative stress metabolism and cell cycle allows avoiding unwanted energy expenses by separating defense mechanisms from the processes of cell division and differentiation.

Thus, an important role of (h)GSH in development and functioning of symbiotic nodules has been identified. Nevertheless, the specificity of action of GSH and hGSH in nodule tissues and at the different stages of its development remains insufficiently studied. A lot of data has been obtained by analysis of only one plant species. In addition, a significant difference is observed between species forming indeterminate (Table 1) and determinate (Table 2) nodules.

1. Synthesis and distribution of glutathione GSH and homoglutathione (h)GSH in organs and tissues of the nodule in legume plants forming indeterminate nodules [36, 45-46, 48, 50, 89]

Thiol, gene, enzyme	Seeds	Leaves	Roots	Nodules						
				WN	zones				C	CB
					M (I)	I (II)	I + II	NF	S	
Thiol distribution										
GSH	+	+	+	+	?	?	+[a]	+[a]	+[a]	?	?
hGSH	–[1]	–[1]	+[2]	+[2]	?	?	+[a]	+[a]	+[a]	?	?
Thiol synthesis gene expression and localization of transcripts											
GSH1	?	+	+	+	+[a]	+[a]	?	+[a]	?	–*	+[a]
GSHS	?	+	+	+	+[a]	+[a]	?	+[a]	?	+[a]	+[a]
hGSHS	?	+	+	+	+[a]	–*	?	+[a]	?	+[a]	+[a]
Activity of thiol synthesis enzymes											
γ-ECS	?	+	+	+	?	?	+[a]	+[a]	+[a]	?	?
GSHS	?	+	+	+	?	?	+[a]	+[a]	+[a]	?	?
hGSHS	?	–	+	+[2]	?	?	+[a]	+[a]	+[a]	?	?

Note. WN — whole nodule, M — meristem, I — infection zone, NF — nitrogen fixation zone, S — senescence zone, C — cortex, VB — vascular bundles; "+" — detected, "–" — not detected, "?" — no data, [1] — can be present in trace amounts or may be the main thiol in the species of *Trifolieae* tribe, [2] — not found in *Vicia faba* and *Lupinus albus*, [a] — data has been obtained for plants of one species. See description of genes and enzymes in the text of the paper.

2. Synthesis and distribution of glutathione GSH and homoglutathione (h)GSH in organs and tissues of nodules in legume plants forming determinate nodules [27, 36, 45, 48, 89, 91]

Thiol, gene, enzyme	Seeds	Leaves	Roots	Nodules							
				WN	NFT	C	VB	CS	P	M	B
Thiol distribution											
GSH	+/–	–[2]	+/–[2]	+[1, 2]	+[a]	+[a]	?	+[a]	?	+[a]	+[a]
hGSH	+	+[2]	+	+	+[a]	+[a]	?	+[a]	?	+[a]	+[2, a]
Thiol synthesis gene expression and localization of transcripts											
GSH1	?	+[a]	+[a]	+[a]	?	?	?	?	?	?	?
GSHS	?	+[a]	+[a]	+[a]	?	?	?	?	?	?	?
hGSHS	?	+[a]	+[a]	+[a]	?	?	?	?	?	?	?
Activity of thiol synthesis enzymes											
γ-ECS	?	?	?	+	+	+	?	+/–	+	–[2]	+
GSHS	?	–[2]	+	+[3]	+	+	?	–[2]	+[4]	–[2]	+
hGSHS	?	+[2]	+	+[2]	+	+	?	+[2]	–	–	–

Note. WN — whole nodule, NFT — nitrogen-fixing tissue, C — cortex, VB — vascular bundles, CS — cytosol, P — plastids, M — mitochondria, B — bacteroids; "+" — detected, "–" — not detected, "+/–" — trace amounts, "?" — no data.

[1] — GSH of bacterial origin, [2] — except for *Vigna unguiculata*, [3] — except for *Vigna radiata*, [4] — contamination from bacteroids,

[a] — data has been obtained for plants of one species. See description of genes and enzymes in the text of the paper.

There are differences and between the species forming the same type of nodules (see Tables 2, 3). Nevertheless, some patterns can be observed. Thus, indeterminate nodules mostly contain GSH as the main soluble tripeptide whereas hGSH is the most widespread tripeptide in determinate nodules [45].

Ascorbic acid. This is a powerful soluble antioxidant acting directly and as a part of the ascorbate-glutathione cycle. Ascorbic acid is present at a concentra-

tion of 1 to 2 mM in nodules [56], 5 to 25 mM in leaves, and 25 to 50 mM in chloroplasts [57] that is consistent with its multiple important functions. The redox state of ASC (ASC + dehydroascorbic acid) controls the cell cycle [58] and plays a crucial role in perception of stress signals in the apoplast and their transduction into cytoplasm. ASC is also a cosubstrate for several dioxygenases participating in hydroxylation of proline and biosynthesis of flavonoids and hormones, i.e. ethylene, gibberellic, and abscisic acids [59]. The importance of ASC for plants is confirmed by the absence of known mutants that are completely defective in ASC synthesis [60]. ASC is mainly synthesized via the D-mannose/D-galactose (Smirnoff-Wheeler) pathway comprising multiple and complex enzymatic reactions, and final reactions is catalyzed by mitochondrial L-galactono-1,4-lactone dehydrogenase [61].

3. Synthesis and distribution of glutathione GSH and homoglutathione (h)GSH in organs and mitochondria of *Vigna unguiculata*, forming determinate nodules [36, 45]

Thiol, enzyme	Leaves	Roots	Nodules	Mitochondria
Thiol distribution				
GSH	+	+	+	?
hGSH	–	+	+/–	?
Activity of thiol synthesis enzymes				
γ-ECS	?	?	+	+
GSHS	+	+	+	+
hGSHS	–	+	–	–

Note. "+" — detected, "–" — not detected, "+/–" — trace amounts, "?" — no data. See description of enzymes in the text of the paper.

Investigation of *P. sativum* showed that ASC content decreases in nodules with age, that correlating with a decrease in the GSH content and nitrogenase activity [23]. It was supposed that ASC is not synthesized *de novo* in nodules but is imported from shoots or roots through the vascular system [23]. However, later it was shown that ASC biosynthesis genes, including *GalLDH* gene encoding L-galactono-1,4-lactone dehydrogenase, are expressed in the nodules of *L. japonicus*. The active enzyme is localized in the mitochondria of beans (*Phaseolus vulgaris*). Investigation of nodules of four legume species: alfalfa (*M. sativa*), pea (*P. sativum*), beans (*P. vulgaris*) and deervetch (*L. japonicus*), showed an increased activity of L-galactono-1,4-lactone dehydrogenase and cytosol ascorbate peroxidase, but also the decreased ASC level in nodules compared to other plant parts [62]. Using FISH, it was shown that mRNA of *GalLDH* is predominantly located in cells of nitrogen fixation zone in the nodules of *M. sativa* and in nitrogen-fixing cells of the central part of the nodule in *L. japonicus*. Also, the maximum content of L-galactono-1,4-lactone dehydrogenase and ASC was found in these cells. At the same time, the enzyme activity was the same in the apex (meristem and infection zone) and in the nitrogen fixation zone, whereas amounts of mRNA were different, that indicates the post-translation regulation [62]. The presence of post-transcription regulation is evidenced by the fact that under the stress condition (treatment with cadmium salts, NaCl, hydrogen peroxide and jasmonic acid), the ASC content changed but the activity of L-galactono-1,4-lactone dehydrogenase remained unchanged in bean nodules [52]. The ageing of symbiotic nodules is possibly accompanied by switching off the ASC biosynthesis that is confirmed by a reduction in the L-galactono-1,4-lactone dehydrogenase activity and ASC content.

The ASC concentration in cells is regulated by a degree of its oxidation and degradation. ASC oxidizes to monodehydroascorbic acid or dehydroascorbic acid during hydrogen peroxide detoxication via ascorbate-glutathione cycle in cytosol, chloroplasts and other organelles. In the apoplast, ASC is oxidized to monodehydroascorbic acid by ascorbate oxidase [63]. The treatment of the bean plants with jasmonic acid, a well-known stress-associated compound, led to translational activation of ascorbate oxidase and post-translational inhibition of dehydroascorbate reductase in nodules that possibly enhances oxidations in apo-

plast and initiates a signal via which nodules may perceive stress and respond to it [52].

Enzymatic antioxidants. *Ascorbate-glutathione cycle*. Ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase participate in this cycle. The regulation of subcellular isoforms each of these enzymes, which have been found in cytosol, plastids, mitochondria, and peroxisomes, is carried out based on the level of ASC and GSH synthesis in these organelles under normal and stress conditions. Each isoform can differently respond to signals associated with stress or developmental process. The regulation of all isoforms of enzymes participated in the ascorbate-glutathione cycle is quite important for maintaining the redox balance in the plant cells under abiotic and biotic stresses [64].

In the nodules, the ascorbate-glutathione pathway was found 30 years ago [56]. Later, the comparison of activity was carried out for enzymes involved in this pathway in the nodules of soybean (*Glycine max*), formed on three genotypes by effective and ineffective strains. The same analysis was carried out for nodules of wild type and ineffective mutant genotypes of *M. sativa* [26]. High activity in effective nodules was demonstrated for all four enzymes (ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase) in both studied species. Also, in effective nodules GSH and hGSH content was higher compared with ineffective ones. At the same time, no such correlation was observed for ASC. Transcriptomic analysis revealed increased expression of genes encoding the enzymes of the ascorbate-glutathione pathway in nodules [65]. Therefore, the ascorbate-glutathione pathway is one of the main mechanisms of their antioxidant defense [26].

In the ascorbate-glutathione pathway, GSSG is formed from GSH that leads to the change in the GSH:GSSG ratio. Some other metabolic pathways can also link the availability of oxidants and alterations in the GSH:GSSG ratio, thus, affecting the signaling transduction via regulation of thiol-disulfide status of the proteins. The key participants of these reactions are Prx, Gpx, Trx and Grx. Some glutathione S-transferases (GST) can also use GSH for reduction of organic hydroperoxides, and others can generate GSSG, possibly, catalyzing the deglutathionylation cysteine residues of proteins [66]. The role of these processes in the formation and functioning of nitrogen-fixing nodule is still insufficiently studied.

Thiol peroxidases and redoxins. Thiol peroxidases (Gpx and Prx) are small proteins without gem, catalyzing reduction of the H_2O_2 or alkyl hydroperoxides (ROOH) to water or corresponding alcohols (ROH) using mostly Trx as electron donors. Trx contains a conservative interaction site (Try-Cys-Gly-Pro-Cys) which reduce disulfide bridges of the target proteins [67]. Grx, with functions similar to Trx, can also act as electron donor; GSH is used for its reduction [21]. Gpx, Prx and Trx are encoded by multigene families, and the corresponding isoforms have different subcellular localization (cytosol, plastids and mitochondria) [14].

In *L. japonicus*, six genes were identified to encode Gpx. Two of these, *LjGpx1* and *LjGpx3*, encoding Trx-dependent hydroperoxidases, are actively expressed in nodules. Immunolocalization showed the presence of Gpx around the starch granules in amyloplasts in infected and non-infected cells of *L. japonicus* nodules, and in infected cell of *M. sativa* nodules that may indicate formation of hydrogen peroxide in amyloplasts as well [68]. Later, using in situ hybridization, a high content of mRNA was shown in infection zone for *LjGpx1* and *LjGpx3* and in nodule cortical cells for the *LjGpx3* as well. Immunolocalization detected LjGpx1 in amyloplasts and in nuclei of infected cells, in cortical cells and in vas-

cular bundles. LjGpx3 was predominantly located in endoplasmic reticulum (ER), cytosol and nuclei [69[. Conclusion, that LjGpx1 and LjGpx3 protect cells from oxidative and salt stresses and membrane damage, was made basing on complementation experiments with yeasts.

PrxIIA (the cytosol isoform of Prx), the amount of which is reduced with nodule age, and mitochondrial isoform PrxIIF, the amount of which does not change were identified in the nodules of *P. sativum*. After exogenous treatment with ASC, the PrxIIA level decreased in young nodules and increased in old ones, the amount of PrxIIF was unchanged in both cases [70]. In *M. truncatula*, *L. japonicus* and *P. vulgaris*, the proteomic analysis of nodules identified PrxIIB, PrxIIE and PrxIIF isoforms (B in cytosol, E in plastids, and F in mitochondria), a cytosol isoform Trxh1, and also GrxC2 and GrxC4, which possibly can serve as electron donors for Prx. Moreover, NADPH-thioredoxin reductases (NTRA/B/C) were found in nodules of these legumes. Most of these Prx isoforms are effectively reduced by Trx and non-plastid Trx is recovered by NTRA and NTRB. The obtained data confirm the presence of this redox regulator network in cytosol, plastids and mitochondria of nodule cells (cytosol forms of PrxIIB, Trxh1 and NTRA are most active).

In *M. truncatula*, two new isoforms of Trx (Trxs), functioning during symbiotic interactions, were found [71]. These ER-addressed Trx are similar to classic ones, but have atypical catalytic sites. *In silico*, using the atlas of *M. truncatula* gene expression in different organs and under various growth conditions, it was shown that *Trxs1* is mostly expressed during nodule formation, whereas *Trxs2* expression is apparently less specific [65, 72]. The analysis of the spatial localization of *Trxs1* and expression of *Trxs2*, using https://iant.toulouse.inra.fr/symbimics/ resource, which gives access to results of laser microdissection of *M. truncatula* nodules combined with RNA sequencing, has shown that these *Trx* are mostly expressed in the infection zone [73]. The expression pattern of *Trxs* indicates their participation in the development of nodule and cell differentiation in the infection zone. In situ hybridization revealed the presence of *GmTrx* mRNA in the root pericycle of *G. max* in 2 days post inoculation and in the infected cells of mature 27-day-old nodules. The transformation of the Trx-defective yeast mutant with the *GmTrx* gene recovered tolerance to exogenous H_2O_2. RNA interference of *GmTrx* led to the absence of nodules or formation of undeveloped nodules that indicates the importance of this gene in their development [74].

Glutathione-S-transferases. These widely distributed enzymes participating in detoxication of xenobiotics, especially herbicides. GSTs act as antioxidants as well, directly trapping peroxides like Gpx. The final products of the peroxide oxidation of lipids alkenals, 4-hydroxynonenal and other α- and β-unsaturated aldehydes, may be removed after conjugation with GSH [75, 76]. In soybean, GSTs are encoded by a multigene family of 25 genes [77]. In the nodules, the expression of 14 GST isoforms was found; gene encoding GST9 showed maximum level of expression [76]. The suppression of the GST9 expression by RNA interference significantly decreased the nitrogenase activity in the nodules [76].

Superoxide dismutases (SODs). SODs belong to the group of metal containing enzymes, catalyzing dismutation of superoxide radical to hydrogen peroxide and molecular oxygen. Depending on metal cofactors, there are Fe-, Mn-CuZn-dependent SODs (correspondingly FeSOD, MnSOD and CuZnSOD). All of these SODs are present in nodules, but with different subcellular localization.

In indeterminate nodules of *M. sativa* and *P. sativum*, the transcripts of the cytosol CuZnSOD, as well as the enzyme itself, are present predominantly in the meristem, the infection zone and distal part of the nitrogen fixation zone

[78]. CuZnSOD was localized in the cytosol in the regions adjacent to cell walls, to the walls of infection threads, and also in the matrix of infection threads. Moreover, the enzyme was found in the intracellular spaces of the cortical cells and cells in the nitrogen fixation zone. The transcripts of MnSOD and the enzyme itself were localized predominantly in the nitrogen fixation zone. The enzyme was present in bacteria inside the infection threads, in bacteroids and mitochondria. In the nodules, the co-localization of H_2O_2 with CuZnSOD was found, but not with MnSOD, i.e. exactly CuZnSOD is one of the most important H_2O_2 sources in nodules [56] and possibly plays an important role in forming matrix of infection threads and their growth [79]. Later, the transcriptome analysis of *M. truncatula* nodules has shown that the genes encoding CuZnSOD isoforms and mitochondrial MnSOD are actively expressed in nodules whereas expression of the gene for cytosol FeSOD remains relatively low [65].

In determinate nodules of L. *japonicus*, the expression of genes of cytosol CuZnSOD and mitochondrial MnSOD, as well as the amount and activity of the enzymes reduced with age. In the young nodules, CuZnSOD is found in dividing cells, in infection threads and in infected cells. The transcription of *FeSOD2* which encodes cytosol FeSOD activated and that of *FeSOD1* encoding plastid FeSOD did not change. With nodule age, the amount and activity of FeSOD increased. At all stages of nodule development, this enzyme was present predominantly in the amyloplasts in the cortical cells, uninfected and infected cells. It was supposed that two cytosol enzymes, CuZnSOD and FeSOD2, are capable to functionally compensate each other at later stages of nodule development. Induction of FeSOD2 is possibly associated with an increase in Fe availability in the senescent nodules, possibly as a result of leghemoglobin degradation [80].

Under salt stress, the total activity of SOD in the nodules of *P. vulgaris* increased [81]; the activity of FeSOD also enhanced, and the activity of CuZnSOD and MnSOD did not change [82]. In the peanut plants under draught, the amount of CuZnSOD transcripts in the nodules increased whereas the activity of SOD, MnSOD I and MnSOD II did not change [83]. The opposite results were obtained for SOD in *P. sativum* and *G. max* nodules at draught, when total SOD activity decreased in *P. sativum* and increased in *G. max* [84, 85]. Such diverse responses of various species stress out a necessity of studying the gene expression and regulation of enzyme activity in more detail.

Catalases. These are tetrameric gem proteins catalyzing the decomposition of hydrogen peroxide to molecular oxygen and water, which are predominantly localized in peroxisomes and glyoxysomes [86]. An important role of rhizobial catalases has been shown for development of effective symbiosis [87]; however, the enzymes of plant origin have been insufficiently studied. In the nodules of white lupine (*Lupinus* albus), the catalase was localized in the peroxisomes of infected cells, and its concentration was reduced during nitrate-induced senescence [88]. Also, the enzyme activity reduced in bean nodules under salt stress [82].

Thus, over 30-year studying antioxidant system of nitrogen-fixing nodules in legumes, a significant progress was achieved. The principles of operation and interaction of antioxidant molecules, enzymes and redox regulatory pathways during the processes of initiation, development and functioning of legume-rhizobium symbiosis were analyzed. The complexity and ambiguity of the functioning of the antioxidant system were identified. To date, it was shown that GSSH participates in signal transduction via alteration of the redox state of the cell and its compartments. This, in its turn, may control the thiol-disulfide status of proteins, i.e. the conformation and activity of enzymes and transcription factors directly or via thiol-dependent peroxidases. Moreover, the oxidation of some

thiol-dependent peroxidases can itself serve as a signal or signal trigger. In 1988, the distribution of GSH and hGSH in plant tissues of 13 *Fabaceae* species from various tribes was analyzed [89]. In the following years, a lot of data was accumulated indicating specific functioning of these thiols, including that among species forming determinate and indeterminate nodules. In 2015, the distribution of GSH and hGSH has been analyzed already in 73 species of three *Fabaceae* sub-families [48]; however, the question of the GSH and hGSH specific functions still remains open. At the same time, the role of GSH in the development and functioning of meristem cells and nitrogen-fixing cells has been proven. Moreover, it was demonstrated that the ratio of hGSH and GSH may activate cell differentiation and dedifferentiation [49]. Data about new components of antioxidant defense in nodules continue to appear, e.g. polyamines, the polycationic compounds capable of modulating concentrations of ROS and RNS, were described. However, the role of these molecules in the functioning and development of nodules requires further studies [90].

Thus, the redox state of a cell and its compartments, determined by the pro- and antioxidant ratio and interaction, controls a variety of processes via changing oxidation/reduction balance under the influence of environmental signals and participating in their transduction and subsequent plant responses. The antioxidant system plays a crucial role in formation and functioning of such a sensitive system as a nitrogen-fixing nodule of legume plants characterized by active metabolism and constant exchange of signal molecules between partners.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 895-904
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 634.232/.233:632.938.1:632.4

doi: 10.15389/agrobiology.2017.5.895rus
doi: 10.15389/agrobiology.2017.5.895reng

GENETIC DIVERSITY OF STONE FRUIT VARIETIES (GENUS *Prunus* L.) RESISTANT TO LEAF SPOT

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported by Russian Foundation for Basic Research (grant № 16-44-230323 p_a) and by the grant from Administration of Krasnodar Krai under the State Program of Federal Agency of Scientific Organizations
Received February 15, 2016

Abstract

The range of disease-resistant varieties and hybrids of stone fruits (genus *Prunus* L.) is quite wide. In Russia, these are the hybrids of sour cherry VP-1, Rubin, Vozrozhdenie № 1, Olymp, (A.F Kolesnikova et al., 1998), Pushkinskaya, Akvarel, Feya, Practichnaya, Zelenoglazka (R.A. Chmir, 2003), sour cherry varieties Livenskaya, Mtsenskaya, Novella, sweet cherry Poeziya, clonal rootstocks V-2-180, V-2-230 (A.A. Gulyaeva et al., 2007), Rusinka, Businka, Yubileynaya 3, Pamyat Sakharova (O.N. Kartashova, 2009), AI, 3-115, 10-15 (A. Kuznetsova et al., 2010), sour cherry samples Hindenburg, Obnovlennaya, Nochka 2, Rannyaya 2, and sweet cherry varieties Muscatnaya Krasnaya, Royyal 23/16, Lamori Gin, Sladkaya Sentyabrskaya, Tseshenskaya Oktyabrskaya (M.S. Lenivtseva et al., 2010). The varieties Anshlag, Lasunya, Lyubimitsa Turovtseva (M.I. Turovtsev et al., 2011) are resistant in Ukraine, sour cherry cultivar Zhivitsa (E.P. Syubarova et al., 2002) is resistant in Belarus. In Lithuania, the resistance of varieties Big Starking, Griot Ukrainskij, Maraska, Samsonovka (D. Gelvonauskiene et al., 2004) is reported. In Poland, Melitopolska, Fortuna, Minister Podbielski, Zagoriewskaja varieties are resistant (G. Hodun et al., 2000; Z.S. Grzyb et al., 2004.). Cherry varieties Kareshova, Kashtanka, Vinka are considered resistant in the Republic of Moldova (E. Cheban, 2005). Also, the resistant samples are clones 5.55, 13.122 (B. Wolfram, 2000), varieties Almaz, Köröser Gierstädt, Coralin and species *P. maackii, P. canescens* (M. Schuster, 2004, 2008; M. Schuster et al., 2004, 2013, 2014) in Germany, cherry varieties Celeste and Giorgia (G. Romanazzi, 2005) in Italy, and Linda, 11/106, Piramis, Csengödi (Z. Rozsnyay et al., 2005; J. Apostol, 2008) in Hungary. In the USA, Almaz, Gisela 6, and *P. canescens* (P.S. Wharton et al., 2003; P.S. Wharton et al., 2005) are reported as resistant. In the review, we discuss a replenishment of effective resistance gene pools using world genetic resources and based on plant resistance studies. It was shown that *Prunus* leaf spot resistance usually dominates and is under monogenic, oligogenic or polygenic control. In hybrids, the manifestation of resistance character depends on whether a resistant parent was male or female one (A.M. Mikheev et al., 1978; O.S. Zhukov et al.,, 1981; A.F Kolesnikova, 1982; N.I Turovtsev et al., 1983; M.V. Kanshina, 2007; J. Apostol, 2000, 2008). Expression of resistance genes derived from the most popular donor, *P. maackii* Rupr., remains quite high in the second and third hybrid generations (I.E. Fedotova et al., 2001). Dominant resistance character is revealed in *P. serrulata* and *P. maximowiczii* (M.S. Chebotareva, 1993). The hybrids 85017, 82990, 83187, 85023 derived from crosses with Kuril, Sakhalin cherry species, *P. maximowiczii* and *P. serrulata* plants are recommended as donors of resistance to cherry leaf spot (N.G. Gorbacheva, 2011). A significant decrease in resistance is noticed in the interspecific hybrid Almaz (with *P. maackii* in the pedigree) which is widely used in breeding for immunity. Genetic diversity of cultivated varieties should be expanded by involving in breeding not only derivatives of *P. maackii*, but also other stone fruit species resistant to leaf spot such as *P. kurilensis* Miyabe, *P. sargentii* Rehd. (M. Schuster, 2004), *P. incisa* Thunb, *P. pseudocerasus*, *P. subhirtella* Mig. (M. Schuster, 2004; M. Schuster et al., 2004), *P. concinna* Koehne, *P. conradinae* (Koehne) Yu. et Li (M.S. Chebotareva, 1986), *P. canescens* Bois. (M.S. Chebotareva, 1986; M. Schuster et al., 2013, 2014; T. Stegmeir et al., 2014), *P. padus* L., *P serotina* Ehrh, *P. asiatica*

Kom., *P. incana* Stev. (M.S. Chebotareva, 1986), *P. glandulosa* Thunb. (M.I. Vyshinskaya, 1984; M.S. Chebotareva, 1986). In breeding, the variability of pathogen populations must be considered

Keywords: stone fruits, leaf spot, resistance, wild *Prunus* L. species

One of the main reasons for the reduction of sweet cherry and cherry acreage is a severe infestation of varieties and rootstocks with cherry leaf spot. The causative agent of this disease is the fungus *Coccomyces hiemalis* (Higg.), the conidial stage *Cylindrosporium hiemale* (Higg.), syn. *Blumeriella jaapii* (Rehm) v. Arx. The most radical method to confront progressing cherry leaf spot diseases is search and creation of the pathogen resistant assortment of sweet cherry and cherry. A rational strategy of breeding for resistance to diseases and pests should involve the extension of the genetic diversity of cultivated varieties. The literature discusses several ways of solving the problem, including the alternation in time of cultivars with different resistance genes, selection of multiline varieties (mixtures of phenotypically similar lines differing in resistance genes), cultivation of varieties with different resistance genes in the area of the pathogen (mosaic), combining different resistance genes in one cultivar (gene stacking). Implementing any of these strategies is based on the study of inheritance of resistance and creation of new donors protected by effective resistance genes.

An intensive study of the resistance of genetic resources of stone fruits to *C. hiemalis* in Russia and the former Soviet Union was started in the late 1960s. Infestation of sweet and sour cherries with cherry leaf spot was analyzed in all the areas of distribution of the causative agent, i.e. in Central Russia [1], North Caucasus [2], Ukraine [3], the Republic of Moldova [4], Belarus [5], the Baltics [6] and Georgia [7]. The resistance of stone fruits was also studied in Germany [8, 9], Poland [10, 11], Romania [12] and the USA [13]. It was found that there are no highly resistant cherry and sweet cherry varieties, and a few varieties characterized by field resistance were selected. These are cherry varieties Pamyati Vavilova, Zhukovskaya, Komsomolskaya, Severyanka and Turgenevka for Central Russia [1], cherry varieties Seyanets 1, Seyanets 2, Zhukovskaya, Glubokskaya, Zvezdochka, Komsomolskaya, Pamyati Vavilova and sweet cherry varieties Aelita, Belobokaya rannyaya, Krasavitsa, Orlovskaya rozovaya and Severnaya for Belarus [5], sweet cherry cultivars Bigarro Oratovskogo, Vinka, Dneprovka, Izyumnaya, Konservnaya, Transportabelnaya and Frantsis for the Melitopol Region of Ukraine [3]. In large-scale screening, the following varieties were considered valuable: sweet cherry Yellow Glass, Schmidt and Emperor Francis from the United States, cherry Karneol, Morina, Safir and Topas from Germany, and cherry Jareniywka, Wloszkowice and Wryble from Poland [8-13].

A study of the total species diversity of stone fruit cultures have amply demonstrated that introgression is the most promising way of expanding the genetic diversity of sweet cherry and cherry for resistance to cherry leaf spot. These wild species are of interest for selection: cherries *Prunus maackii* Rupr. [syn. *Cerasus maackii* (Rupr.) Erem. et Simag., *Padus maackii* Komar., *Laurocerasus maackii* Schnaid.] [5, 14-20], *P. kurilensis* Miyabe [syn. *C. nipponica* var. *kurilensis* (Miyabe) Erem. et Yushev, *C. kurilensis* Kaban. et Vorobiev, *P. nipponica* var. *kurilensis* Wils.], *P. sargentii* Rehd. [syn. *C. sargentii* (Rehd.) Erem. et Yushev, *Cerasus sachalinensis* Komar. et Klob. Aliss.], *P. maximowiczii* Rupr. [syn. *Padellus maximowiczii* (Rupr.) Erem. et Yushev, *C. maximowiczii* Kom.] [5, 14, 17-20], *P. serrulata* Lindl. [syn. *C. serrulata* var. *lannesiana* (Carr.) Erem. et Yushev, *C. serrulata* G. Don.], *P. incisa* Thunb. [syn. *C. incisa* Lois.], *P. pseudocerasus* Lindl. [syn. *C. pseudocerasus* G. Don.], *P. canescens* Bois. [syn. *C. canescens* (Bois.) Erem. et Yushev], *P. subhirtella* Mig. [14, 17-22], *P. concinna* Koehne, *P. conradinae* (Koehne) Yu. et Li [17]; bird cherry *P. padus* L. [syn.

Padus racemosa Gilib.], *P. serotina* Ehrh. [syn. *Padus serotina* Borkh.], *P. asiatica* Kom. [5, 17]; micro-cherry *P. incana* Stev. [syn. *Cerasus incana* Spash., *Microcerasus incana* Roem., *Microcerasus incana* var. *araxina* (Pojark.) Erem. et Yushev] [17], *P. glandulosa* Thunb., [syn. *Cerasus glandulosa* Lois., *Microcerasus glandulosa* (Thunb.) Roem.] [5, 17].

As a result of selection and breeding, a substantial fund of disease-resistant varieties, hybrids, clonal rootstocks of sweet cherry and cherry has been created in Russia. There are interspecific hybrids and cherry varieties among them: VP-1, Rubin, Vozrozhdenie 1, Olimp, 30014, 31414 [23-24], Pushkinskaya, Akvarel, Dzhussi frut, Brilliant, Feya, Praktichnaya, Pamyati Vavilova, Kharitonovskaya, Ustoichivaya, Zelenoglazka [16]; the sour cherry varieties Livenskaya, Mtsenskaya, Novella and Orlitsa; sweet cherry Poeziya; the clonal rootstocks V-2-180, V-2-230, V-5-88, V-5-182 [25], Rusinka, Businka, Yubileynaya 3, Shakirovskaya, Ballada, Polyanka, Pamyat Sakharova [26], 3-90, 11-17, AI, 3-115, 10-15 [27]; the sour cherry samples Hindenburg, Ujfehertha Fürthosh, Podbelskaya, Obnovlennaya, Lunered Mont Burhholder, Nochka 2 and Rannyaya 2; the sweet cherries Muskatnaya krasnaya, Orlovskaya (3734), Planeta, Polyanka 1043, Royal 23/16, Lamory Guigne, Sladkaya sentyabrskaya and Tseshenskaya oktyabrskaya [28].

In recent years, resistant varieties Anshlag, Lasunya, Lyubimitsa Turovtseva derived using sweet cherry Tseshenskaya Oktyabrskaya have been included in the Ukrainian State Register of Plant Varieties [29]. Sour cherry cultivars resistant to cherry leaf spot have been selected in Belarus (cultivar Zhivitsa) [30] and in Lithuania (cultivars Big Starking, Griot Ukrainskij, Maraska, Samsonovka, Oblacinska, Vytėnų žvaigždė and Recta) [31]. Cherry variety Karešova is moderately resistant to cherry leaf spot in Czechoslovakia [32]. In Poland, the resistant cherry varieties are Melitopolska, Fortuna, Minister Podbielski, Zagoriewskaja, Stevensbaer, Pomiati Vavilova and Oblacińska [11, 33]. The sweet cherry varieties Kareshova, Kashtanka, Vinka, Maslovskaya and Pozdnyaya Lermontova have been created in the Republic of Moldova [34]. In Bulgaria, the sweet cherry cultivar Patriotca Krima is resistant [35]. In Germany, the cherry clones 5.55, 13.122 and 19.130 [9] have been selected, as well as the varieties Almaz (Russian breeding), Köröser Gierstädt, Korai Pipacsmeggy, Coralin and *P. maackii* [18, 20, 36, 37]. In Italy, the sweet cherry varieties Celeste and Giorgia [38] have been created; in Hungary these are Linda, 11/106 and Piramis [39-41]. In the USA, Almaz and Gisela 6 [21, 22] are considered resistant. The cherry samples Almaz, Novella, Zhukovskaya, Pamyati Vavilova, Luch, Stepnoi Rodnik, Feya, Kharitonovskaya, VP-1, Rubin, Oblacinska and others are disease-resistant in different regions of Russia and abroad. The sweet cherry cultivars Sladkaya sentyabrskaya and Tseshenskaya oktyabrskaya are disease-resistant in the Krasnodar Krai, the Crimea and Ukraine [42].

This review is the first summarizing studies of genetic control of stone fruits resistance to cherry leaf spot in Russia and abroad.

It was shown that in combinations of cherry varieties resistant × resistant most of the seedlings had increased immunity to cherry leaf spot, and in families, obtained by crossing the susceptible samples, the plants with strong development of the disease dominated. The number of susceptable seedlings varied from 23.4 % (Lyubskaya × Shirpotreb) to 65.7 % (ground cherry × Shirpotreb). In direct and inverse crosses of resistant varieties with the susceptible ones, in most families more than half of the hybrid progeny was characterized by increased resistance to the pathogen of cherry leaf spot. The number of resistant plants varied from 52.9 % (Tserapadus No. 1 × Lyubskaya) to 95.4 % (family Griot Moskovskij × Tserapadus No. 2) [43]. Similar results were obtained by A.A. Melesh-

kevich in Belarus [44]. When resistant varieties were parents (Kistevaya × Novodvorskaya 2/3), the hybrids showed relative resistance, in combinations of susceptible × susceptible parents, the susceptible seedlings prevailed.

Relatively resistant varieties Solnechnyi shar, Vinka and Melitopolskaya rozovaya have been obtained (Ukrainian Research Institute of Irrigated Horticulture, Melitopol), using the cherry variety Frantsis as s female parent. It is assumed that the influence of the maternal genotype on the manifestation of the resistance character in seedlings was more pronounced than of the paternal one. Crossing susceptable cultivars resulted in a high percentage of susceptible seedlings [3].

Sweet cherry seedlings in the hybrid families 8-14 × 3-36, 3-36 × 6-72, 3-36 × Yantarnaya had the highest resistance in Bryansk Province. The proportion of highly resistant seedlings in these families ranged from 23 to 50 %. In the analysis of 15 hybrid families of sour cherry, a high overall genetic diversity was revealed. It is due to the additive effects of genes in eight hybrid families, and to the nonadditive effects in seven ones. According to the author, resistance to cherry leaf spot is under control of oligogenes and polygenes [45].

In the works on the genetic control of cherry resistance to leaf spot and breeding, the experiments with the Maack cherry (*P. maackii*) are mainly described. This species was involved in breeding by I.V. Michurin. When crossing the ground cherry with the Maack bird cherry (cherry), Michurin obtained Tserapadus 1. Later, a number of cerapaduses and padoceruses were created. From a hybrid population (Padocerus M × cherry-sweet cherry Novosyolka) × Pamyati Vavilova, the hybrid A-135 (Almaz) was selected (I.V. Michurin All-Russian Research Institute of Genetics and Breeding of Fruit Plants — ARRIGBFP, Michurinsk). O.S. Zhukov and L.A. Shchekotova [46] selected about 50 % of highly resistant seedlings in the progeny from a cross of this hybrid with highly susceptible varieties. This hybrid was recommended as a source of monogenic resistance to cherry leaf spot. Analyzing crossing with the variety Lyubskaya revealed splitting 1:1, i.e. the resistance gene to cherry leaf spot was in a dominant state. The resistance gene of the hybrid Almaz is marked by the authors with the symbol *A* [46]. In the further study of the progenies of this hybrid compared to relatively resistant variety Zhukovskaya and the highly susceptible variety Lyubskaya, the highest percentage of resistant seedlings was observed in the hybrid progeny, middle percentage was in variety Zhukovskaya and the lowest one was in cultivar Lyubskaya. Cherry varieties, differing in resistance, proved to be heterozygous by this character, therefore, the degree of cherry leaf spot infestation is controlled polygenically [46]. Adaptive donors, rootstocks and varieties resistant to cherry leaf spot were created on the basis of the hybrid Almaz. Subsequently, the cherry-bird cherry hybrids Brilliant, Korall, Luch, Stepnoi Rodnik, Feya and Kharitonovskaya were obtained, which, along with resistance to this dangerous disease, have a good quality of the fruit [47]. According to R.A. Chmir [16], the samples Padocerus B, Pushkin, Pushkinskaya, Akvarel, Dzhussi frut, Brilliant, Feya, Praktichnaya, Pamyati Vavilova, Kharitonovskaya, Ustoichivaya, Zelenoglazka are characterized by polygenic resistance to cherry leaf spot.

Crossing Maack cherry plants with sour cherry varieties (All-Russian Research Institute of Breeding Fruit Crops — ARRIBFC, Orel Province) resulted in hybrids F_1 and donors of resistance to cherry leaf spot VP-1, Rubin and 28889 [48]. Given the nature of the progeny splitting, the resistance of sample Tserapadus 28768 (VP-1) derived from self-pollination and free pollination is heterozygous. In the offspring of this sample there was a dominance of the characteristic, as the ratio of resistant and fragile seedlings was 3:1 [49].

As donors of resistance to cherry leaf spot and adaptability, the hybrids of cherry Vozrozhdenie 1 (Zolushka × VP-1), ELS PI 15-21 15-21 (VP-1 × Muza F_2) and ELS PI 14-1 (Muza × Vozrozhdenie 1 F_3), tetraploid 1-36 (Shokoladnitsa × *P. serrulata* Hally Tolivetta), triploids 1-13 (Shokoladnitsa × *P. incisa*) and 2-13 (Shokoladnitsa × *P. kurilensis* Dolinsk 5) [50] may be suggested. Based on backcrossing cherry cultivars Pamyati Vavilova, Lyubskaya, Vladimirskaya, Zolushka, Muza, Turgenevka with derivatives of the Maack cherry, the ARIBFC hybrid fund was created, and promising seedlings were separated and multiplied. Differences in resistance to cherry leaf spot between the reciprocal hybrids F_2 and F_3 were not detected. Ageing and declined physiological condition of trees, as well as mass accumulation of the fungus led to a decreased resistance of the hybrids. However, Maack cherry retained high resistance in backcrosses not only in the second but also in the third generations [24].

According to E.N. Jigadlo and A.A. Gulyayeva [51], VP-1, 28889, Rubin, 30013, Olimp, 30020, Vozrozhdenie 1, Vozrozhdenie 2 (31414) and Dolgozhdannaya (ARIBFC) created on the basis of the Maack cherry are of interest for use in breeding as donors of mono-genic resistance to cherry leaf spot. The assessment of resistance of the sour cherry seedlings obtained from backcrossing with Maack cherry revealed a very small number of highly resistant plants. Thus, highly resistant progeny amounted 7.4 % in VP-1 families from self-pollination, 11.1 % in VP-1 × Vladimirskaya combination, 0.3 % in VP-1 × sweet cherry Muskatnaya, 0.7 % in Lyubskaya × 28889 cross, 2.1 % in Lyubskaya × Rubin cross, 2.6 % in Lyubskaya urozhainyi klon × Rubin cross, and 7.7 % in 31414 × 33585 combination. A large part of resistant seedlings were observed in families obtained with a participation of VP-1. According to the authors, the use of distant hybrids of sour cherry with the Maack cherry in backcrossing with sour cherry leads to a decrease in resistance to cherry leaf spot in hybrid offspring. Hybrids resistant to cherry leaf spot were produced at Sverdlovsk breeding station of horticulture, i.e. 1-53-86, 1-35-89 and 1-39-89 with VP-1 involved, and 7-15-83, 8-37-82 with Almaz involved [52].

In crossing immune sample *P. serrulata* var. *lannesiana* (No. 2) with the susceptible sweet cherry varieties Krepysh, Kuban, Lyubimitsa Duki and after free pollination, resistance dominated in F_1 in the field experiments and under artificial inoculation. Obviously, the sample *P. serrulata* var. *lannesiana* No. 2 has a dominant resistance gene (genes). In combination *P. serrulata* var *lannesiana* No. 1 × *P. avium* L., there was complete dominance of resistance, however, in reciprocal crossing incomplete dominance was marked, which can be explained by the influence of the maternal cytoplasm. In families from crosses of Frantsuzskaya chernaya × *P. sargentii* (Sakhalin cherry 3/75) and Frantsuzskaya chernaya × *P. serrulata* (Batumi 1), all seedlings were highly susceptible to the fungus. Afterwards, crossing *P. serrulata* var. *lannesiana* No. 2 with the susceptible sweet cherry variety Frantsuzskaya chernaya showed complete dominance of resistance. When using this sample as a male parent in crossing with the susceptible cherry variety Zhagarskaya, incomplete dominance of the trait was marked. The dominance of resistance, except for *P. serrulata* var. *lannesiana* No. 2, was also found in a sample of *P. maximowiczii* (the Maksimovich cherry) [17, 53].

Involving diploid species of the Kurile, Sakhalin, Maksimovich cherry and *P. serrulata*, hybrids 85017, 82990, 83187, and 85023 were obtained, which are recommended as donors of resistance to cherry leaf spot [54]. The study of cherry leaf spot resistance of seedlings obtained by I.E. Fedotova and A.F. Kolesnikova in crossing *P. incisa*, *P. serrulata*, *P. kurilensis*, *P. sargentii* at hard infection loads showed that resistant seedlings from all the combinations used aver-

aged 65.45 %, of which 32.93 % were immune. The amount of immune seedlings depends on the interaction of polygenes of the maternal plant (variety) with oligogenes of the male parent (wild species). The seedlings obtained by crossing Rovesnitsa possessing high polygenic resistance with *P. serrulata* were infested by the fungus least of all [55].

In the United States, the Almaz variety obtained with the use of *P. fruticosa* 18-6-44 from Krymsk (Krasnodar region), as well as the sample GI 148-1 with a resistant species *P. canescens* in the pedigree, showed high resistance to cherry leaf spot after free pollination [21, 22, 56]. Note, Almaz variety was obtained using the Maack cherry, and GI 148-1 was created uisng *P. canescens*, that is, the resistance of these forms is probably under control of different genes. When infected by five monosporous fungal isolates collected from plants of different species and genera (cherry, sweet cherry, *P. serotina*, mahaleb cherry and plum), Almaz and GI 148-1 plants showed a slight injury caused by fungi from cherry, sweet cherry and mahaleb cherry. In Hungary, in studying inheritance of resistance to cherry leaf spot in variety Csengödi, Cultivars Érdi bötermö, Meteor korai, Érdi nagygyümölcsü, M 221, III-43/60, IV-2/152 were used as female parents; Csengödi, Érdi bötermö, Meteor korai and IV-2/152 served as male parents. A total of 35 resistant seedlings were identified of which 13 ones were tolerant. Cherry resistance to cherry leaf spot is determined by the recessive polygenes [57-60]. In Serbia, genotypes Feketicka demonstrated different field resistance to cherry leaf spot [61]. In Germany, the inheritance of resistance to cherry leaf spot of species *P. canescens, P. maackii* is being studied [62].

Thus, resistance to cherry leaf spot pathogen in genus *Prunus* L. usually dominates and is under monogenic, oligogenic or polygenic control. Manifestation of the trait depends on the use of resistant plants as female or male parents. The dominance of resistance in *P. serrulata* and *P. maximowiczii* (the Maksimovich cherry) is revealed. Expression of cherry leaf spot resistance of the most popular donor, the Maack cherry, was observed in its hybrids in the second and third generations. The range of stone fruit varieties and hybrids resistant to the disease is quite wide. The Russian State Register of Selection Achievements admitted to use includes resistant varieties Novella, Rusinka, Businka, Kapelka, Feya, Kharitonovskaya created on the basis of the Maack cherry, the clonal rootstocks V-2-180, V-2-230, V-5-88, V-5-172, and sweet cherry varieties Anshlag, Lasunya, Lyubimitsa Turovtseva with sweet cherry resistant variety Tseshenskaya Oktyabrskaya in the pedigree. The sample GI 148-1 with a resistant species *P. canescens* in the pedigree showed high resistance to cherry leaf spot after free pollination. Note, most breeders use only derivatives of Almaz variety (with Maack cherry in pedigrees) as donors. However, as shown by our studies, clones of the fungus which can greatly infest this variety are sufficiently high frequent in the pathogen populations. It is necessary to improve genetic diversity of cultivated varieties by involving in breeding not only the Maack cherry derivatives, but other stone fruit plants resistant to cherry leaf spot such as *P. kurilensis* Miyabe, *P. sargentii* Rehd., *P. incisa* Thunb., *P. pseudocerasus, P. subhirtella* Mig., *P. canescens* Bois., *P. concinna* Koehne, *P. conradinae* (Koehne) Yu. et Li, *P. padus* L., *P. serotina* Ehrh., *P. asiatica* Kom., *P. incana* Stev., *P. glandulosa* Thunb. In breeding, one should take into account the variability of this pathogen populations.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 905-916
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 635.655:631.527.8

doi: 10.15389/agrobiology.2017.5.905rus
doi: 10.15389/agrobiology.2017.5.905eng

GENETIC SOURCES REQUIRED FOR SOYBEAN BREEDING IN THE CONTEXT OF NEW BIOTECHNOLOGIES

(review)

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The authors declare no conflict of interests
Supported by a subsidy from Federal Target Program (agreement No. 14.575.21.0136 of 26.09.2017)
Received June 11, 2017

Abstract

Soybean is a strategic crop of multipurpose use. Production and consumption of soybeans are increasing year by year, with new uses appeared. Soybean can become one of the key plants in bioeconomics. Food, fodder, technical, medical and pharmaceutical use of soybeans is diversified and requires specialized varieties with the target traits. This poses new challenges for breeders and, accordingly, for holders of germplasm collections that supply source material for breeding. VIR soybean collection for many years serves as a genetic source for breeding. Based on long-term phenotyping, the accessions are systematized by a number of traits. Rapid development of new molecular technologies, e.g. marker-assisted selection (MAS) and genomic breeding are targeted to optimize both creation of new varieties and searching for the necessary genotypes. A number of agronomically important quantitative trait loci (QTL) have been found for soybean (Y. Xu, J.H. Crouch, 2008; D.C. Leite et al., 2016; Y. Ma et al., 2016; H. Liu et al., 2017), and putative candidate genes have been determined (E.Y. Hwang et al., 2014; J. Zhang et al., 2015; J. Zhang et al., 2016). This allows quick and targeted search for genotypes in germplasm collections, and necessitates relevant knowledge of the gene pool, i.e. the trait variability, the industrial uses, including the use of alternative values, etc. The purpose of this paper is an overview of the genetic diversity of VIR soybean collection in the context of modern breeding needs, in particular the creation of specialized varieties for target use, taking into account the crop studying and diversifying in the world, as well as developing new breeding technologies. It is shown that the VIR soybean collection contains genetic sources for high grain quality, i.e. high in protein and low in antinutritional substances, improved in oil and soy milk characteristics, etc. Breeding early maturated varieties for all soybean producing regions based on relevant gene sources is urgent. For all the traits discussed, the paper gives the modern data on genetic control, genomic organization and mapping genes and QTL. It is concluded that the range of soybeans uses should be based on a diversity of specialized varieties with specified parameters for target use and different adaptive abilities.

Keywords: *Glycine max* (L.) Merr., soybean, VIR collection, genetic resources, initial material, QTL, genes, breeding, specific uses, grain quality, early maturation

Soybean *Glycine max* (L.) Merr. is a strategic crop for multipurpose use. The production of soybeans is steadily increasing in the world and in the Russian Federation. In 2016, the world soybean areas were 120.31 million hectares [1]. In the Russian Federation over the last 10 years, they have increased by almost 1,500,000 hectares (up to 2,228,485 hectares in 2016). Commercial crops have expanded, including regions for which soybeans were not previously traditional. For 10 years, the crop yield has also increased from 9.2 centner/ha in 2007 to 15.6 centner/ha in 2016 (Official Internet Portal of the Federal Service of State Statistics, http://www.gks.ru/). To a large extent, this is due to the success of breeding.

Currently, 210 soybean varieties are registered in the State Register of Selection Achievements admitted to use in the Russian Federation. Their significant share (32 %) comprises varieties of foreign origin [2]. Meanwhile, there are at least 43 institutions engaged in the selection of soybean in the Russian Federation. The geography of these institutions, like the soybean acreage, has a certain dynamics. Since the 1990s, most of the institutions working with soybeans are in the European part of Russia, while earlier production and selection of soybeans in our country were considered the prerogative of the Far East. An increasing variety of soil and climatic conditions makes specific demands on the source material in connection with the need to create adaptive varieties, as for each region, genotypes with a certain photoperiodic sensitivity, requirements for heat, moisture, tolerance to edaphic stressors of the terrain, etc. are needed.

As science develops, along with new production and processing capacities, new directions of soybean use arise. The soybean use is also diversified within its traditional range as a food, fodder and technical crop. The need for pharmaceutical and medical purposes is increasing. Soybean, as a raw material for the production of biofuels and organic fibers, can become one of the key vegetative objects in developing a bioeconomy. According to scientists and the US Department of Energy, biodiesel from soybeans is the only alternative fuel that meets all environmental requirements [3]. The production of fibers from soy protein has long been a reality. Fabrics from it are called textiles of the 21st century [4, 5]. All these innovations require the creation of new varieties that determine the quali-milk, okara and tofu-based products, which are increasingly recognized among the population, especially in southern Russia. In many countries of the world, interest in the use of soybean as a vegetable crop, which was popular in the ancient times in the East, is reviving, the direction so far little known in the Russian Federation. Soybean grain is also suitable for products with preventive and therapeutic properties in a number of diseases. Therefore, since 1990, soybean is classified as functional food [6, 7]. Along with the use of traditional soybean feed (meal, cake, fat soy flour, feed phosphatides, straw, chaff, green mass, etc.) for many types of farm animals and poultry, such feeds are increasingly used in unprecedentedly fast-growing industry of fish farming, as evidenced by the reports of the international conference 'Aquaculture of America 2017' held in early 2017 in Texas [8].

Herein, we have given an incomplete list of soybean use, but those already listed make us reconsider the traditional way of deriving new varieties from existing ones which are considered good in this or that region and often called universal. Selection should focus on specialized varieties with high quality and an increased level of target ingredients. All this requires a parental material with identified traits of interest specific for the intended purpose [9, 10].

However, perhaps the main reason to know the existing diversity of the gene pool is the radical change in breeding methods with the inevitable transition from traditional to genomic technologies on the threshold of which we are standing. Soybeans have being subjected to transgenic manipulation for a long time, but in this review we will focus on those molecular technologies that generate new conceptual level for searching genotypes of interest in germplasm collections. Soybean has already been involved in MAS, based on description and mapping many quantitative trait loci (QTL) in *Glycine max* genome which determine, in particular, grain quality, resistance to diseases, tolerance to some abiotic stressors, vitamin E content, seed weight, etc. [11-14]. The genomic selection of soybeans has been laid [15]. To date, whole-genome sequencing revealed candidate genes determining seed protein and oil content [16], seed size [17], and made it possible to find several new loci and clarify linkage groups of

known ones associated with early flowering, early maturity and plant height in early ripening soybeans [18].

The gene pool stored in the VIR collection was systematized basically on phenotypic characters obtained in studding the accessions in different soil and climatic conditions and in laboratory tests. However, the task for a curator is to know the world trends and priorities, both recent and future, in use and selection of a crop. It is necessary for seeking parental forms and donors to be involved in deriving varieties for the intended use. The possibility of such a search is based on the wide variability of traits revealed in the VIR soybean collection, and knowledge about the differentiation of the gene pool on these traits.

The purpose of this paper, in the context of current needs in varieties for target use, is to overview genetic diversity of the VIR soybean collection, which for many years served as a source of material for breeding, with a focus on the world's achievements in modern breeding technologies, soybean studying and diversified use.

The quality of raw materials is the main requirement for agricultural crops, regardless of their use. For soybean, it is primarily the grain quality, i.e. the composition and content of protein and oil, antinutrients. In VIR collection, soybeans have traditionally been evaluated for protein, oil and trypsin inhibitors in seeds. Very often breeders want to find in the gene pool sources for breeding a variety with high levels of both protein and oil, using their total value. It was found that over the last 30-40 years this value increased from 49.7 to 66.3 %, mainly due to a decrease in the proportion of the seed coat, and practically reached the biological limits of the crop [9]. However, the accumulation of protein and oil in the soybean seed usually reveals a strong negative relationship that cannot be broken [19]. The negative correlation between the traits can vary from $r = -0.25$ to $r = -0.93$ [20] at high heritability of 0.89-0.93 [21]. Moreover, the total plant productivity often negatively correlates with the protein content, although this relationship is weaker than that between protein and oil levels [9, 22]. It is shown, however, that there are no permanent metabolic barriers between these traits. The amount of seed proteins can be increased by backcrossing. Thus, a 4.7 % increase required eight cycles of recurrent selection [23]. It was also reported about a 5.6-6.9 % increase in the portion of proteins without reducing seed oil level and grain yield under univariate intravarietal selection [24].

It is known that modification variability of protein and oil levels in seeds is high, and oil content is much less influenced by environment conditions than protein content [25]. According to the assessment of biochemical indicators of 936 soybean samples (Kuban branch of the Vavilov All-Russian Institute of Plant Genetic Resources — VIR, steppe zone of the Kuban plain, Krasnodar Krai), the seed protein content ranged from 23.5 to 48.0 %, and the oil content ranged from 13.8 to 27.2 %. The relationship between the protein and oil content was negative ($r = -0.57$). In this, 24 donors of high protein content (45.1-50.0 %) and 111 donors of high oil content (24.1-27.1 %) were identified. A total of 12 samples were simultaneously high in protein and oil (from 20.1 to 22.0 %), and 22 samples of different ripeness groups showed high oil content and high seed production, and all samples with high protein content had an average or low productivity [26]. When assessing 189 samples in the Adler branch of the VIR (Black Sea coast, Krasnodar Krai), the seed protein content was higher (34.4-51.0 %), and the oil content was lower than in the conditions of Kuban (14.7-24.8 %). The relationship between the protein and oil levels was also negative ($r = -0.63$). An apparent correlation between seed productivity and protein and oil content was not found [27, 28].

Since the seeds protein and oil level is resultant of interaction of multiple

genes and loci, influenced by the environment, traditional selection for this trait in a number of world breeding centers is accompanied by an analysis of linkage groups to identify QTLs that determine oil and proteins in different genotypes and under different environmental conditions [29-31]. Several QTLs affecting seed protein content have been detected. Loci located on the Gm20 chromosome have been identified in many mapping populations. The genes present in these loci are described in the offspring from crossing NILs (near-isogenic lines) with the contrast trait values [32]. In particular, the genes encoding the potential protein regulator of Mov34-1 family, heat shock protein Hsp22.5 and AT synthetase were identified. QTLs associated with protein synthesis and related traits in combination with genomic analysis may contribute to the rapid selection of soybean QTLs that are significant for protein accumulation and the identification of candidate genes regulating seed protein content [33].

As noted, differentiation that determines the variety character exists within a certain use, for example, for food. A variety of products from soybean is difficult to list. This is oil, soy milk and its derivatives, flour, protein concentrates, isolates, textures, lecithin, etc. However, the relevant specialized varieties are still often produced mainly on the basis of any highly productive forms. In particular, for the varieties suitable for soy milk production, the breeders use large-seed samples with a light seed coat, light hilum and high protein content. These are the characteristics of the soybean variety Donskaya, which is commonly referred to as "milky" (All-Russian Research Institute of Grain Production), and Lakta (All-Russian Research Institute of Oil Crops). Such varieties should also have low trypsin-inhibiting activity, good extractability of solids and improved taste. The assessment of the above traits is rather time-consuming. It seems more constructive to screen the collection to reveal the soybean gene pool polymorphism of the level and proportion of glycinin (11S) and β-conglycinin (7S) protein fractions which constitute 70 % of the total soybean protein. The need for such assessment is determined by the fact that soy milk production necessitates soybean varieties rich in conglycinin as an emulsifier. Varieties with an alternative trait, in turn, should be suitable for protein texturates produced from soybean seeds [34]. Thus, tofu should be manufactured only from seeds with the prevalence of 11S fractions [35]. Soybean varieties are significantly polymorphic on content of these fractions [36-39], which allowed conclusion about the possibility of selection for the predominance of a fraction or even its subunits without reducing the total amount of protein. Such data can also be useful for the tactics of fodder production, since varieties containing more conglycinin are more suitable to fattening pigs, while cattle need more glycinin fractions [40]. The variability of this trait in gene pool may be assessed using molecular screening, since the families of genes encoding for these protein fractions are well known, their genomic organization has been studied and QTLs have been found and mapped [41-43].

When creating oil-bearing varieties, the characterization of the parental forms for the quality of the oil is also not always performed. In the VIR collection, the sources of valuable traits are found and corresponding genotypes are identified to optimize the oilseed selection. These valuable forms are those with high oil content (above 25 %), high levels of linoleic acid (50-52 %) and polyunsaturated acids, with *fan* gene recessive allele, determining the content of seed linolenic acid, and *fap1* gene recessive allele, determining the seed palmitic acid level [43]. The accumulation of oil in soybean seeds is under multiple genes control. Thus, about 110 corresponding QTLs have been mapped. Alleles of 14 genes, which control significant differences in fatty acids content, have been identified in the regions containing QTLs, and the greater number of "high oil"

alleles, the higher oil content is [44]. Interestingly, in samples adapted to high latitudes, the seed oil level is higher than that for those growing in low latitudes, which also correlated with the number of "high oil" alleles. This indicates the additivity of the action of genes controlling the synthesis and quality of oil in soybean. The study of the 10 most widely distributed varieties in China showed that they do not carry all the identified alleles for high oil content, which gives prospects for pyramiding additional alleles.

Modern technologies of improving storability, taste and nutritional qualities of soy oil suggest regulating proportion of certain fatty acids. Therefore, screening the gene pool polymorphism for fatty acids and tocopherols (vitamin E) content is constructive in searching for the parental forms for soybean breeding. In soybean seeds, there are γ-, δ- and α-tocopherols, amounting 60-70, 20-25 and less than 10 % of the total seed tocopherols, respectively [45-46]. It is α-tocopherol that is the most active as vitamin E in mammals. To date, a QTL has been identified for high α-tocopherol content, a mapping of this region has been carried out and *γ-TMT3* gene was identified which appears to be responsible for increased α-tocopherol accumulation [47]. In the VIR collection, the search for genotypes with high α-tocopherol based on identification of *γ-TMT3* and QTL is a matter of a near future.

The quality of soybean oil is largely depends on concentration of lipoxygenase (coenzyme Q). Lipoxygenase catalyzes synthesis of peroxide products (hexanal, etc.) from unsaturated fatty acids, which determine the "bean" flavor of raw soybean. Soybean seeds are considered as the richest natural source of lipoxygenase [48]. Three lipoxygenase isozymes are described of which lipoxygenase 2 mainly contributes to flavoring. Low content or inactivation of at least one isoenzyme eliminates bitter taste, improving the organoleptic characteristics of soy products. Forms with a low amount of lipoxygenase are also used for selection of edamame, the vegetable soybean which is very popular in Japan and the USA [49]. The presence of lipoxygenase fractions is controlled by the dominant *Lx* gene, and their low content or absence is controlled by the recessive *lx* allele. In the VIR collection, there are samples with *lx* recessive alleles; adding them to the genome of commercial soybean genotypes will reduce lipoxygenase activity to get products that are less prone to undesirable oxidation. To date, these genes have been sequenced, and molecular markers can be used to speed up the search for the desired genotypes [50].

Sugars are also important characters of soybean grain quality. Water soluble sugars of soybean are mainly sucrose disaccharide and stachyose and raffinose trisaccharides. In most varieties, the content of sucrose is 4.0-4.5 %, reaching 7-11 % of dry matter in some. The increased content of sucrose is desirable both for vegetable and feed use [51, 52].

Till now, targeted selection has been carried out for concomitants of soybean oil (phospholipids, tocopherols), biologically active substances (oligosaccharides, isoflavones) and for improving organoleptic parameters [9], so screening of the VIR soybean collection by these indicators has not been done yet.

Increasing the nutritional and fodder value of agricultural crops, endowing food varieties with the qualities of highly functional products is the prerogative of selection technologies, referred to as "bio-fortification". The creation of varieties containing more vitamins, bioactive substances, antioxidants, oils with an optimized fatty acid composition, a reduced content of antinutrients, etc. is aimed at combating the so-called hidden hunger. This is facilitated by high variability in the content of macro and micronutrients in plants. Unfortunately, in Russia this field has not yet found a proper development and the gene pool has not been screened for these characteristics. However, the impressive results of legume biofortification

obtained abroad (including traditional methods of selection) are known. As for soybean, the most famous example of bio-fortification is high-oleic oil, and also oil with a reduced content of saturated fatty acids. DuPont Pioneer obtained a variety for high-oleic food oil (80-85 %) through transgenesis. Varieties for technical purposes also have been created with containing up to 50 % linolenic acid in oil in contrast to 3 % characteristic of food varieties. Usually, the proportion of oleic acid in the soybean oil does not exceed 23 %. The high-oleic variety does not differ from the control ones in total proteins, oil, hydrocarbons, coarse fiber, certain amino acids, vitamins, ashes, minerals, trypsin inhibitors, oligosugars [53]. By traditional selection, low palmitic acid lines were created [54, 55]. In Japan, after chemical mutagenesis and x-ray treatment of seeds, lines with a high content of saturated fatty acids were revealed to increase the oil stability and to produce margarines and other solid and semi-solid fats. In total, the proportion of palmitic and stearic acid in soybean is usually 5 %, reaching 38 % [55] for new lines. A form with increased vitamin E content, more resistant to herbicides, was obtained by physical mutagenesis in the United States [56].

Molecular study revealed 35 QTLs associated with accumulation of Ca, Mg, Fe, Zn and P in soybean seeds. It creates the prerequisites for biofortification by MAS [57]. In addition, the researchers explore possibilities to improve utilization of these elements from soil by plants. For example, the QTL qPE8 was identified on the GM08 chromosome, which also contains gene *GmACP1* encoding the acidic phosphatase, a putative candidate gene for high phosphorus utilization. Overexpression of this gene in soybean root hairs leads to a 2.3-fold increase in acid phosphatase activity and an 11-20 % increase in the efficiency of phosphorus utilization compared to the control [58].

Our brief overview of successes in soybean biofortification is done in order to emphasize the need to study the soybean gene pool, i.e. variability of traits and the branches of industry in which they can be used, including alternative trait manifestations. In this, the variability of proteinase inhibitors in soybean seeds can be seen as an example. The study of the VIR collection accessions carried out earlier showed high activity of these antinutrients in the samples of cultivated and Ussuri soybean, and their low level (several times less) in wild Australian species [59, 60]. These species, constituting the tertiary gene pool, were often considered as agronomic potential for crosses with cultivated soybeans [61]. However, presently, the samples with an alternative (high) activity of trypsin inhibitors may be of great importance, since the participation of protease inhibitors in plant protection from pests and diseases has been shown, and most importantly, anticarcinogenic and radioprotective functions of protease inhibitors have been proven [62, 63]. Both of these properties are most pronounced in the Bowman-Birk protease inhibitor. In biochemical studies of the polymorphism of soybean varieties from VIR collection with regard to Bowman-Birk protease inhibitor and Kunitz-type soybean trypsin inhibitor, using a developed methodological approach to mass screening, the sources of raw material for the pharmaceutical industry have been identified. In addition, it has been shown that it is more reliable to identify soybean varieties by proteinase inhibitor electrophoretic spectra than by reserve proteins or DNA fragments detected by RAPD (random amplification of polymorphic DNA) [64].

A key role in expanding the geography of soybean cultivation plays high-yield varieties with a shorter vegetation period. The reaching of ripeness is relevant for all regions of soybean cultivation in Russia. In the southern regions, the early-ripening soybean varieties will optimize time of harvesting. Early ripeness can also ensure the growth of soybean in the more humid northern areas. This problem, posed by N.I. Vavilov in the early 1930s [65],

becomes especially relevant now due to warming and frequent droughts.

Expansion of soybean crops to the north is a worldwide trend. The main soybean country in the world, the United States, has long come to the need to create fast-ripening varieties, which led to the emergence of a new classification of ripening groups. If in the 1980s there were 10 such groups and the classification began with MG1 (maturity group 1), the appaeance of early maturing forms led to an increase in the number of ripeness groups to 13 and the appearance of groups MG0, MG00, MG000 [66]. In recent years, due to expansion of soybean crops to the high-latitude regions of China, MG0000 category with the fastest ripening to date has been added to this classification [67].

Crop adaptation to the cultivation zones is ensured by the time of flowering and maturation. The molecular genetic basis of precocity is not yet fully understood, but 10 loci determining the periods of flowering and maturation and designated as *E* loci have been identified by classical methods. Of these, four (*E1*, *E3*, *E4* and *E7*) are classified as photoperiodic genes. It was suggested that allelic variants and combinations of these genes determine the variability of maturation period [68]. The dominant alleles *E6, E9* and *J* lead to early blooming, while the dominant alleles of other genes, on the contrary, slow the flowering and the onset of ripeness [69]. The *E1, E2, E3, E4* and *E9* loci were categorized as QTLs. The casual genes of these loci are, respectively, the genes of the DNA-binding protein *Q3*, *GIGANTEA*, *Phytochrome A3*, *Phytochrome A2* and *GMFT2a* [69-73]. Casual genes of other loci have not been identified. In the field, the locus *E1* [74] exerts the greatest influence on the flowering time. Thus, molecular screening on the basis of early maturity is still a matter of the future. However, we have data of phenotypic assessment reflecting soybean ripeness periods in different Russian regions, including the northernmost point of the world's soybean cultivation (VIR, Leningrad Province). Breeders are given varieties corresponding to one or another region with regard of ripening groups, including ultra-ripe genotypes of the "northern ecotype", which, in our opinion, corresponds to the American ripeness groups MG00-MG000 [75-77].

So, at the present development of molecular selection, the diversity of soybean genetic resources from the VIR collection should be used more efficiently. The variability of the traits in gene pool and the molecular methods that optimize identification of gene pool differentiation by the target properties contribute to purposeful and targeted selection of parental forms for breading. The range of soybean application areas should be based on the varieties with specified parameters for different uses and different adaptive capabilities.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 917-927
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633/635:57.086.83:579

doi: 10.15389/agrobiology.2017.5.917rus
doi: 10.15389/agrobiology.2017.5.917eng

ENDOPHYTES, AS PROMOTORS OF in vitro PLANT GROWTH
(review)

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Received June 29, 2017

Abstract

In vitro plant propagation is a developed biotechnology, however until now there are no effective protocols for many perennials, especially for trees. High contamination of mature explants during tissue culture initiation, low multiplication and rooting during following passages are the main challenges. Aseptic culture of explants is associated with stress due to tissue damage and exposition to aggressive disinfectants and antibiotics during initiation. These could be the reasons of virulence of endophytes in following propagation. Plant-associated microorganisms were until recently seen as a problem for micropropagation, leading to contamination of in vitro explants. However recent studies showed that colonization of endophytes often play crucial role for increasing viability of in vitro and ex vitro plants. Most endophytes affect positively plant growth, providing nutrients and exhibiting antagonism to pathogens, as well as decreasing stress effects on plants. Beneficial effects were obtained in using *Beauveria bassiana* (J. Akello et al., 2007), *Piriformospora indica* and other members of family *Sebacinales* (P. Sharma et al., 2014), *Fusarium oxysporum* (A.S.Y. Ting et al., 2008), *Ophistoma*-like fungi (M. Mucciarelli et al., 2003), *Phialocephala fortinii* (M. Vohnik et al., 2003), *Trichoderma harzianum* and other *Trichoderma* species (P. Franken et al., 2012). Of bacteria, *Acetobacter diazotrophicus* (C.O. Azlin et al., 2007), *Achromobacter xylosoxidans* (A. Benson et al., 2014), *Azospirillum brasilense* (E.E. Larraburu et al., 2015), *Azotobacter chroococcum* (E.E. Larraburu et al., 2007), *Bacillus subtilis* (M. Vestberg et al., 2004), *B. megaterium* (P. Trivedi et al., 2007), *Burkholderia phytofirmans* (E.A. Ait Barka et al., 2000), *B. vietnamiensis* (M. Govindarajan et al., 2006), *Enterobacter* sp. (M.S. Mirza et al., 2001), *Klebsiella variicola* (C.-Y. Wei et al., 2014), *Microbacterium* sp. (M. Quambusch et al., 2014), *Pseudomonas fluorescens* (J. Thomas et al., 2010) и *P. putida* (R. Lifshitz et al., 1987) also can beneficially influence plants. But until now it is unclear which factor is a trigger switched endophytes from mutualism to virulence. The only way to control such a change is to develop optimal conditions (time of obtaining explants, culture media composition and pH, temperature, etc.) in view to save in vitro mutualism with benefit for both host plant and the endophyte. Studies of many perennials showed the in vitro biotization to be helpful in microclonal propagation and plant rooting. Particularly, arbuscular micorhyza, ectomicorhyzal fungi, ericoid micorhyzal fungi, and wide range of bacteria influence positively plant micropropagation. Bacterial and fungal endophytes could stimulate plant growth due to activation of plant protection mechanisms, induction of systemic resistance to pathogens, phytohormone synthesis and better transport of water and nutrients. In this, the difficulties of classification and obtaining pure cultures of microorganisms are the main problems faced with.

Keywords: micropropagation, endophyte, plant culture media, growth regulators, phytohormones

In vitro plant propagation is a developed field of biotechnology; however, until now there are no effective protocols for many perennials, especially for trees [1, 2]. Low yield of aseptic explants for tissue culture, low multiplication and rooting of microshoots during passages are the main challenges. Surface sterilization of explants and treatment with antibiotics do not relieve plant tissues from endophytic microflora, but often provoke the virulence of latent microorganisms [3]. In the culture, bacteria and fungi may appear in the first or, what happens more often, after a few passages (the so-called secondary infection) [4-

6]. Moreover, even in the absence of visible traces of microorganisms in the explant culture the efficiency of multiplication and rooting of the perennial microshoots is often low [7, 8] which can also result from a misbalance of endophyte bacterial communities [3].

Endophytic microorganisms contribute to growth and development of the host plant through production of phytohormones, better transport of water and nutrients, activation of biological protection mechanisms and induction of systemic resistance to phytopathogens [9].

The metabolic activity and the nature of the plant cell wall play a key role in the colonization of the host plant by microorganisms [10]. At different stages of colonization, the plant innate immunity is suppressed by phytohormonal signaling, which leads to better compatibility between the endophyte and the plant [11]. According to the latest data, plants and microorganisms coevolve, and, probably, even in "aseptic" in vitro tissue culture, there is no plant free from microorganisms [12, 13].

This data has been confirmed in numerous works, including in our studies, which mark visually the appearance of microorganisms in aseptic *Pelargonium*, *Citrus* spp., *Camellia sinensis*, *Hydrangea macrophylla* cultures and many other perennial species in multiple subcultivation [14, 15] The localization of microorganisms in the host plant is different: they occur in the apoplast, the intercellular spaces, the lumen of differentiated dead cells (sclerenchyma and xylem cells) of organs (roots, branches, leaves, flowers, fruits and seeds) and very rarely inside the cell [16].

The first reviews related to the application of microorganisms in cultures of in vitro plant tissues were published by J. Novak [17] and M.K. Rai [18]. J. Novak coined the term "biotization" and summed up the positive examples of its use in the plant micropropagation. The second article gives examples of mycorrhization in cultivated in vitro plants, deals with the problems of finding strains, obtaining a pure culture and the possibility of using mixed fungal cultures. The recently published review of the Russian colleagues focuses on identification and classification of bacterial microorganisms, their possible role in the culture of in vitro explants [5]. After that, the reviews on the positive results of the bacterial and fungal endophytes use in multiplication of in vitro plants have not been published. Filling the gap, we have summarized the progress made in recent years in biotization of in vitro plant cultures by fungal and bacterial microorganisms, and have identified promising areas of research in this field. In addition, in our review we briefly touch upon mechanisms to foster plant growth and defense reactions with the help of endophytic microorganisms.

Biostimulation and bioprotective potential of in vitro microorganisms. For plant micropropagation, the researchers used a wide range of microorganisms, fungi and bacteria. They evaluated the effect of arbuscular mycorrhiza [19], ectomycorrhizal fungi [18] and ericoid mycorrhizal fungi [20]. Beneficial effects were obtained in using *Beauveria bassiana* [21], *Piriformospora indica* and other members of family *Sebacinales* [22], *Fusarium oxysporum* [23], *Ophiostoma*-like species of fungi [24], *Phialocephala fortinii* [25], *Trichoderma harzianum* and other *Trichoderma* species [26]. A meta-analysis of the influence of root fungal endophytes showed that woody plants generally respond negatively to their presence, while herbaceous monocotyledons often respond positively to inoculation [27]. Of bacteria, *Acetobacter diazotrophicus* [28], *Achromobacter xylosoxidans* [29], *Azospirillum brasilense* [30], *Azotobacter chroococcum* [31], *Bacillus subtilis* [32], *B. megaterium* [33], *Burkholderia phytofirmans* [34], *B. vietnamiensis* [35], *Enterobacter* sp. [36], *Klebsiella variicola* [37], *Microbacterium* sp. [38], *Pseudomonas fluorescens* [39] and *P. putida* [40] were studied. Bacterial in-

oculation resulted in increased fresh and dry weight of shoots and roots, plant height, leaf area and rhizome mass [19, 20], better in vitro rooting (number and length of roots) [30], best adaptation (the percentage of acclimatization, the appearance of the plant) [41], early flowering and increased number of flowers, increased resistance to stress and immunity [42]; moreover, there were differences in profiles of metabolites [43].

The beneficial impact of microorganisms on the growth and biomass accumulation is due to the improvement of nutrient absorption by plant tissues and the production of various secondary metabolites, growth regulators [45], chitinolytic enzymes involved in protection against pathogens [46] and osmoprotectants, by which plants overcome abiotic stresses [47]. Below, some mechanisms and examples of biostimulation will be discussed in more detail.

Plant growth can be improved directly due to secondary metabolites and phytohormones produced by the microbial endophyte cell. For example, *Streptomyces atrovirens* ASU14 uses tryptophan and synthesizes indoleacetic acid IAA (22 rg/ml) [47]. Auxin-like activity was also observed in pteridic acid, which is synthesized by *S. hygroscopicus* TP-A0451 — endophyte of *Pteridium aquilinum* plant (L.) Kuhn ex Decken (Commom Bracken Fern) [48]. This substance stimulates the elongation of roots and the formation of adventitious roots in hypocotyls of *Phaseolus vulgaris* pinto bean. Another class of compounds produced by certain endophytes is gibberellins [49]. Strong growth stimulating influence of many endophytes is also due to the fact that they can turn plant exudates and macromolecules into forms digestible by other growth stimulating microorganisms, which is one of the mechanisms of plant growth biostimulation [50].

Microcuttings of *Pinus pinaster* Sol. and *P. sylvestris* L. pines rooted more effectively in the processing by *Hebeloma cylindrosporum* Romagnesi strains [51], and spontaneous rhizogenesis of *Larix eurolepis* larch microcuttings obtained from somatic embryos increased significantly in the presence of four ectomycorrhizal fungi, while the length and the degree of root branching increased [52]. Another study shows the influence of *Achromobacter xylosoxidans* AUM54 and indolyl-3-butyric acid (IBA) on the growth of in vitro *Naravelia zeylanica* (L.) DC medicinal plant. *A. xylosoxidans* is a diazotrophic endophytic bacterium, which showed a pronounced ability to enhance the uptake of NO_3^- by roots and reduce the content of ethylene (presumably due to the production of deaminases) [53]. Plant processing by these endophytic bacteria in combination with IBA improved the growth of shoots propagated in vitro, increased the length and the number of roots, the content of chlorophyll, nitrogen, antioxidant enzymes (peroxidase and superoxide dismutase) and enhanced resistance to stress (ethylene level) compared to the indicators in the untreated control. In the separate application of bacteria and IBA, a positive effect was much weaker [29]. In case of in vitro plant inoculation of *Elaeis guineensis* Jacq oil palm with *Acetobacter diazotrophicus* and *Azospirillum brasilense* diazotrophic rhizobacteria, there was strengthening of roots and shoot growth through fixing atmospheric nitrogen [28]. *A. brasilense* was more effective than *A. diazotrophicus*. Inoculation of micropropagated *Handroanthus impetiginosus* (Mart. ex DC.) Mattos plants with *A. brasilense* strains of Cd and Az39 stimulated in vitro rooting, reducing the need for auxin by 49 % on MSG half medium [30]. On this medium, Cd strain in combination with IBA (30 μM) induced root formation in 98 % of the shoots 21 days earlier than in the control without inoculation. The biomass index of inoculated shoots increased from 127 to 286 %.

Plant inoculation with endophytic microorganisms influenced positively not only the root formation, but the increase of in vitro plant biomass, the multiplication coefficient and the synthesis of biologically active substances. Thus,

subtracting nitrogen-fixing bacteria from sugar beet and their positive impact on the growth of micropropagated plants was described [36]. Two nitrogen-fixing bacterial isolates (SC11 and SC20; 10^6 CFU/g of dry weight) were obtained from shoots and two ones (SR12 and SR13; 10^7 CFU/g of dry weight) — from roots of plants in the open ground. Isolates identified as *Enterobacter* sp. produced IBA in pure cultures, and its synthesis increased on a nutrient medium with tryptophan. These isolates were used for inoculation of micropropagated plants. The maximum increase in the weight of roots and shoots and the most active nitrogen fixation were observed in case of SC20 strain. In the study of in vitro growth and the terpene synthesis, *Mentha piperita* peppermint in response to the inoculation of leaves with fungal endophytes had an increasing plant growth, increased leaf area, dry matter content, root biomass and higher menthol content [24].

Non-pathogenic strains of *Paenibacillus glucanolyticus*, *Curtobacterium pusillum* and *Methylobacterium extorquens* bacteria were isolated from the tissue culture of hosta and raspberry plants [54]. With these bacteria, the microshoots of chrysanthemum *Chrysanthemum* × *hortorum*, gerbera *Gerbera jamesonii*, hosta *Hosta japonica* and rose *Rosa* sp. were inoculated. *C. pusillum* bacteria stimulated the formation of lateral shoots in all studied genotypes. In the inoculation of *M. extorquens*, the number and the length of shoots and roots of gerbera and hosta, and the number of shoots of chrysanthemum were higher; the length of shoots of chrysanthemum and rose and the length of roots of rose were lower than in the noninoculated control. *P. glucanolyticus* affected the number and the length of shoots of chrysanthemum and gerbera, but the number of roots of gerbera and hosta was lower than in the control without inoculation; the length of roots of rose was just 0.2 cm. All three bacteria strains assimilated atmospheric nitrogen, and *M. extorquens* and *P. glucanolyticus* also synthesized IBA.

Biofertilization with microorganisms increases the vitality of multiplied in vitro plants at the stage of ex vitro acclimatization. For example, in studies of Czech scientists [20] from the roots of several host plants belonging to *Ericales* (*Vaccinium* sp., *Calluna* sp., *Rhododendron* sp., *Empetrum* sp., etc.) row, over 200 strains of endophytic fungi have been allocated. In these experiments, 10% of the identified strains proved to be effective and influenced positively the growth of micropropagated plants of rhododendron species (*Rhododendron* sp.) during ex vitro acclimatization in peat substrate. None of the isolates had a negative effect on the growth of host plants. In another study [55], in the optimization of a scheme for in vitro production propagation of wild indigo *Baptisia tinctoria* medicinal plant, it was shown that the application of arbuscular mycorrhizal fungi increased the acclimatization percentage of microshoots and rooted microplants.

So, according to the research results, cocultivation of plant microshoots and endophytic microorganisms may be an effective method of overcoming the difficulties encountered in some species during in vitro micropropagation.

Bioprotective activity of endophytes. The study of the potential of *Pseudomonas* sp. PsJN rhizobacteria strain as growth promoters and an increase in resistance of *Vitis vinifera* L. grape to gray rot caused by *Botrytis cinerea* showed that inoculation leads to a significant increase in plant growth, making them more resistant and viable [56]. The observed effect enhanced in transplantation. Cocultivation with *B. cinerea* led to significant differences in the pathogen aggressiveness of inoculated and intact plants. In the presence of the studied strain, the plants became more resistant to the pathogen.

Another good example of bioprotective potential of endophytes is the banana tissue culture [42]. One of the most serious banana viral diseases is

caused by *Banana bunchy top virus* (BBTV). The treatment of in vitro banana microplants by *Pseudomonas fluorescens* and *Bacillus* sp. microbial inocula isolated from the banana roots increased the resistance of plants to biotic and abiotic stresses. For this, the micropropagated banana plants were inoculated with *Pseudomonas fluorescens* Pf1 and CHA0 strains in combination with EPB5 and EPB22 (Pf1 + CHA0EP + B5 + EPB22) endophytic bacterial strains, which limited significantly the development of BBTV infection in the field (the frequency of contamination was 33.33 %, or by 60 % less than in the control). Production of protective enzymes and proteins was greater, and morphological and physiological characteristics were better in the plants treated with rhizospheric and endophytic bacteria (in particular, the plants responded to treatment with increased growth). In this report, induction of systemic resistance in banana with the help of associated bacteria was demonstrated, which may have practical importance for the development of methods for culture banana protection against BBTV virus [42].

Some authors have noted that the systemic bioprotective effect depends on the degree of tissue colonization by the microbiota [57]. Thus, in wheat, the observed antagonism of endophytes against pathogenic microflora was more a consequence of the protection mechanisms activation in the host plant, but not the result of direct antagonistic relations in the microbiota [58]. In experiments to study the effect of *Neotyphodium lolii* endophytic fungus on the induction of specific protection mechanisms, the inhabited plants were much less susceptible to *Fusarium poae* infection. In perennial grasses inhabited by endophytes, the number of chitinases is significantly higher than in intact plants, and depends on the time of inoculation [59].

Some endophytes can affect positively the resistance of plants to adverse abiotic factors [60]. Thus, mycorrhizal fungi improve the sodium neutralization under salt stress [61], which may serve as a mechanism for increasing the tolerance of plants in salinization. A similar technique is used in tissue culture to enhance the plant adaptability to abiotic stress (particularly, to salt one), and some endophytes are considered as useful and effective tools [62].

Achievements and problems of identification and use of endophytes in biotechnology. The development of modern methods of microscopy and molecular technologies (e.g., omics technologies) enabled a deeper understanding of interaction in the system of plant – endophytic microorganisms, mechanisms of mutualism and pathogenicity, which is clearly shown by P.R. Hardoim et al. [63], ecologically and evolutionary justifying the term "microbial endophytes". Sequencing of DNA and RNA has radically changed the approach to the study of microbial communities [64, 65]. The result of the application of these methods has received a lot of new data on the plant-associated microorganisms [66]; however, there is a problem with the interpretation and the analysis of this huge volume of genetic information for its effective use [67]. Full sequencing of the endophytic metagenome remains a challenge, as it requires separation of the genome of host plant from metagenome of endophytes [68]. A relatively easy technique is the analysis of the composition of endophytic microbial communities using polymerase chain reaction (PCR) enabling to determine the taxonomic composition of such a community and its structure [69], which, in turn, may reflect functional modifications in the groups of microorganisms [70].

As an example, we present a study of endophytic bacterial communities of six *Prunus avium* L. genotypes differing in the growth pattern during in vitro micropropagation [38]. For the analysis of uncultivated fractions of endophytic bacteria, a clone library of amplified 16S rDNA fragments was compiled. The

bacterial diversity was investigated using the analysis of restriction fragment length (restriction fragment length polymorphism — RFLP) in ribosomal DNA with clone sequencing for each certain taxonomic unit. For this purpose, 799f and 1492r-Y primers were used to separate amplified 16S rDNA fragments. The purified PCR products were cloned into pJet1.2 vector and were transferred into *Escherichia coli* DH10B. *E. coli* ampicillin resistant colonies were selected and tested in PCR using HpaII, HhaI, and BsuRI restriction enzymes. In the result, the dominant group of endophytes proved to be *Mycobacterium* sp. mycobacteria identified in clone libraries from all analyzed genotypes of *Prunus* genus. Other dominant bacterial groups of easily propagated genotypes were *Rhodopseudomonas* sp. and *Microbacterium* sp. The structure of the endophytic communities differed largely from the easily and hardly propagated in vitro genotypes: in the first one, bacteria groups were identified that stimulate plant growth.

As for the industrial use of endophytes in biotechnology and production of medicines-bioinoculants, the main problem is to find the most effective strain or a combination of strains. Over 80 % of endophytes are not detected in seeding on conventional nutrient media [71], which creates difficulties in obtaining a pure culture, identification and use of many strains. Besides, you must be sure that the selected endophyte will inhabit the internal plant tissues again and will have a positive effect. Another difficulty is the compatibility of endophytes isolated from one species of plants with plants of another species.

So, one of the innovative approaches recognized by the international experts as promising for an agricultural model under formation is the application of biologized technologies based on natural processes occurring in the soil – plant system. In this connection, plant-associated microorganisms and products of their metabolism are considered as a resource in the development of biotechnologies and their application for effective adaptation and rooting of microclones, as well as in plant protection. During the introduction of plant explants in aseptic culture, they are exposed to stress effects due to tissue damage and treatment with aggressive sterilizing agents, antibiotics, etc. This may be the cause of the sudden appearance of malicious endophytes during following passage. It is unclear until now what factor changes the nature of the interaction of an endophyte with its host, leading to the development of the pathological process instead of mutualistic relationship. The only way to control processes in such systems is the choice of the optimal passage time, optimal cultivation conditions and nutrient medium content to maintain mutualistic symbiosis beneficial for both the host plant and its endophytes.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 928-939
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Genetic resources, introgression, immunity
(towards 130th Anniversary of N.I. Vavilov)

UDC 633.31:631.461.52:577.21

doi: 10.15389/agrobiology.2017.5.928rus
doi: 10.15389/agrobiology.2017.5.928eng

A COMPARATIVE ANALYSIS OF GENOMIC CHARACTERS OF REFERENCE *Sinorhizobium meliloti* STRAINS, THE ALFALFA SYMBIONTS
(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported by Russian Science Foundation (grant № 17-06-01095)
Received August 2, 2017

Abstract

Plant-microbial symbiotic interaction is a unique highly specific biological system for fixing atmospheric nitrogen and its transformation into compounds accessible to living organisms. A fundamentally new approach may be the creation of a system for genetic monitoring of the stability of economically valuable strains in microbioms of agroecosystems. By comparison of the genomic characteristics of symbiotically active strains functionally significant marker sequences could be identified and the basis for genetic monitoring system will be created. In the review, we compared genomic characteristics of the symbiotic active strains obtained on the basis of *Sinorhizobium meliloti* 425a and SU47 strains, used for production of biologicals. Strain 425a was isolated from alfalfa nodules in the mid-1970s in the Almaty region (Kazakhstan), which is a part of the Central Asian primary center of cultivated plant origin designated by N.I. Vavilov. Strain SU47 was isolated from alfalfa nodules in the late 1930s in Australia, which is the secondary center of the diversity of cultivated plants. Strains CXM1-105 (CXM1) and Rm1021 (Rm2011) are widely applied as a reference strain as they have been used to develop or adapt a wide range of symbiogenetics methods. Genomes of both the original strains and their derivatives consist of a chromosome (SMc) and two megaplasmids (SMa, SMb), and do not contain cryptic plasmids. However CXM1-105 genome, unlike Rm1021 genome, did not contains 508 protein encoding ORFs (open reading frames) of which 242 are located on SMa, 121 are on SMb and 145 are on SM, as it was found using DNA biochips. This indicates significant differences in the structure of all three replicons in the reference strains CXM1-105 and Rm1021. Chromosome of CXM1-105 (CXM1), as well as of 425a did not contain sequences of phage origin (the «genomic islands») described in Rm1021. It was found that 62 ORFs of genomic islands are similar or homologous to those of the members of the same species or genus, as well as of phylogenetically distant bacterial classes. However, in the CXM1-105 chromosome there are sites for the integration of genomic islands (EU196757, EU196758 and EU196759), which are 99-100 % homologous to appropriate sequences of Rm1021 (Rm2011). We studied the occurrence of *S. meliloti* strains harboring type SMc_{Rm1021} or $SMc_{CXM1-105}$ chromosome (presence or absence of genomic islands, respectively) in native populations. The significant prevalence of strains inherited SMc_{Rm1021} was shown for the area, belonging to the Middle-Asian gene center of cultivated plants, while strains harboring the chromosomal type $SMc_{CXM1-105}$ were dominant in the area of extremely saline soils next to Aral Sea area ($P < 0.05$). Consequently, the presence of additional «foreign sequences», which could participate in horizontal gene transfer, is typical of native *S. meliloti* isolates abundant in

the primary center of the diversity of their host plants whereas those sequences are lost under an abiotic stress (salinity) impact. In addition, strains harboring different chromosome types, according to structural differences in the intergenic sequence of *rrn-rrl* operons, can be referred to divergent clonal lineages. According to the discussed data, it was suggested to consider strain 425a and its derivatives as the model *S. meliloti* strains to create a system for genetic monitoring of practically valuable strains in agrocenoses.

Keywords: symbiosis, alfalfa, reference strains, *Sinorhizobium meliloti*, molecular genetic analysis, genomic islands, sites for specific integration, accessory genome

Plant-microbial symbiosis is a unique highly specific biological system for fixing atmospheric nitrogen and its transformation into compounds available for living organisms. An important role in increasing the productivity of plants is performed by the microbial component [1], the importance of which is often underestimated [1-3]. It is known that the microorganisms that form the basis of biopharmaceuticals must have certain properties, including virulence, specificity, competitiveness, symbiotic activity and efficiency (productivity), and also must meet a number of technological requirements [4]. In order to comply with these requirements and to preserve the properties, strains should be subjected to supporting selection, because their economically valuable properties deteriorate or are lost over time [5-6]. One of the reasons may be that genes that determine the symbiotic properties of nodule bacteria are localized, as a rule, on plasmids, the availability and structure of which may be unstable (for example, under the influence of an abiotic stress factor), which, in turn, can lead to a decrease or loss of efficiency (productivity) of inoculated plants [7, 8].

A fundamentally different approach is to create a system for genetic monitoring of the stability of economically valuable strains and to study the pangenome of nodule bacteria of alfalfa and the microbiome of agroecosystems [9-11]. The comparison of the genomic characteristics of symbiotic active strains will allow identifying functionally important marker sequences and can become the basis for creating such a system [12]. One of the stages of this study is a comparison of the properties of production strains that can be recommended as model in the development of a system for monitoring the stability of agroecosystems.

This review includes the first analysis of the strains of *Sinorhizobium meliloti*, obtained on the basis of the commercial strains 425a and SU47, which have been studied for a long time in domestic and foreign laboratories dealing with the issues of symbiogenetics.

Origin of the 425a, SU47 strains and their derivatives. A modern taxonomic name for the strain *Rhizobium meliloti* 425a is *Sinorhizobium* (*Ensifer*) *meliloti*. The strain was isolated as highly active from the nodules of alfalfa in the Almaty Region (Kazakhstan, AS No. 549454 dated May 25, 1977). In 1986, on its basis, a highly efficient, resistant to streptomycin (StrR) strain 425a-str-6, or CXM1 [13] was obtained, from which subsequently, as a result of UV mutagenesis, the strain CXM1-105 [14] was obtained. The strain *R. meliloti* SU47 was isolated from the nodules of *Medicago varia* in New South Wales (Australia) in 1937 [15]. On the basis of SU47, the strain Rm2011 was created. The strain Rm1021 is its StrR derivative. Also, the derivatives of Rm2011 include the strain RCR2011. All strains obtained from SU47 were maintained in different laboratories of the world, which caused differences in their names.

Alfalfa is one of the oldest cultures used by mankind. It is mentioned in the Babylonian texts relating to 700 BC [16]. The main centers of the formation and distribution of the more ancient diploid species of blue and yellow alfalfa are Asia and Middle Asia, included, according to the theory of N.I. Vavilov [17-19], in different primary centers of origin of cultivated plants (gene centers). These centers, in which natural and artificial selections are intensively and jointly act-

ing, serve as a source of the diversity of natural plant genotypes [20-21]. The Almaty Region in Kazakhstan, where the 425a strain was isolated from the nodules, is adjacent to the Central Asian gene center, while Australia (the place of isolating the SU47 strain) does not belong to the primary center of diversity of perennial alfalfa species; however, a secondary gene center of the genera *Medicago* L. was formed in this continent. Perennial alfalfa first arrived in the territory of Australia from France (at the end of the 18th century), and then from the US [19].

Taking into account the historical ways of distributing alfalfa as a host plant, it can be assumed that strains-microsymbionts have also undergone similar distribution. It is possible to assume that the strain SU47 could be introduced with seeds or soil from the territory of the Central Asian center. This does not contradict the conclusions of the researchers that the chromosomal genotype of the strain RCR2011 (derived from SU47) has a wide geographical prevalence [22]. Thus, it is possible to assume that the strains 425a and SU47 considered could have common historical origin.

Symbiotic effectiveness of the strains 425a, SU47 and their derivatives. The SU47 strain was available as a symbiotic effective inoculant for di- and tetraploid forms of alfalfa in Australia for farms since 1955 and was used there on an industrial scale since the 1960s, and in New Zealand since 1973-1975 [23]. On the basis of the strain Rm2011, the biopreparation Nitrogin [24] was created. The symbiotic properties of Rm1021 and Rm2011 have been studied during long-term model microvegetation and field experiments, which served as a rationale for the classification of these strains as referent strains [25-26]. The strain 425a is used to prepare the Rhizotorphin biopreparation. The average increase in the yield of alfalfa during inoculation with this strain, as it was stated (AC No. 549454 of May 25, 1977), was 14.5%. Field and plot experiments conducted using different varieties of alfalfa (Yakutskaya, Agniya, Pastbishchnaya 88, Vega) in the continental and transitional conditions of the temperate climatic belt (Tyumen, Vladikavkaz, Leningrad Region), as well as in the Far North in the Republic of Sakha (Yakutia) in the period from 1999 to 2016, showed an increase in the yield of green alfalfa mass by 9.8-48.4% when inoculated with the strain 425a ($P < 0.0015$) [27]. Its derivatives – the strains CXM1 and CXM1-105, have also been studied for economically valuable properties, such as productivity (symbiosis efficiency), nitrogen-fixing (acetylene-reductase) activity, host specificity, competitiveness in model and plot experiments [13, 14, 28]. According to the results of independent micro vegetation and vegetation experiments performed in different years, it should be concluded that the strains CXM1 and CXM1-105 stably retain high symbiotic activity. The average increase in dry weight in inoculated plants was 90.8-100.2% ($P < 0.0015$) with respect to the control without inoculation and 28.6-32% ($P < 0.0015$) compared to the strain Rm1021. Differences between the strains under study in terms of the ability to form a symbiosis with the model diploid species of alfalfa – M. *truncatula* of the Jemalong variety in model micro vegetation experiments was identified. The strain CXM1-105 formed effective pink nodules, and the increase in the dry weight of plants with respect to control without inoculation was 31.5% higher compared to similar plants inoculated with the strain Rm1021. The fact that Rm1021 does not form an effective symbiosis with *M. truncatula* A17 was shown by Australian researchers [29]. At the same time, in the conditions of poor salinity (0.3% NaCl), significant differences ($P < 0.05$) between the strains under study in terms of symbiotic efficiency were not found with either *M. truncatula* or *M. varia* (V.S. Muntyan, personal message). Therefore, the strains obtained on the basis of 425a (CXM1, CXM1-105) are used as reference

ones: they have higher indicators of symbiotic activity compared with the strain Rm1021 and stably exhibit them under standard (typical) conditions.

Methods of molecular genetic analysis developed on the basis of reference strains *S. meliloti*. Using the reference strains listed above, various models have been developed or adapted to study the formation and functioning of symbiotic systems and for their molecular genetic analysis. The methods of UV and chemical mutagenesis, protoplast fusion, transduction, conjugation, and transformation were developed on the basis of derivatives of the strain 425a [30, 31]. Using the species-specific region of the ISRm2011-2 chromosome of the strain Rm2011, a system for typing genomes (fingerprinting) of natural strains of nodule bacteria of alfalfa has been developed [32]. In the genome Rm2011 (Rm1021) there are 12 copies of ISRm2011-2, in the genome of the strain CXM1 — 11 copies [33]. A significant step in the study of symbiotically important genes was the development of systems of general en masse and directed Tn5-Mob and mini-Tn5 mutagenesis. A collection of 12,000 mini-Tn5 mutants was obtained on the basis of the reference strain Rm2011, an analysis of more than 9,000 of which showed that insertions of mini-Tn5 into genes encoding peptides occurred at a frequency of 0.6 [34, 35].

More than 20 genes involved in the control of the synthesis of poly- and lipopolysaccharides, symbiotic efficiency, competitiveness, salt tolerance, and acid resistance have been sequenced, and their phenotypic manifestation in the derivatives of the CXM1 strain has been studied [36-41]. The genomes of the strains Rm1021 and Rm2011 were sequenced in 2001 and 2013 respectively and are presented in databases (https://iant.toulouse.inra.fr/S.meliloti, https://iant.toulouse.inra.fr/S.meliloti2011). Further, this allowed proposing methods of modern genomic analysis using phenotypic, DNA and expression biochips [42-43] to simultaneously study the work of more than 14,000 genes and intergenic regions, and compare their expression and phenotypic manifestations [35, 44].

Comparative genomic analysis of reference strains. The genomes of the considered reference strains are similar; they have three replicons (a chromosome and two megaplasmids) and do not contain additional cryptic plasmids. According to the data of the genome-wide sequencing, in Rm1021 the chromosome size (SMc) is 3.5 million bp, the sizes of the megaplasmids SMa and SMb are 1.35 and 1.68 million bp, respectively, which is accepted as a typical characteristic of the genome of nodule bacteria of the species *Sinorhizobium meliloti*.

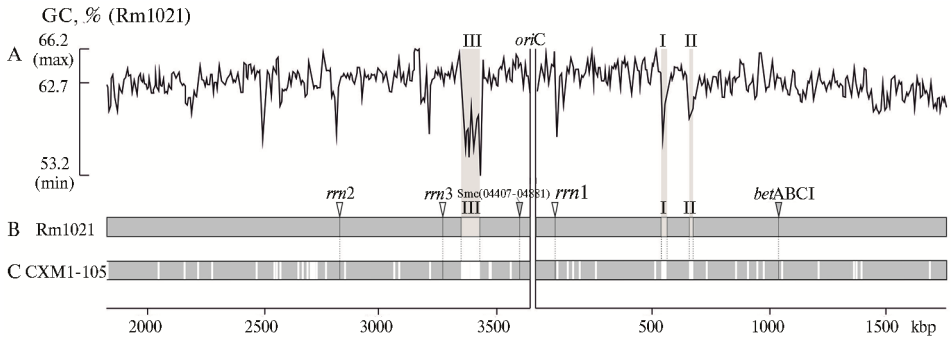
The replicon SMb is considered as a small chromosome Rm1021, since it has an average composition of GC pairs of 62.4%, which is 0.3% lower than that of the chromosome. 1,570 genes are located on this replicon (open reading frame, ORF), which are functionally related to 20 different COG-groups (clusters of orthologous groups) [45]. However, mainly these are the genes responsible for the carbohydrate metabolism and synthesis of polysaccharides necessary for successful microbial-plant interaction and for the saprophytic existence of bacteria in the soil or in the rhizosphere. The second mega-replicon Rm1021-SMa contains 1,293 genes (ORF), and its nucleotide composition contains 60.4% of GC pairs on average, which is significantly lower than in SMc and SMb. In this replicon, there is a zone of 90 thousand bp including clusters of *nod, nif, fix* genes that determine the process of formation and functioning of nitrogen-fixing symbiosis with the host plant.

Comparative analysis of the genomes of the reference strains CXM1-105 and Rm1021 using the DNA biochips SM6kOligo showed that 242 ORFs, localized on SMa, and 121 ORFs, localized on SMb, were changed or absent in CXM1-105 [46]. This indicates significant differences in the structural organiza-

tion of mega-replicons in the reference strains CXM1-105 and Rm1021.

We identified 69 genes belonging to five different groups that are involved in the control of such features of symbiosis formation as virulence (*nod*), nodule formation and its specificity (*nol*, *noe*), and the process of nitrogen fixation (*fix* and *nif*), and analyzed them using the technique of DNA-biochips [46]. As a result, it was found that in both model strains 65 ORFs are similar, including *nodD1*, *nodABC*, *nodEFGH*, which also had similar PCR-RFLP types (the analysis of polymorphism of the length of restriction DNA fragments) [46]. The remaining four ORFs were not detected in CXM1-105 [46], which meant the absence or significant alteration of these sequences (divergent sequences). Three of them are localized on SMa and belong to the fix-genes group, and the fourth is localized on SMb and presumably encodes the acyltransferase (EC 2.3.1.-), that belongs to the proteins gene family *cysElacA/lpxA/nodL*.

The Rm1021 chromosome contains 3,341 open reading frames, whose products are involved in the functioning of information systems – replication, transcription, translation, and are also responsible for the key pathways of metabolism and the formation of cellular structures [45]. Therefore, genes located on the chromosome are often called core.



Comparative analysis of the chromosome structure in the strains *Sinorhizobium meliloti* Rm1021 (A, B) **and CXM1-105** (C)**:** A — the content (%) of GC pairs in Rm1021 chromosome determined by the moving average method (10,000 bp window, 5,000 bp step, Unipro UGENE program); B and C — graphics images of the chromosome structure in Rm1021 and CXM1-105; *oriC* — the point of the origin of autonomous replication of the chromosome; I, II, III — the genomic islands Sme21T, Sme19T, Sme80S, respectively; ▒ — the projection of the regions of localization of genomic islands on the graphic image of chromosomes Rm1021 and CXM1-105; ▒ — sequences that are homologous in Rm1021 and CXM1-105, □ — sequences, that are not detectable in the CXM1-105 genome; ▽ — *rrn*-operons; ▽ — loci *betABCI* and SMc04407-SMc04881 (for details, see the text of the article).

We analyzed 5 loci in chromosomes Rm1021 and CXM1-105, which are located in remote regions of the chromosome and can give an idea of the structure of a vital replicon. This is the intergenic sequence SMc04407-04881 with a length of 1,280 bp between the genes SMc04407 and SMc04881, which is located to the left of the origin of replication of oriC (Fig.). The second sequence is the locus, which includes the part of the bet operon located at a distance of 1,039 million bp to the right of the oriC (see Fig.). This sequence of 1,544 bp included 1,400 bp of the *betC* gene, an intergenic region of 1 bp and 143 bp of the *betB* gene, which are involved in the synthesis of osmoprotectant glycine-betaine. PCR-RFLP analysis did not identify differences in loci SMc04407-04881 and *betCB* in the referent strains under consideration [47]. Three other regions correspond to taxonomically significant intergenic sequences of ribosomal operons (*rrn*, see Fig.). According to the data of PCR-RFLP analysis, all three intergenic sequences of *rrn* operons in the Rm1021 strain, 1,307 bp long each, are of the a-type [48], and similar regions of CXM1-105 are b-type, which

may indicate that the genomes of the strains under consideration belong to phylogenetically remote divergent clonal lines [48, 49].

The regions in which the content of GC pairs is 6-8% lower than the average content (62.73 %) are of particular interest in the structure of the chromosome Rm1021. Such regions have less structural rigidity [50] and are evolutionarily younger in comparison with the core part of the chromosome. In the chromosome Rm1021 (Rm2011) there are three such sequences (Sme80S, Sme21T and Sme19T), which are considered as so-called genomic islands (see Fig.). These structures are atypical extended mobile elements that contain sequences of phage origin, IS elements and functionally significant genes, as well as more than seven dozen sequences from which non-coding RNAs are transcribed. The islands can actively participate in the horizontal transfer of genes [51]. It is necessary to consider in detail the structural arrangement of the islands in Rm1021 relative to the origin of replication of the chromosome *oriC* (see Fig.) The two islands (Sme21T and Sme19T) are located adjacent to the right of the *oriC* and have a similar length (20.7 and 18.6 thousand bp). Sme21T is located at a distance of 541 thousand bp from the oriC, Sme19T — at a distance of 98.6 thousand bp on the right of it (see Fig.). The third island (Sme80S, length 80.2 thousand bp) is located to the left of the *oriC* and is 216.4 thousand bp away. Such an arrangement of islands in regions close to oriC, as well as to ribosomal operons, indicates that these sequences can have important functional significance, since they are replicated in the first place. Structures analogous to genomic islands were not detected by us in the chromosomes of the strains 425a, CXM1 and CXM1-105. In addition, according to data obtained using DNA-biochips, 145 protein-coding ORFs [46] are absent in the structure of the chromosome CXM1-105. The figure above illustrates the differences between the chromosome structure in the reference strains Rm1021 and CXM1-105.

All three genomic islands, like the so-called pathogenicity islands, which are most well studied, have site-specific embedding. The places of integration of the Sme21T and Sme19T islands are direct nucleotide repeats located at the 3′-ends of the two isoacceptor tRNA of threonine (tRNA-Thr), and in the case of Sme80S at the 3′-end of the tRNA of serine (tRNA-Ser) [52]. Based on the complex in silico analysis of the site-specific integration of genomic islands in *S. meliloti* Rm1021, a system for their in vivo detection by PCR and original pairs of primers was developed [52]. According to the technical parameters of the PCR method, the PCR products can be synthesized with primers for amplification of the outer border regions of the islands (regions of integration) only in the absence of islands, as, for example, in the strain CXM1-105 [52]. Sequences obtained as a result of PCR and corresponding to the border regions in the chromosome CXM1-105 are sequenced and deposited in GenBank (EU196757, EU196758 and EU196759). A comparative analysis of these sequences in CXM1-105 and the integration regions of genomic islands in Rm1021 made it possible to study their structure and to estimate the degree of homology. It has been found that the sequence EU196757 (1,230 bp) contains a site for the specific integration of the Sme21T island with a length of 16 bp (direct repetition), to the left and right of which there are sequences of 880 and 334 bp, respectively, which are 99% homologous in Rm1021 and CXM1-105. The sequence EU196758 (561 bp) has one direct repeat of 31 bp in length — the site for the specific integration of the island Sme19T, and to left and right of it there are sequences of 53 and 530 bp with 100 % homology in the reference strains under consideration. The site for the specific integration of the third island Sme80S — a direct repeat of 15 bp in length is identified in the sequence EU196759 with a

length of 826 bp. To the left and right of it there are sequences of 397 and 417 bp respectively, which are 100 % homologous in Rm1021 and CXM1-105. On the left in the region between the SMc03748 gene and direct repeat, the Rm1021 also has an IS element of TRm11, which is not present in CXM1-105. An analysis of the border regions of the islands shows that in the CXM1-105 genome, sites for specific integration/insertion of existing genomic islands are preserved. In addition, it is possible to assume that the absence of islands can contribute to a greater structural stability of the chromosome in CXM1-105, as well as in CXM1 and 425a; however, in order to confirm this, appropriate studies are required.

The island detection system was used to identify structural types of chromosomes similar to those of Rm1021 ($SMc_{Rm1021,}$ have islands) or CXM1-105 ($SMc_{CXM1\text{-}105,}$ do not have islands) in four geographically remote regions. It was established that the natural strains of *S. meliloti*, isolated in the southern region of Uzbekistan, which is part of the Central Asian gene center, had the chromosome type SMc_{Rm1021}, which has a frequency of 0.72. Strains from the northern region of the Caucasus, adjacent to the Front Asian gene center, which played a leading role in the formation of cultural diploid alfalfa, as well as from the modern center of introgressive hybridization of alfalfa [21] located in the foothills of the Mu-dozhary in the north of Kazakhstan, also predominantly had the chromosome type SMcRm1021. The frequency of these types was similar, but was lower (0.54) than in the Central Asian center [52]. Only in the territory located at a distance of 500 km from the modern shore of the Aral Sea, which was subjected to extreme salinization, strains isolated from the liquorice rhizosphere or from saline sands predominantly had a chromosome type SMcCXM1-105 with a frequency of 0.62, that is, they had no genomic islands. Differences between samples of strains from the Central Asian gene center, in which strains with the chromosome type SMc_{Rm1021} predominated, and from the Aral region where strains with the chromosome type $SMc_{CXM1\text{-}105}$ prevailed were significant ($\chi^2 = 4.388$; $P < 0.05$). Consequently, strains that had islands were significantly more frequent in the area of the primary gene center of alfalfa, whereas under the influence of the stress factor (salinization), loss of islands occurred.

The evaluation of the functional significance of genomic islands in Rm1021 was of interest. The islands contain copies of functionally important genes which, for example, can participate in the protection of cells from foreign DNA (locus *hsdRSM*), and can also affect the resistance of cells to the specific soil conditions (involved in the synthesis of the melanin pigment, proline osmoprotectant). In addition, one of the islands contain the *fixT3* gene − a copy of the *fixT* gene [52], which, as previously shown [53], is involved in the control of nitrogen metabolism, is influenced by the two-component global regulation system FixJL and is induced at a low oxygen content, as well as in nodules. However, the functional role of a significant portion of ORFs is not known. We conducted the search for sequences similar (homologous) to those in the strain Rm1021, in the closely related genomes, as well as in the taxonomically distant species. It was found that there are 62 ORFs in the Rm1021 islands, the sequences of which are similar or homologous to those in bacteria — representatives of 22 genera from 4 fillets, as well as from uncultivated bacteria. In most cases (33%), the islands contained OFRs, whose homologues were detected in the α-proteobacteria. They were functionally related to the storage of information (K, L; 21 %), metabolism (E, P, G, M; 33 %) or belonged to the group of poorly characterized (R, S, 17 %). ORFs that are homologous or similar to those in taxonomically distant representatives of the β-, γ- and δ-proteobacteria, were predominantly belonged to the group involved in cellular pro-

cesses and to the signaling group (O, T, V; 17 %). Differences in ORF distribution between the indicated bacterial taxa were significant ($\chi^2 = 11.02$, $P = 0.01$). Thus, the Rm1021 gene has an “additional genome” that includes functionally significant ORFs that are similar or homologous to those in representatives of the predominantly the same species or genera, as well as in phylogenetically remote classes of bacteria. Such diversity of ORFs is the result of high activity of horizontal gene transfer, which could take place both in the soil microbiome and in planta in the nodule. In the latter, according to a recent publication [54], different representatives of phylogenetically remote groups of bacteria can be present simultaneously. Indirect evidence of the possibility of horizontal transfer of genetic determinants in the nodule can be the fact, discovered by us, of the presence of a high phylogenetic diversity of OTF in the genomic islands of the strain Rm1021 – typical representative of saprophytic bacteria that form a non-obligate mutualistic symbiosis with leguminous host plants. Currently, it is not possible to conclude that the genomes of the reference strains Rm1021 (Rm2011) and CXM1-105 (CXM1) differ in terms of the presence of an "additional foreign genome", since "foreign" genes can be fixed in the core part of the chromosome CXM1-105; however, such a statement can be confirmed with full genome sequencing.

Thus, summing up the results of the comparative analysis of chromosome structures in the reference strains obtained on the basis of the production strains 425a and SU47, it should be concluded that these strains can relate to the evolutionarily diverged lines of nodule bacteria that were distributed in the Central Asian center of the origin of cultivated plants. Based on the set of data presented, the derivatives of the strain 425a-CXM1 and CXM1-105 should be considered as model high-performance strains of the *Sinorhizobium meliloti* genera, which are genetically different from Rm1021/Rm2011, which can be used to develop methods for obtaining new genetically stable strains of nodule bacteria that promote the formation of highly productive and stress-resistant plant-microbial symbiosis, and to create an “umbrella” system for genetic monitoring of the stability of economically valuable strains in microbiomes of the agroecocenosis.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 940-951
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 635.24:631.522/.524

doi: 10.15389/agrobiology.2017.5.940rus
doi: 10.15389/agrobiology.2017.5.940eng

BREEDING OF JERUSALEM ARTICHOKE WITH THE DESIRED TRAITS FOR DIFFERENT DIRECTIONS OF USE: RETROSPECTIVE, APPROACHES, AND PROSPECTS

(review)

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The authors declare no conflict of interests
Received June 11, 2017

Abstract

In the last decade, a new direction has been widely developed for industrial and food use of Jerusalem artichoke (*Helianthus tuberosus* L.). At the same time significantly expanded the cultivated areas of this crop, especially in Asian countries. Relevant extension studies therefore becomes focused on breeding of new varieties, including those with high content of certain biochemical components in tubers or leaves and stems. The future of J. artichoke as energy source for biofuels, as well as a source of fiber, sugar substitute for people, who require insulin is very promising. Despite the presence of a large number of varieties (more than 300) of J. artichoke in different countries, its genetic diversity is not so wide (P.P. Wangsomnuk et al., 2011; R. Puttha et al., 2013), because all breeding varieties are based on intraspecific hybridization, or as result of selection of seedlings from self-pollination. In addition, due to the very low self-fertility, the breeding of J. artichoke and its generative reproduction has yet little success. The experience on multi-year studies of J. artichoke diversity and breeding work in many countries shows, that the effect of high-directed breeding on desired traits can be achieved only through inter-specific hybridization. The crossing J. artichoke with sunflower, allows transmitting at new generation the characters and properties of the initial forms and improved through heterosis (L. Natali et al., 1998; C. Breton et al., 2010). Thus, we can say with great certainty about reality of J. artichoke breeding to achieve the desired traits using inter-specific hybridization. The inter-specific hybridization of artichoke J. artichoke with sunflower (*Helianthus annuus* L.) can be successfully use as a breeding method for creation of varieties with the desired traits for specific uses. Given the current demand for different directions of use products from J. artichoke, it is likely that the breeding of J. artichoke will be focused on creation of special varieties - for food, for medicinal purposes, for processing in the inulin, purposes of animal feeding, for the production of bioenergy, technical and environmental goals etc. (M. Baldini et al., 2004; G.J. Seiler et al., 2004; R. Puttha et al., 2012; S. Favale et al., 2014). We can say with confidence that there are enough initial material for all areas of breeding. For these, it is necessary to extend the researches to find the possibilities of using the existing gene pool of artichoke in many gene banks. Today, there are a different of modern methods for this, including molecular genetics. One has to stress for breeding J. artichoke the importance of molecular genetics technologies towards the existing gene pool of artichoke in many gene banks.

Keywords: Jerusalem artichoke, sunflower, breeding, hybridization, selection, target traits, food and forage use, raw for use, inulin production, bioethanol production

To breed varieties for a new use, it requires to start on a wide genetic basis to ensure enough diversity in the future crop. In the past, several crops have been transformed from a specific use to undergo new uses. Several examples could be developed and among them are the crops, devoted to

industrial uses such as sunflower (*Helianthus annuus* L.), cotton, safflower, fiber crops, linseed to make them an oil crop for food and recently extant to bio-fuel. The second example is the root beet transformed into sugar beet in France at the beginning of the eighteen century to provide sugar because of the blockade set up by England to cut France from its colonies to import sugar from sugar cane. Louis de Vilmorin (1850) has initiated sugar beet breeding and the main issue of the research has been to harvest sugar beet plants separately, and not in mixture enabling selection of individuals with with improved sugar quantity. Root beet was devoted to feed livestock, it contains by 10 % sucrose and lower than in table beet, but only Root beet was chosen because it was white devoid of anthocyanins that makes white sugar, while because table beets that may content more sugar are frequently colored, they were carefully not used to get sugar.

Sunflower story may shed light on this process and may give back what we have to do on J. artichoke (*Helianthus tuberosus* L.) before starting a biotechnological platform to industrial production of inulin. Confectionery sunflower has been transformed into an oil crop between 1860 to 1920 by Russian peasant selection to give fat, because confectionery sunflower was not on the list of forbidden plants to eat during the Orthodox Lent. During the Lent period confectionery sunflower provided fats. Moreover, the seeds were commonly eaten after baking and salted. Thus, scientists from the VNIIMK (All-Union Research Institute of Oil Crops, former USSR) decided by the 1930's to screen through mass selection, populations thriving in plenty of gardens for those with the most oil content. Each center had constituted one population by mixing all the sources. The history of oil sunflower remains unclear because most of the geneticists involved did not agree Lysenko theories and have disappeared in jails. These tasks done in four main research centers have released all oil Russian sunflower populations which are now designed as old Russian populations (ORP).

For researchers not specialized in plant breeding it may appear superfluous to spend time on this reflexion because the diversity of *J. artichoke* is widely kept in several worldwide institutes [1-5] and hundreds of clones appeared sufficient to carry out breeding programs. However, there is no genetic diversity in a J. artichoke clone and thus the diversity is only between clones. The number of clones to handle for breeding J. artichoke should be therefore very important. The diversity can come only from new J. artichoke varieties screened in the progenies of crosses between J. artichoke clones. Wild and feral J. artichoke thrive as clones, but they may also come from seeds [6], that was rarely observed from feral J. artichoke [7]. Seeds of J. artichoke are tiny, they are as small as for wild sunflower, with the same troubles to induce germination because of their dormancy, due to integuments. Thus it is required to make an incision onto the integument and to bath seeds in Ethrel or GA3 to enable fast germination. The novel protocol for seed germination improvement of J. artichoke has been published by P. Wangsomnuk [8] which demonstrated that the exogenous supplementation of growth regulators and temperature improved germination of dormant seeds under in vitro and in vivo conditions.

The length of the vegetative period from a tuber put in soil to blossoming is determined by the sum of temperature of each day. One clone has a threshold genetically determined and when the sum of temperature from each day, and as long as the threshold is not reached the plant cannot blossom. For a given threshold the time length to get blossoming depend on the average sum of temperature and as high the temperature as short the delay to blossoming.

Today Jerusalem artichoke, due to its richness of biochemical and mineral components, is rapidly growing demand for its multilateral use [9-11].

As J. artichoke was commonly used for animal feed the concentration of minerals has been studied [12-14]. Also, in the last two decades, interest in Jerusalem artichoke has resumed in many countries, as a plant from which cheaper alcohol can be obtained [15-17], both from tubers, as well as from above-ground biomass [18, 19]. The economic benefits of the production of Jerusalem artichoke alcohol have been proven in many countries [19-22]. Accordingly, for this direction, no doubt, we need varieties of Jerusalem artichoke with the highest content of sugars. The demand for raw materials from Jerusalem artichoke for biofuel production is noticeably increasing in various countries [23].

However, for future uses, the main storage compound in J. artichoke is inulin that is synthesized in leaves by photosynthesis and accumulates in the vacuole of each cell. Starch is poorly present in leaves, thus, the inulin induces osmotic pressure depending on its polymerization degree [24]. Among J. artichoke clones the trait "inulin content" displays a wide range of variation and "inulin yield at harvest" is the trait to improve for a biotechnological platform. However, not all environmental factors that influence on inulin content at harvest time are known yet, but such influence factors were confirm manifold [25-29], including irrigation [13, 30,] as well as the harvesting dates, methods and conditions for storing tubers [27, 31, 32].

The range of variation between different clones is due to genetic differences in their potential. Thus, the targeted screening of breeding material should release new varieties with regular higher inulin content, which justifies the cost of the breeding program [33].

The choice of parents to obtain new progenies remains random because we have no knowledge on the complementary effects between J. artichoke varieties to release new varieties with improved inulin content at harvest time. We have to define strategies to help breeders to make the best crosses by choosing the adequate parents in order to succeed in the breeding program. Sunflower has been improved partly by crosses with *J. artichoke* such as ♀ sunflower × ♂ J. artichoke because in this direction the progenies provide most annual plants without tubers. To transfer valuable genes from J. artichoke to sunflower, crosses have to be made in the other direction, i.e. ♀ J. artichoke × ♂ sunflower that is much more difficult to carry out, but ensure most plants with tubers [34].

Another difficult feature to breed J. artichoke is that it is hexaploid carrying 3 times more chromosomes than sunflower [34]. It has been shown that J. artichoke is probably auto-polyploid carrying 3 different elementary genomes named H, C and P [35], as other polyploid species, *H. tuberosus* displays any regular meiosis et is partially female fertile. Moreover, J. artichoke displays a strong self-incompatibility system that prevents a plant to produce seeds after self-pollination. Because S-alleles in J. artichoke have not been identified success or failure of crosses between clones cannot be predicted and several crosses have to be done to ensure some will succeed [34].

Although it was a long way, the two successes for transformation to adapt crops to be used for other purposes revealed the difficulties, which the breeders will face in J. artichoke adaptation to new uses. Thus, it is important to explain which difficulties may be encountered with J. artichoke, for higher inulin content at harvest.

Diversity: phenotype and genotype. The Jerusalem artichoke originated in the east of the North America at latitude (between 45° North to 27° South). Depending on the latitude origin of the clone once transferred to Europe, in Montpellier as example (latitude 42° North) plants that had origin in the North start the flowering earlier than those from South. Clones flowering between the end of June to October, and clones that have not flowered in Octo-

ber indicate the presence of phenotypic diversity. The date of flowering is important and has to be as late as possible, that will avoid plants to loose energy for flowering [36]. Wild forms of J. artichoke have tiny tubers with more or less cylindrical shape. Seeds from wild J. artichoke display difficulties to germinate because of integument dormancy. Seeds from cultivated J. artichoke display the same difficulties to germinate and have to be treated as wild seeds. The tuber shape and size displayed by one J. artichoke clone depends both on the latitude and the average temperature of days during the growing season (between planting to harvest) [28].

In the former USSR the majority of J. artichoke varieties and hybrids were produced by a clone selection method from local populations and forms of foreign origin and by selection from the achene – grown seedlings from free pollination of cultivated varieties. In 1966-1972 at VIR's Maycop experimental station an inter-varietal hybridization was carried out, the tests showing its favourable prospects for J. artichoke breeding [37]. Among the seedlings grown, a large diversity of hybrid forms was noted, comprising positive characters of initial parental forms and suitable to be used for crop production as well. Ethanol production has been a goal

For the first time, the breeding work on J. artichoke began in USSR by the 1934-1936 years, when several seedlings were obtained from inter-varietal crosses, the greatest success achieved [38-40]. Their breeding method was based on the inter-varietal hybridization followed by selection of seedlings. The scheme of the selection process included 5 stages: 1) the obtaining seeds from open pollination and artificial hybridization; 2) growing seedlings and evaluation in the first year of life; 3) comparison of the vegetative progeny of seedlings in the second year of life; 4) a preliminary test of selected clones; and 5) other types of tests. N. Schibrya [38] in the one of his works noted about importance to attract attention to necessity of crosses certain parental forms, bearing the desirable traits. And the most interesting F_1 plants then easily can be fixed in vegetative propagation. The experience in VIR breeding works, including experiments under the leadership of the breeder Nicolay Pas'ko [40], shows that some Caucasian and French forms (Var), e.g. Blanc precoce, as well as 3 wild species, *H. mac*rophyllus Willd, *H. subcanescens* (Grau) Wats and *H. rigidus* (Cass) Desf., should be involved as donors. Since J. artichoke, as cross pollinator, is heterozygous, than the breeder can always expect to obtain from the use self-pollination of plants segregation to recessive forms that can be practically valuable.

An evaluation of a wide diversity of J. artichoke 315 varieties from 24 countries, collected during 80 years in N. Vavilov Institute (VIR) collection [3], showed that some varieties have the same high seed productivity as well as sunflower and not all forms have a low fertility. The analysis of studies in inter-varietal crosses showed that the formation of germinal seed of J. artichoke cultivars in a pairwise crossing varies from 0 to 98 % [40]. The crossability in some combinations reaches 75-98 %, each pollinated inflorescence gives 20-30 seeds, but the average statistical accounting the achenes germ forming reaches no more than 11 %. Most important factors are the place and time, air temperature and humidity. For example, in foothills of the Caucasus Mountains, the best time for crossing is the second decade of July until the first decade of September [37].

Presently, the diversity in J. artichoke crop is represented by about 300 clones and it may have redundancies between collections based on the same names of a clone or deformation by translation of names. Even though the phenotypic diversity is apparently huge as shown in results of some studies [40, 41], the genotypic diversity is insufficient to start a breeding program on this basis. Studies published by R. Puttha et al. [26] which have studied about four tens of

clones, showed that the molecular diversity — supposed to be neutral — is low between clones. In contrast, each J. artichoke seed will generate a putative clone, and thus the phenotypic and molecular diversity of wild J. artichoke propagated by seeds is much more than the diversity of clones, but of course, wild J. artichoke have not been screened for the quality of their tubers. In the same way seeds from J. artichoke clones will lead to plants with a range of diversity on tubers color and shape. Seeds are obtained on to the female plants by crossing with an unknown male unless the cross has been controlled and the male was identified. In INRA J. artichoke collections, we have harvested thousands of seeds (200 g) that were conserved in a cold room as genetic resources by institute. Noticeable is that on a J. artichoke head a few of flowers give seeds, and we found broadly 5 seeds per head whereas the number of flowers is by 60. We hypothesized that this is due to the self-incompatibility system in J. artichoke and also due to its polyploid structure. Several authors have studied meiosis in J. artichoke and they did not reveal abnormalities such as laggard segregation [42]. However, if most pollen grains are rejected, this cannot be due to the self-incompatibility system only, and we should hypothesize that pollen grains do not carry the adequate chromosome number.

The important point to increase seed productivity is the choice of varieties-pollinators. For example, var. Vengersky at crossing with var. Tambovsky krasnyi gives the highest percentage (98 %) of seed setting. Of course, the germ forming depends from the fertility of maternal form. According to our observations, at VIR's Maycop experimental station the best parent varieties, providing the highest percentage of seed setting are Vengersky, Tambovsky krasnyi, Gorno-Altaiskyi. Moreover, in order to predict the level of achene germ forming and plan the work for their obtaining, when are going to select the parental pairs for crossing, there is necessary to know their combining ability for seed productivity. Also in breeding work, when doing the selection of varieties for the cross-pollination, it is very important to know the extent of pollen fertility, because it increases the seed productivity of maternal forms of *J. artichoke*. The increase in a percentage of achene germ forming depends on involved pollinator varieties with high pollen fertility. N. Pas'ko [40] proved that the larger the pollen, the higher the fertility is.

The analysis of inheritance of desired traits in the progeny of intervarietal crosses shows that there are different degrees of trait dominance and compulsory segregation of characters. Therefore, the most likely way for success is selection of seedlings in the first generation with further testing in second vegetative reproduction

The variability on inulin content at harvest time. The Jerusalem artichoke varieties are characterized by a wide variability in inulin and sugar content [23-26, 32, 33] and elements productivity, which depend on environmental conditions significantly [43-46]. As for all traits that follow quantitative variation, inulin content at harvest should be evaluated for a series of putative parents of future J. artichoke clones by crossing them following the diallel design as male and female. The method provides information on the value of each clone in the assay as male and female parents. Moreover, the method allows further choosing parental clones with complementary parameters favorable to the higher content in inulin at harvest time. Obviously, the clones and a few of progenies can be evaluated simultaneously for other traits such as disease and abiotic stress tolerance. Clearly, this experiment is heavy but without this information obtained from each clone all mates will be made in blind and successes may be low. In addition, in order to assess the potential of selected seedlings for the manifestation of characteristics, the evaluation of the selected clones of seed-

lings should be carried out comprehensively, in different ecological and geographical conditions, as well as in various backgrounds - including with the irrigated method of cultivation. The results of the applied research of the last decades on Jerusalem artichoke show the influence of various factors both on the productivity of Jerusalem artichoke and on the biochemical content of tubers and aboveground biomass [23, 25, 47-50].

Variability on inulin content in tubers. Plenty of studies have released variation for inulin content at harvest in J. artichoke [14], but none has release which clones could be the best parents to improve the trait inulin content at harvest. The yield in inulin levels out at 19 % of the tuber raw material. The upper theoretical limit is probably around 22-25 % as judged by other crops (chicory) that produce inulin in roots.

Variability of inulin in leaves and stems. Most J. artichoke growers harvest only tubers and leave the stems to dry and if harvested it is for fire wood. Stems are rich in cellulose and could enhance profits from the crop. They also contain inulin that could be extracted by water treatment and thus the yield of the crop is indeed enhanced. The same design described previously could serve to estimate the potential of each J. artichoke clone as a parent one.

A guide to mate Jerusalem artichoke clones. To mate two J. artichoke clones requires to envelope with a paper bag one head of the plant, chosen as female, to prevent unwanted pollination and when the stigmata rose above the tubular hermaphrodite flowers, the pollen from another plant has to be spread with a small brush onto the stigma surface. The pollen is harvested onto the one or several heads from the male plant that has enveloped in a paper bag. The pollen is introduced into the paper bag of the female heads. Ten to fifteen heads of the female have to be treated for one cross to ensure to have enough seeds. Seeds are mature one month and half later and as birds are very fond of them, they have to be protected under the bag until harvest.

Another method consist in leaving all heads free pollinated (at the condition that none sunflower plants may blossom in the area). The J. artichoke clones, which have been evaluated for the inulin production, could be mixed in a plot for seed production. Bees are able to transport pollen up to 5 km from the source. In general, around Montpellier sunflower is not cropped in the fall and protection of heads is useless. Without bag protection, the yield in seeds is higher, i.e. several grams can be obtained and the diversity in the progenies is ensured. Seeds could be harvested on the clones of female plants that display the higher ability for inulin content. To obtain seed germination is a challenge, one has to break dormancy, both due to integuments and to hormone balance. Several methods for seed treatments have been published [51, 52] by scarification and/or hormonal treatments. Once the root has come through the integument the seedling is immediately transferred in a pot with sandy soil without too much moisture to avoid mold and rotting diseases [53]. Each plant is single, and all are different from each other and thus before evaluation each plant able to give tubers has to be retained. The evaluation plots should consist in a field plots with repeats of tubers and random distribution of them. The field plot is costly and has to be repeated each year for new plants. The bottleneck for getting enough genetic diversity is at this level and thus the step cannot be shifted.

If progenies from hybridization between *J. artichoke* and sunflower are looking for, in Montpellier we planted sunflower rows (different hybrids) besides the J. artichoke clones. Once seeds have been harvested and cleaned, they are separated based on the size (the upper size will be enriched in hybrid seeds).

Field experiments. Pre-screening of clones able to produce tubers in the local field conditions is not sufficient for an acceptable agronomic behavior of

clones and requires verification in usual seeding. Conventional agronomic plots should be carried out for their evaluation. Each evaluator has different evaluation criteria because of the agronomic environment and the aim of the production he (her) works with. The screening for hybrids between J. artichoke ($2n = 102$) and sunflower ($2n = 34$) requires cytological controls such as chromosome counting (the hybrids should display 68 instead of 102 chromosomes). Such plants are called Topinsol. The diversity of Topinsol forms is scarce because crosses have not been made with breeding aims. Their potential is practically unknown, but in J. artichoke collections, Topinsols are detected only by cytological analyses that means they probably yield tubers as J. artichoke clones.

Use inter-specific hybridization. The Russian breeders were the first in the world who created the inter-specific hybrids between J. artichoke and sunflower in 1935 [37-39]. It was a new artificial species tuber-bearing crop. Genetically, J. artichoke and sunflower are members of polyploid series. J artichoke is hexaploid ($2n = 102$), sunflower is diploid ($2n = 34$). In the somatic cells of *J. artichoke* and sunflower hybrids there were 68 chromosomes, which is consistent with the theoretical assumption of 51 chromosome from J. artichoke and 17 chromosomes from sunflower [54-56].

Since the most of J. artichoke varieties involved in breeding have a late and middle maturity, to accelerate the flowering it is possible to use a method of reducing the day length providing the creation of seeds to be held in a more favorable time for this. For this, it needs to tie the leaves from the stalk to reduce their photosynthesis to 10 h of daylight. This method allows accelerated flowering from 2 to 60 days and a successful crossing with sunflowers. At VIR's Maykop experimental station in 1966-1980, more than 25 varieties of artichoke, 3 of Helianthus wild tuberous species and 13 varieties of sunflower *H. annuus* L. were involved in crossing and has been cultivated over 10,000 seedlings of inter-specific hybrids (F_1). The level of achene germ forming from crossing J. artichoke with sunflower is small — 0.1-4.5 % [57-59]. In VlR, Dr. N. Pas'ko [37, 40] assessed J. artichoke varieties on ability as pollinators and he singled out the most fertile var. Patat Vilmorin, Maykopsky 33-650, Vengersky, which provide 42-46 % seed germ forming. The efficiency of seed productivity of J. artichoke depends on the fertility of parent forms pollinators of sunflowers. More fertile forms of J. artichoke at their pollination by the same varieties of sunflower annually have a tendency to retain their best cross ability. Conversely, the varieties with low fertility produce weak achenes germ forming [40, 60]. Analysis of inheritance degree of traits shows that the first generation of F_1 hybrids between J. artichoke and sunflower thanks to heterozygosity present a wide diversity of forms. The analysis of traits of hybrid plants shows that their inheritance has an intermediate character or character of one of the parental forms. So, the height and thickness of stem, and leaf number are inherited mainly from the expression of heterosis [60]. The leaves of most hybrids become larger than that of J. artichoke.

The tuber-creation of inter-specific hybrids in F_1 appears as a dominant trait, and therefore almost all progenies (94-98 %) create the tubers. Also it was found that the obtained hybrid plants on the most morphological traits tubers retain maternal type, but at the same time increases the mass of tubers per plant, so the average weight of a tuber. In some combinations has created the plants with better tuber shape than the parental forms. Furthermore, it is important to note that the biochemical analysis indicates on increased sugar content in tubers of hybrid plants. The long-term evaluation has shown that the resulting heterosis effect persists in further propagation by tubers. For example at Maikop experimental station of VIR during many years inter-specific hybridization in 1984 was

created var. Vostorg (Delight, in Russian) (3M-1-156), which was obtained by crossing of French var. Commun (high yield of tops of plants and high sugar content in it) with sunflower var. Gigant 549 (silage type). As a result, this hybrid has a yield of tops reaches up to 90 tons per hectare, with average of plant height up to 3.8 meters, and tuber yield 32 tons/ ha. The sugar content in the stems and leaves reached 16 -18 % [37, 60]. Even greater success was achieved with the creation at VIRs experimental station in 1986 of inter-specific hybrid, which later became a famous whole country variety Novost' VIR (News of VIR, in Russian). The data of this class exceeded all previously created inter-specific hybrids. All the main agronomic and biochemical properties: yields of tops at experimental fields up to 100 tons per hectare and sugar content in them was 16 %, while at the same time, the tuber yield reached 60 tons per hectare, when the sugar content in the tubers was 18 % and inulin was 14 %.

The future of J. artichoke as energy source for bio-fuel as well as source of fibers and as sugar substitute for people, lacking of insulin is promising. However, because J. artichoke has never been improved for these uses as its genetic basis is narrow and cultivars are clones, we suggested to improve J. artichoke for inulin content before looking for new varieties. This strategy has been found efficient to change confectionery sunflower in oil sunflower and to modify root beet in sugar beet.

Thus, it is safe to approve the reality of selective breeding to achieve a desired trait using inter-specific hybridization. Inter-specific hybridization of J. artichoke (*Helianthus tuberosus* L.) with sunflower (*Helianthus annuus* L.) may be successfully used as a method of breeding directed to the improvement of the J. artichoke plant and creating Topinsol comprising characters and properties of initial forms with the expression of heterosis. Given the current demand for different directions of using products from J. artichoke, it is likely that the breeding of J. artichoke is aimed at the creation of special varieties — for food, for medicinal, for processing, for inulin, for bioenergy, for feeding purposes etc. And we can say with confidence that there is sufficient initial material for all directions of breeding, the only need is to expand research in finding the possibilities of using the existing gene pool of Jerusalem artichoke in many gene banks. Today there are different modern methods for it, including molecular genetics.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 952-963
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.174:631.527.56:577.21

doi: 10.15389/agrobiology.2017.5.952rus
doi: 10.15389/agrobiology.2017.5.952eng

POLYMORPHISM OF GRAIN SORGHUM FROM VIR WORLD COLLECTION FOR THE CHARACTERS ASSOCIATED WITH THE CMS-*Rf* GENETIC SYSTEM

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The authors declare no conflict of interests
Acknowledgements:
The sequencing was carried out using equipment of the ARRIAM Center of Genomic Technologies, Proteomics and Cell Biology (St. Petersburg).
The authors thank A.G. Pinaev (ARRIAM, St. Petersburg) for assistance in DNA sequencing
Received July 3, 2017

Abstract

Seven different types of cytoplasmic male sterility (CMS) are known for the grain sorghum (*Sorghum bicolor* L. Moench), however, only A1 (milo) is used in heterotic hybrid breeding. The genetic control of pollen fertility restoration of CMS A1 is complex and determined by two or three *Rf* (*Restoration of Fertility*) genes, and also by a number of modifiers. It is very little known about molecular mechanisms of CMS A1 and fertility restoration. Only one gene, *Rf1*, is identified at the molecular level (R.R. Klein et al., 2005). In the present paper we have demonstrated for the first time the nucleotide polymorphism in the coding regions of the recessive and dominant alleles of *Rf2* gene and also of the candidate *RFL-PPR* gene homologous to the rice *Rf1* gene. . Here, we studied polymorphism of the CMS-*Rf* genetic system related traits in sorghum accessions from the VIR collection, including the fertility restorers k-928 and k-929; a half-restorer k-1362, the sterile lines A-10598 and A-83 (CMS A1) and their fertile analogs, the F_8-F_{12} BC_1-BC_2 sister lines resistant to *Schizaphis graminum* Rond. which have been isolated among the hybrids derived from crosses between the sterile (CMS A1) line N-81 and lines k-929 and k-928, and also hybrids between the sister lines. For investigating variability of candidate genes associated with the CMS-*Rf* genetic system the reference sequences were selected from the bioinformatic database (http://www.ncbi.nlm.hih.gov), the eighth specific primers were designed, and the fragments amplified on the DNA of genotypes differing by the ability to suppression of the CMS phenotype were sequenced. In the CMS lines and fertility restorers a significant polymorphism (18 polymorphic sites) was revealed in the 825 bp fragment of the *Rf2* coding region (reference fragment XM_002459403.1, chromosome SDI02) and also in *RFL-PPR* candidate gene located in the chromosome 3 (reference fragment XM_002458104.1). The sequenced regions of the structural nuclear gene *ALDH2b* encoding mitochondrial aldehyde dehydrogenase, the maize *Rf2* gene homolog, and also of the mitochondrial F0F1 ATPase alpha subunit were identical in the CMS and fertility restorer lines. Variability of pollen fertility indices was studied using acetocarmine stained cytological preparations. The lines resistant to *S. graminum*, and their hybrids differed in the percentage of stained (fertile) pollen grains, the presence of anomalous large pollen grains (54-70 μm in diameter), giant pollen grains (up to 84 μm in diameter) and deformed pollen grains. In the fertile F_8-F_{12} BC_1-BC_2 lines which derived from the hybrids produced in crossings with fertility restorers, the frequency of stained pollen grains was relatively high and reached 72.2-83.8 % for k-929, and 57.4 and 63.4 % in two lines, respectively, for k-928; large pollen grains occurred at different frequency in five lines, and the giant ones were observed in two lines. The variability in pollen fertility could be due to the differences in the alleles derived from the recurrent parent.

Keywords: *Sorghum bicolor* L. Moench, grain sorghum, CMS, fertility restoration, *Rf*, pollen fertility, candidate genes, nucleotide polymorphism

The phenomenon of cytoplasmic male sterility (CMS) was discovered in the United States in 1931 by M. Rhoades and in the USSR by M.I. Khadzhinov (All-Union Institute of Plant Industry — VIR). To date, it has been described for more than 150 species of plants [1, 2]. CMS is widely used in the production of hybrid seeds of many crops (maize, rice, rape, cotton, sunflower, cabbage, etc.). The creation of heterotic hybrids based on CMS is considered as a priority of modern breeding programs for sorghum (*Sorghum bicolor* L. Moench), an important cereal crop widely spread in arid and semi-arid regions of the planet. The first mention of heterotic hybrids of sorghum dates back to 1927 [3, 4]; however, commercial hybrids were made only after the discovery of stably inherited cytoplasmic male sterility A1 (milo) in Kaffir sorghum and the sources of pollen fertility restoration [5]. Later, alternative types of CMS — A2-A6, 9E [6] were identified, but because of the difficulties in obtaining reliable sources of fertility restoration genes, as well as the epigenetic variability of the trait depending on environmental conditions [7-9], CMS A1 only is used for breeding programs [10]. At least two main genes, *Rf1* [11] and *Rf2* [10], whose dominant alleles are responsible for the restoration of the A1-milo CMS fertility, have been identified by hybridological analysis. Their manifestation depends on the environmental conditions and the action of the modifier genes. It is also suggested that in the case of CMS A1, pollen fertility restoration is controlled by the dominant alleles of the main and two duplicated complementary genes, whereas it is determined by the dominant alleles of the three genes interacting complementarily in CMS A2 and A3 [12]. The *Rf5* gene and a number of modifiers that restore the pollen fertility of CMS A1 and A2 have been found [13]. Fertility restoration with CMS A3 is controlled by dominant alleles of *Rf3* and *Rf4* genes at gametophyte level [14], and is explained by paramutations of *Rf* genes at sporophyte level [15]. The molecular mechanisms of CMS manifestation and fertility restoration in sorghum have been little studied. It has been shown that, like in other plants, sorghum CMS is caused by aberrant mitochondrial genes [16].

Most of the *Rf* genes characterized to date (in petunia, maize, rice, radish) encode proteins that contain repeating motifs of 35 amino acid residues (PPR, pentatricopeptide repeats) and regulate the coordinated work of the nucleus and mitochondria. *PPR* genes with the fertility restoration function are allocated to a separate subfamily *RFL-PPR* (*Restoration of Fertility Like-PPR*). The structural and functional diversity of *PPR-RFL* genes is supported by the variability of PPR motifs, as well as by the complex cluster organization of *Rf* locus in the genome [17-19].

Only one gene for pollen fertility restoration, *Rf1*, has been characterized for sorghum at the molecular level [11]. It was found that the locus *Rf1* is in the linkage group 08 and includes four open reading frames (ORFs) that code for Ca^{2+}-ATPase of the plasma membrane, cyclin D-1, and also an unknown mitochondrial protein containing 13 PPR motifs and belonging to the E-type of *PPR* genes subfamily. There are 19 polymorphic sites in the coding sequence and in the 5′- or 3′-end flanking regions of the dominant and recessive alleles of the candidate gene *PPR13*. Sorghum *PPR13* gene differs significantly from other representatives of the *PPR-RFL* genes subfamily in the structure of PPR motifs [19-21]. Another candidate gene (*Rf2*) is located on 236,219 bp region of SDI02 chromosome. The region includes 31 ORFs, including one *PPR* gene characterized by high similarity to the rice gene *Rf1* [10]. The polymorphism of the nucleotide sequences of the dominant and recessive alleles of the Rf2 locus has not yet been studied thus limiting the development of specific molecular markers for their identification. Other sequences of sorghum genome potentially associated with pollen fertility restoration were not identified, too.

The VIR collection of sorghum totals about 9 thousand accessions. In the collection, there are CMS A1 (milo) sterile lines, fertility restorers and sterility maintainers. In sorghum, like many other plants, cytological analysis of pollen in F_1 hybrids is used, in addition to seed setting rate under self-pollination, to assess the male fertility restoration [8, 13, 22]. However, the variability of this trait and the polymorphism of genomic sequences potentially associated with the CMS-*Rf* genetic system have not yet been studied yet in the samples of VIR collection.

In this study, we first reported on significant nucleotide polymorphism in coding regions of *Rf2* gene recessive and dominant alleles (the reference fragment XM_002459403.1, chromosome SDI02), and also of the candidate gene *RFL-PPR* (the reference fragment XM_002458104.1) homologous to the rice gene *Rf1*.

In order to elucidate the character of variability associated with the CMS-*Rf* genetic system in sorghum lines, we studied nucleotide polymorphism of the candidate genes associated with the genetic system CMS-*Rf*, and compared the formation of pollen in fertile forms and plants with a sterile cytoplasm of type A1 (milo).

Techniques. Studied sorghum samples were the VIR collection accessions which differ in their ability to restore pollen fertility, i.e. fertility half-restorer k-1362; restorer lines k-928 and k-929; sterile lines A-10598 and A-83 based on CMS A1 (milo) and their respective fertile counterparts B-10598 and B-83; sister lines F_8-F_{12} BC_1-BC_2 resistant to *Schizaphis graminum* Rond. which were derived from the hybrids produced by crossing sterile line (CMS A1) Nizkorosloe 81 (N-81) with the restorers k-928 and k-929; F_1 hybrids from crosses of the sterile and the fertile lines [23]. The lines k-928 and k-929 selected from the samples of grain sorghum Dzhugara Belaya from Western China possess different alleles of for resistance to *S. graminum* [24]. The sample k-1362 (Dzhugara Belaya, Syria) is a donor of resistance to *S. graminum* and the fertility half-restorer. The sterile lines of grain Kaffir sorghum A-10598 and A-83 and their fertile analogues B-10598 and B-83 entered the VIR collection from India in the 1980s. The lines and hybrids were grown in 2014-2016 (field tests, VIR Kuban Experimental Station, Krasnodar Krai).

For estimation of pollen fertility, mature anthers were collected early in the morning in the period of mass flowering and fixed in 70 % ethanol. The proportion of pollen fertile grains was counted according to Navashin method [cited from 25] in preparations stained with aceto-carmine glycerol (Zeiss Axioplan 2 imaging, Carl Zeiss, Germany). The percentage of fully stained (fertile), weakly stained and unstained pollen grains (PGs) was calculated based on at least 30 fields of view at a magnification of ×20 and considering pollen diameter, uniformity in diameter and the presence of abnormal PGs.

DNA was extracted according to a CTAB protocol [26]. Eleven sequences were identified as a result of bioinformatic search (GenBank database, NCBI, USA) (http://www.ncbi.nlm.hih.gov). These sequences have a homology to the nuclear *Rf* genes and mitochondrial genes associated with CMS in sorghum [27] and other plant species. Eight pairs of specific primers flanking the full-sized and internal fragments were designed based on four selected reference sequences (Table 1). Amplicons produced with the developed primers using template DNA of genotypes, contrasting in pollen fertility, were purified in 1 % agarose gel and sequenced on a genetic analyzer ABI 3500xl (Applied Biosystems, USA). The alignment and analysis of the sequences were implemented using Mega 5.1 software (http://www.megasoftware.net/) [28].

The means (M) and standard error of means ($\pm m$) were calculated for

the fertile pollen grains number.

Results. To determine the variability of *Rf2*, one of the main genes for CMS A1 fertility restoration located on chromosome 8, we studied the polymorphism of fragments amplified using designed primers specific for the *S. bicolor* genomic sequence (XM_002459403.1) which contains PPR motifs and is the closest [10] to the sequence of rice *Oryza sativa* L. *Rf1* gene restoring the fertility of CMS type BTII (Boro II) (Table 1). In this, we revealed a similarity of the reference fragment with several fragments of sorghum genome (presumably, the gene *Rf1* sequences) and also with the predicted fertility restorer genes of *Setaria italica* (L.) P. Beauv. and *Zea mays* L. The reference fragment length was 951 bp and the sequenced fragment size was 825 (positions 76-901). The length of in silico translated sequence was 275 bp.

1. Primers designed to amplify fertility restoration gene homologs in *Sorghum bicolor* L. Moench

Reference fragment (length)	Gene, protein	Primers, nucleotide sequence 5′→3′	Sequenced fragment, bp	Positions in the reference sequence
XM_002458104.1 (2801 bp)	Not identified, PPR protein	02458104fw1: CACCCAATTCTCCAGACCAT 02458104rev1: ACATCTGCCGGTACATAGCC 02458104fw2: GGCTATGTACCGGCAGATGT 02458104rev2: GATGGGATCAAATGGAATGG 104_inner_fw: TTGCTTGCATGGAGAAATTG 104_inner_rev: CTGCGAGATCACAGCAGTTG	818	301-1119
XM_002459403.1 (951 bp)	*Rf2*, PPR protein	2459403fw: CAGGGGCCAAATGTTGTTAC 2459403rev: CACAGTTTTATATTTTCCGTGATAGTG	825	76-901
AJ278689.1 (1324 bp)	*atpA*, α subunit of ATPase synthase F0F1	AJ278689fw: AACTTTTACACGAATTTTCAAGTGG AJ278689rev: TGACAGCAGCATAAATAACAACAA AJ_inner_fw: TCCTATAGGCCGTGGTCAAC AJ_inner_rev: CGTCTCCAGCTTGTGTTTCA	1183	60-1243
AB084898.1 (2159 bp)	*ALDH2b*, mitochondrial aldehyde dehydrogenase	AB084898fw: TTCTGGTTTTGGCCCTACTG AB084898rev: CTCTTCTAACAAATGTTTTTTCATAAT AB_inner_fw: AACCATACGAATAAAGCCTTGC AB_inner_rev: CTCGCATTTGCCCTCTTAAT	738	858-1596

For the restorer lines k-928 and k-929, presumably carrying dominant alleles of *Rf2*, the studied sequences differed in six nucleotide substitutions, whereas sterile lines A-10598 and A-83, the carriers of recessive alleles, differed in three nucleotide substitutions. The reference sequence which originates from of BT × 623 line, a maintainer of sterility with a putative genotype *rf2rf2*, was more similar to the sequences of the sterile lines A-10598 and A-83 and differed from them in two and four polymorphic nucleotide positions, respectively. At the same time, the sequenced fragments of k-928 and k-929 genomes differed from the reference fragment and fragments found in sterile lines in 18 nucleotides substitutions and 7 amino acid substitutions (Fig. 1). It is known that the polymorphism of the coding sequences of *Rf* genes of plants, particularly *Rf1* gene in sorghum, is associated with their functional status [11, 18]. We can assume that the polymorphic sequences identified in CMS lines and fertility restorers, are different allelic variants of gene *Rf2* which products are involved in editing mitochondrial RNA. In the sequences, we found six and five PPR repeats for the carriers dominant and recessive *Rf2* alleles, respectively. In general, the *Rf2* locus polymorphism in the dominant and recessive allele carriers was relatively high (2.2 % of polymorphic sites).

We studied polymorphism of 2,801 bp XM_002458104.1 fragment located on chromosome 3, using three pairs of primers (Table 1, Fig. 2). XM_002458104.1 contains 17 PPR repeats and is the closest to nuclear gene *Rfo* of radish *Raphanus*

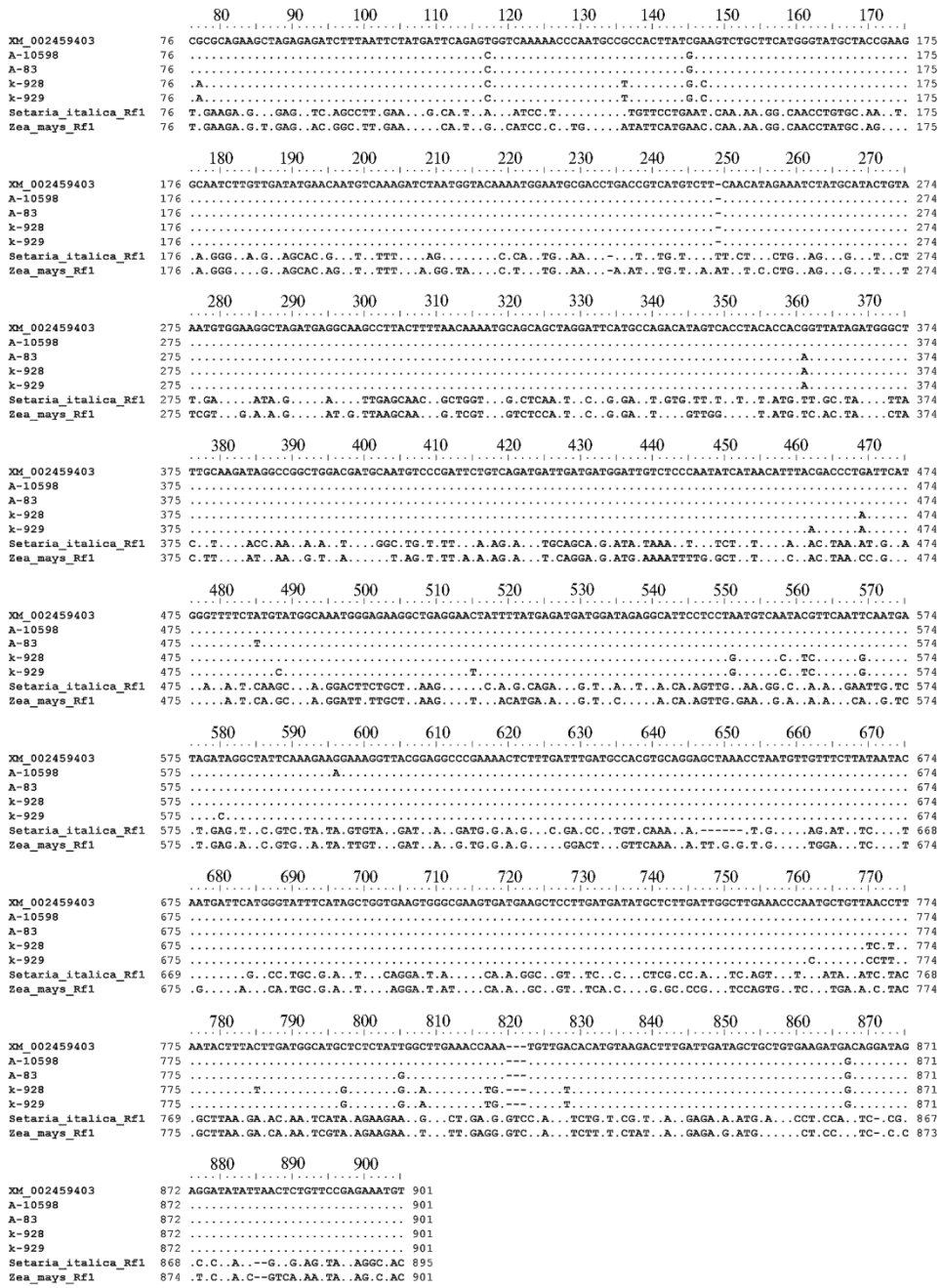

Fig. 1. Alignment of the nucleotide sequences of the candidate gene *Rf2* of *Sorghum bicolor* L. Moench lines from the world VIR collection: k-928 and k-929 — carriers of dominant allele, A-10598 and A-83 — carriers of recessive allele; XM_002459403 — reference fragment. For comparison, homologous fragments of the genes *Rf Setaria italica* and *Zea mays* are given.

sativus (L.) Domin., restoring the fertility of CMS Ogura [29], and the gene *Rf1* of rice *O. sativa* Japonica Group. The reference fragment and 818 bp DNA fragment of line A-83 genome were identical, but differed from the DNA fragment of the fertility restorer k-929 (17 polymorphic sites and 8 amino acid substitutions revealed) (Fig. 2). The highest similarity (90 % identical nucleotide positions) was found between the identified fragment and XM_008677263.2 sequence from Gen-

Bank database for *Zea mays* PPR protein.

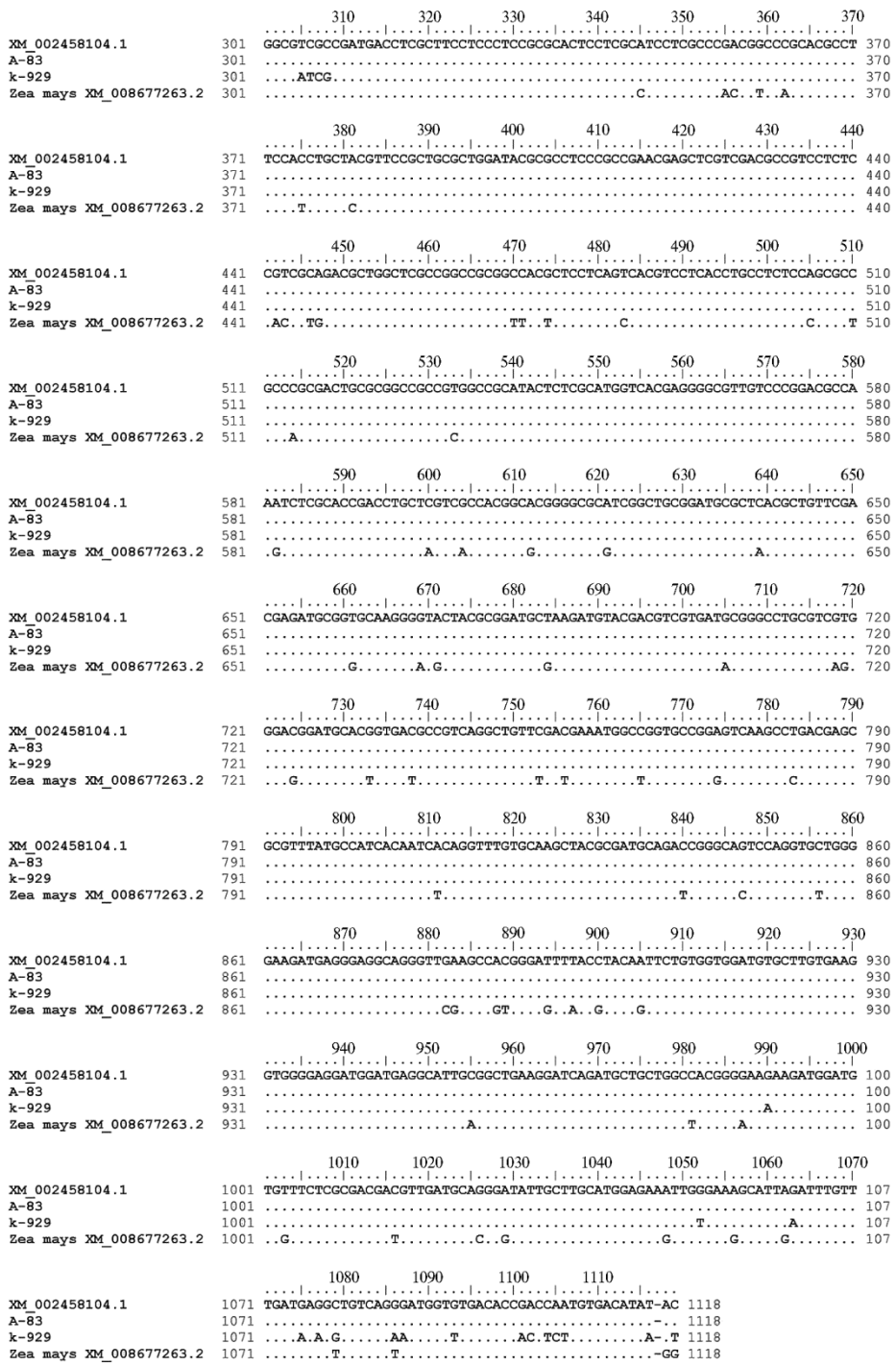

Fig. 2. Alignment of the nucleotide sequences of the locus encoding mitochondrial PPR protein in *Sorghum bicolor* L. Moench lines from the world VIR collection: k-929 — fertility restorer, A-83 — sterile line; XM_002458104.1 — reference fragment. For comparison, the homologous sequence of *Zea mays* is given.

Rf2, one of maize genes restoring fertility of T-type CMS line, encodes the aldehyde dehydrogenase (ALDH), the enzyme catalyzing oxidation of alde-

hydes. It is known that ALDH is involved in the detoxification of acetaldehyde, produced during the development of pollen, also ALDH may be involved in cell energy metabolism, especially during anther development, and possibly interacts with the mitochondrial protein URF13 related with the T-type CMS in maize [30, 31]. It is believed that the action of ALDH as a fertility restorer controlled by gene *Rf2* is due to the tunnel cavities on the surface of the enzyme molecule, through which the protein can bind long-chain ligands of different lengths and/or potentially toxic products of the mitochondrial CMS gene expression which are harmful for pollen development [32].

To test the hypothesis about the possible involvement of sorghum aldehyde dehydrogenase gene in control of pollen fertility restoration, we designed primers specific for coding sequence of the enzyme based on *ALDH2b* mRNA of *S. bicolor* (AB084898.1) from GenBank database. In sorghum *ALDH2b*, a gene of subfamily 2 of the extensive ALDH superfamily of higher plants, nucleotides located at the positions 858-1596 was homologous to the ALDH sequences of other cereal species including maize (94 % identical amino acid residues). At the same time, we found no differences between the fertility restorers and CMS lines in nucleotide sequences of this fragment. It should be noted that we succeeded to determine the sequence of an extended 2055 bp region of *ALDH2b* gene of line A-83, but sequenced and reference fragments differed considerably in the N-terminal regions. It may be due to a considerable variability of residues in the specified part of the molecule [32] or the presence in the amplified product of an intron or introns information of which is still lacking for sorghum and maize ALDH genes.

It is known that promoter regions of ATP-synthase genes or parts of these genes are found in many mitochondrial loci associated with CMS. For example, gene *urf13-T* associated with T-type CMS in maize contains 59 nucleotides from the regulatory region of the *atp6* gene. Gene *orf221* linked to and cotranscribed with *urf13-T* was identified as a fragment of gene encoding a subunit of F0 component of ATP synthase F0F1 [16]. We used AJ278689.1 sequence (GenBank database) of gene *atpA* encoding the α subunit of ATP-synthase F0F1 of line CS3541, the fertility restorer for CMS A1, as a reference sequence to analyze fragments of *S. bicolor* mitochondrial genome potentially associated with CMS A1. Aligned fragments had a length of 1183 bp and were identical in epy forms with sterile (A-83) and fertile (k-929) cytoplasm types. The translation products of these sequences in the CMS line and the restorer line, as well as the reference sequence AJ278689.1 and sequences coding α subunit of ATP-synthase F0F1 in maize, rice, *Triticum aestivum* L., *T. durum* L., and *Secale cereale* L. were highly homologous.

Thus, in this work, the polymorphism of nucleotide sequence fragments of a candidate gene *Rf2* which controls fertility restoration for CMS A1 in sorghum was revealed for the first time. The greatest number of polymorphic sites was found when comparing these sequences in sterile lines and restorers. In particular, the nucleotide polymorphism was found in the fertility restorers k-928 and k-929 which served as parental forms for *S. graminum* resistant lines F_8-F_{12} BC_1-BC_2. These lines have sterile cytoplasm and, apparently, differ in allelic composition of *Rf* genes derived from the male parent. Despite the high homozygosity of the lines F_8-F_{12} BC_1-BC_2, their pollen fertility varied significantly.

The studied genotypes differed significantly both in the amount of fertile (stained) pollen grains and in their diameter (Table 2). It has been reported [33] that normally the diameter of fertile PGs in diploid sorghum does not depend on the year condition and varies within 37.5-54.2 μm. PGs of more than 54.2 μm in diameter (large pollen grains) occur in diploid sorghum quite rarely and, appar-

ently, contain an unreduced number of chromosomes. B-10598 line, the fertile analog of CMS A-10598 line, as well as k-1362 and k-928 (75.5 %, 100.0 % and 83.3 % of fertile PGs, respectively), was characterized by high pollen quality. At the same time, k-1362 and k-928 had a large number of large PGs with a diameter of 55 to 70 μm. Seven fertile lines F_8-F_{12} BC_1-BC_2 resistant to *S. graminum* which were selected from the hybrids N-81 × k-928 and N-81 × k-929, showed a relatively high percentage of fertile PGs. This index was slightly higher for five lines in which k-929 served as the donor of the *Rf* genes (72.2-83.8 %, respectively) than that for two lines from crosses with k-928 (57.4 % and 63.4 %, respectively). Different frequency of large PGs of 55-70 μmin diameter showed five lines, and two lines had giant PGs of up to 84 μm in diameter.

2. Pollen grains of sorghum (*Sorghum bicolor* L. Moench) lines with different types of cytoplasm from the world VIR collection

Sample	Type of cytoplasm	Genotype	Characterization of pollen grains					
			F PGs, % ($M\pm m$)	D PG, μm		G PGs, %	L PGs, %	D uniformity/abnormal PGs
				min	max			
k-1362 Dzhugara Belaya	F	Fertility half-restorer	100	51.8	68.9	0	70.0	+/–
k-928 Dzhugara Belaya	F	Fertility restorer	83.3±3.73	40.1	60.5	0	60.0	–/–
B-10598	F	Sterility maintainer	75.5±9.71	42.2	48.3	0	0	+/–
2146/15	S	Fertile line F_9BC_2 (N-81 × k-929)	75.3±4.65	19.9	50.3	0	0	–/–
2148/15	S	Fertile line $F_{10}BC_2$ (N-81 × k-929)	76.6±5.56	26.5	58.5	1.8	0	+/+
2149/15	S	Fertile line $F_{10}BC_2$ (N-81 × k-929)	75.6±3.30	27.6	52.2	0	0	+/–
2150/15	S	Fertile line $F_{10}BC_2$ (N-81 × k-929)	83.8±3.66	28.7	84.3	2.7	8.2	–/–
2151/15	S	Fertile line, $F_{12}BC_2$ (N-81 × k-929)	72.2±6.34	16.4	57.2	0	2.1	–/–
2152/15	S	Fertile line $F_{12}BC_1$ (N-81 × k-928)	63.4±3.90	20.8	56.1	0	2.8	–/+
2153/15	S	Fertile line $F_{12}BC_1$ (N-81 × k-928)	57.4±3.20	28.5	56.1	0	3.1	–/–
73/16	S	Hybrid F_1 between sterile line and fertility restorer	27.0±5.88	13.4	52.5	0	0	–/–
74/16	S	Hybrid F_1 between sterile line and fertility restorer	58.3±8.96	28.5	60.2	0	3.0	–/–

N o t e. F PGs — fertile (stained) pollen grains, D PG — diameter of pollen grains, G PGs — giant pollen grains (diameter > 70-80 microns), L PGs — large pollen grains (diameter > 55 microns); "+" or "–" mean presence and absence of traits.

Only the line 2149/15 of seven analyzed sister lines had PGs uniform in the diameter. Since fertile lines have sterile cytoplasm inherited from the maternal form N-81, it can be assumed that their genotypes contain alleles of nuclear *Rf* genes derived from the male parents which to varying degrees influence pollen fertility restoration under sterile cytoplasm. Deformed PGs were encountered in two lines. The hybrids from the crosses of sterile lines resistant to *S. graminum* with presumably fertility-restoring sister lines also significantly differed in the proportion of fertile pollen, from 9.0 % to 58.3 % for different F_1 plants. One of the hybrids from crossing the sterile line with the sister maintainer line was completely sterile and did not form pollen while the other had pollen in the anther, which, however, was not stained, that is, was sterile. Thus, the variability in pollen fertility rates observed in the *S. graminum* resistant sister lines and in the interlinear hybrids derived from crossing with these lines indicates differences in the alleles of genes participating in fertility restoration (possibly minor genes or modifier genes), which is consistent with the hypothesis of complex genetic con-

trol of the trait [13].

Thus, the nucleotide sequence of *Rf2*, which is a candidate gene for fertility restoration of CMS A1 in sorghum, is polymorphic in the sterile lines A-10598 and A-83 and in the pollen fertility restorers k-928 and k-929 (Dzhugara Belaya). A 825 bp fragment of coding sequence of *Rf2* gene in the carriers of recessive and dominant alleles differs in 18 polymorphic sites, and the translated sequence differed in seven amino acid substitutions. The genomic fragment of one of the *PPR* genes homologous to the rice gene *Rf1* (XM_002458104.1) is highly polymorphic in the lines differing in ability to suppress CMS phenotype, and contains 17 polymorphic sites. Identified polymorphism can be used in the development of allele-specific molecular markers for *Rf2* locus. The sequenced 1183 bp fragment of mitochondrial gene *atpA*, which encodes ATP synthase F0F1 subunit, and the 738 bp fragment of *ALDH2b* aldehyde dehydrogenase nuclear gene are identical in the studied lines. Lines F_8-F_{12} BC_1-BC_2 resistant to *Schizaphis graminum*, which were selected among hybrids derived from crosses of CMS line Nizkorosloe 81 with fertility restorers k-928 and k-929, as well as hybrids between these lines differ in pollen fertility and the presence of large, giant and abnormal pollen grains. Since these lines have sterile cytoplasm, it can be assumed that they carry different alleles of genes derived from the recurrent parent that affect the manifestation of pollen fertility restoration. Grain sorghum lines resistant to *S. graminum* can serve as a model for studying mechanisms of pollen fertility restoration.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 964-975
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.491:631.527.51:577.21

doi: 10.15389/agrobiology.2017.5.964rus
doi: 10.15389/agrobiology.2017.5.964eng

FACILITATION OF INTROGRESSIVE HYBRIDIZATION OF WILD POLYPLOID MEXICAN POTATO SPECIES USING DNA MARKERS OF *R* GENES AND OF DIFFERENT CYTOPLASMIC TYPES

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The authors declare no conflict of interests
Acknowledgements:
Studies of plant resistance to late blight in the epiphytotic 2016, pollen fertility and molecular screening in 2016-2017 (VIR, St. Petersburg, Russia) were supported by Russian Science Foundation (grant № 16-16-04125). Hybridization and assessment of plant resistance to late blight and Potato virus Y were carried out in 2012-2015 (Swedish University of Agricultural Sciences — SLU, Sweden) with the financial support from the E. & I. Nilssons Foundation
Received June 27, 2017

Abstract

Nowadays potato breeding is targeting to develop genetically divers high yielding varieties with multiple pathogen resistance traits. Interspecific hybridization jointed with marker-assistant-selection (MAS) can effectively combine the *R* genes from different resistance sources. Additionally to effective pyramiding the target genes, MAS allows to restrict introgression of genetic factors conferring the undesirable traits, for example, male sterility of interspecific hybrids associated with *Solanum stoloniferum*-derived W/gamma cytoplasm that complicate the traditional breeding. Current study is targeting to search for the opportunities to improve the efficiency of introgressive hybridization between common potato and Mexican polyploid species *Solanum. neoantipoviczii* (=*S. stoloniferum*) and *S. guerreroense* using MAS with DNA markers for different cytoplasmic types and markers associated with major *R*-genes to the most harmful potato pathogens. DNA-based markers of genes for late blight resistance (*R2 like*, *R3a*, *Rpi-blb1*, *Rpi-sto1*), for extreme resistance to Potato virus Y (PVY) (*Ryadg*, *Rysto*, *Ry-fsto*) and for *H1* gene for resistance to the root cyst nematode (*Globodera rostochiensis*, pathotype Ro1) were used in this study. Based on the MAS, hybrid genotypes with different combinations of these markers were selected. Among them, there were the clones with high field resistance to late blight and to PVY. Of 29 hybrid clones from different combinations of crossing with polyploid Mexican species used as the maternal forms, 15 had a W/γ cytoplasmic type and were male sterile; both these traits were maternally inherited. The remaining hybrids with W/α cytoplasm produced fertile pollen and were used in interspecific crosses as pollinators. Selection of resistant clones with W/alpha cytoplasm and elimination of genotypes with sterile W/γ cytoplasm among wild species germplasm could increase the probability of obtaining male fertile introgressive lines. This approach allows to obtain the multi-species hybrid genotypes that combine *R* genes for resistance to pathogens from different Mexican species and to avoid various types of male sterility in breeding. The joint use of two systems of DNA markers, i.e. nuclear markers associated to *R* genes, and cytoplasmic markers for male sterility factors, could reduce costs and increase efficiency of target gene pyramiding programs.

Keywords: *Solanum* spp., potato, DNA markers, *R* genes, cytoplasmic types, interspecific hybridization

Based on studying wild and cultivated potato accessions from the VIR col-

lection (All-Russian Institute of Plant Genetic Resources), the formation of which began during expeditions of 1926 to 1933 [1-3], Russian scientists had developed a theory on the centers of origin and diversity of potatoes [1, 2] and for the first time proved and realized introgressive interspecific hybridization as a new trend in the world potato selection [2-4]. By the end of 20th century, interspecific hybridization became the main potato selection technique owing to the developed methods for overcoming pre- and post-zygotic interspecific incompatibility, such as planning crosses based on EBN (endosperm balance number) of parent species, variation of the ploidy of crossing samples, search for bridge species, reciprocal crossbreeding for leveling nuclear-cytoplasmic conflicts, which allowed involvement of wild species from secondary and tertiary genetic pools in breeding [5-9].

Nowadays, potato selection is focused on broadening the genetic variety of the genetic sources and donors of commercially valuable traits to produce cultivars which combine high productivity with complex and group pathogens resistance. These problems are solved be means of interspecific hybridization, marker-assisted selection (MAS) [10-12] and developed biotechnological methods [13]. MAS drastically increases the efficiency of stacking target genes/QTLs for pathogen resistance, and facilitates control of spreading unwanted genetic determinants. Thus, the hybrids with *R* genes introgressed from Mexican polyploid *Solanum stoloniferum* and *S. demissum* often inherit features which complicate traditional selection [14, 15]. Many hybrids and varieties possessing immunity to the potato virus Y (PVY) are, at the same time, male-sterile, that is, forming fully sterile pollen grains with anomalous morphology [16, 17]. This feature hindering the selection of crossings pairs is transmitted via the maternal line from wild species *S. stoloniferum* which is the sources of gene Ry_{sto} for the extreme YBK resistance [16, 17]. Markers associated with α, β and γ mitotypes have been developed, and a statistically significant relationship between the γ mitotype (W/γ cytoplasm type) and cytoplasmic male sterility has been established in the varieties and hybrids having *S. stoloniferum* in their pedigree [18]. These results found their confirmation in the works of other researchers [16, 17]. At the same time, PVY resistant cultivars with the W/α cytoplasm type are mostly fertile [16]. The hybrids which conferred *R* genes for race-specific resistance to late blight and cytoplasmic determinants from *S. demissum* Mexican species are another examples of joint transfer of target genes and male sterility [17, 19]. The hybrids and cultivars with D cytoplasm (W/α) from *S. demissum* can form morphologically normal pollen grains which, however, are functionally sterile [17, 20]. It is obvious that, while transfer of sterilizing cytoplasm types to various introgressive forms, the *R* gene stacking may have serious limitations.

In the present paper, we report on improving efficiency of introgressive hybridization based on screening parental forms for DNA markers of cytoplasm types and *R* genes encoding potato plant resistance to the most harmful pathogens. Hybrid clones selected within the segregating populations of two- or multi-species hybrids derived from polyploid Mexican species *S. neoantipoviczii* (=*S. stoloniferum*) and *S. guerreroense* due to their resistance to pathogens and tuber characteristics were involved in MAS for the first time. For the *S. guerreroense* species, the relationship of male sterility and W/γ cytoplasm type has been shown for the first time as well.

The aims of this study were i) to breed the progenies of the multi-species hybrids derived from wild polyploid Mexican potato species, and ii) to screen obtained progenies for the type of cytoplasm and resistance to the most dangerous potato plant pathogens, *Phytophthora infestans,* potato virus Y (PVY) and golden potato nematode (GPN), using DNA markers.

Techniques. The initial parental forms of the original interspecific hybrids

were the accessions of Mexican and South American species, breeding clones and varieties from VIR and SLU (Swedish University of Agricultural Sciences) collections, which were selected due to high foliar and/or tuber resistance to late blight and PVY resistance [21-23]. The VIR accessions were the clones of the Mexican wild species *S. neoantipoviczii* (=*S. stoloniferum*) (K-8505) (nan) and *S. guerreroense* (K-18407) (grr). These clones possess high foliar resistance to late blight and also are PVY resistant: *S. neoantipoviczii* is resistant to three strains, PVY^0, PVY^{N-Wi}, PVY^{NTN}, and *S. guerreroense* is resistant to two strains, PVY^0, PVY^{N-Wi} [22]. Most likely, the Ry_{sto} gene of *S. neoantipoviczii* K-8505 is in the homozygous state, because PCR analysis revealed Ry_{sto} gene marker YES3-3A in each of 20 individually tested seedlings [24]. The South American wild species were the clones of accessions with high (*S. microdontum*, K-20320) (mcd) and partial (*S. tarijense*, K-10712) (tar) foliar resistance [22, 25] or tuber resistance (*S. kurtzianum*, K-12488) (ktz) to late blight [22]. The clones of *S. kurtzianum* (K-12488) and *S. tarijense* (K-10712) at long day conditions are capable of forming tubers with good morphologic characteristics. A part of the plants from population of cultivated Andian species *S. tuberosum* subsp. *andigenum* (selected from K-8077) (adg) showed tuber late blight resistance. The fertile and productive *S. tuberosum* breeding clones from SLU collection, with good tuber characteristics, were involved in hybridization. These clones were SW-0906512 which shows field resistance to PVY, SW93-1015 of unknown origin, with resistance to PVY [23] and high field late blight resistance of constitutive type [26] conferred by *R2 like* gene [27], and SW93-1015 having an increased content of α-chaconine in tubers [23]. In this evaluation, the breeding clone NZ2010-10nb with foliar late blight and PVY field resistance derived from *S. stoloniferum* and the potato varieties Campina (nematode resistance), Desirée, Sarpo Mira (high late blight resistance) and Superb from SLU collection were also used in crossing in order to improve the agronomic traits of developing forms. All interspecific hybrids were developed by Dr. N.M. Zoteeva. Earlier some hybrids derived from two-species crossing were evaluated in the field and laboratory phytopathological tests [28-30]. In order to develop multi-species hybrids, the genotypes selected for PVY and late blight resistance were involved in further crossings. The current study examined a total of 35 hybrid clones (11 cross-combinations) which were split into three groups having different initial maternal forms (the cytoplasmic determinant donors).

The DNA was isolated by modified CTAB method from leaves of field grown plants [31].

The primers used in molecular screening for PVY [16, 32-33], foot rot [27, 34-37] and golden potato nematode [38] resistance markers were chosen based on the analysis of the parental forms of wild Mexican species.

The cytoplasm types of hybrids was determined as per K. Hosaka and R. Sanetomo [17] using a kit they proposed for four markers to detect various plastid DNA sites (*ndhC/trnV*, *rpl32/trnL-UAG*, *cemA*, *rps16/trnQ* loci) and two mitochondrial genome sites (*rps10* and *rps19* loci).

The PCR was carried out in 20 μl reaction mixture containing 10 ng total DNA, 1× buffer (Dialat Ltd., Russia), 2.5 mM $MgCl_2$, mixt of dNTPs at a final concentration of 0,4 mM each, forward and reverse primers (0.2 μM each), 1 unit of Taq DNA polymerase (Dialat Ltd., Russia). For ALM4/ALM5 primers, the dNTPs concentration was 0.6 mM. PCR was carried out in a Mastercycler® Nexus Gradient (Eppendorf, Germany) at annealing temperatures reported earlier (see the *Results* section). PCR was repeated at least 3 times for all markers except CAPS, and at least 5 times for ALM4/ALM5 primers.

The restriction was carried out in 30 μl reaction mixture according to the manufacturer's protocol (NPO SibEnzyme, Russia; http://russia.siben-zyme.com).

The electrophoresis was carried out in the TBE buffer and 2 % agarose gel followed by ethidium bromide staining and UV visualization. The molecular weight marker 100 bp + 1500 bp + 3000 bp (NPO SibEnzyme, Russia) was the standard.

Fertility of the hybrids was determined by staining the pollen with acetocarmine and by crossing method.

Foliar field resistance to late blight was assessed in 2014-2015 under the strong infection pressure (SLU, Sweden) as well as in the epiphytic season of 2016 in the experimental field of VIR Pushkin labs (Leningrad Province). Each year, the assessment was carried out from the first infection symptom manifestation on susceptible varieties (Desire and Bintje, SLU collection) until the end of vegetation. The nine-grade estimation scale was used, with 1 for totally diseased plant and 9 for the absence of disease symptoms.

The field resistance of the hybrids to PVY was assessed in 2012-2014 (SLU) when high infection spreading had been initiated by strong aphid invasion on untreated plantations. In the same field, the susceptible variety Magnum Bonum (SLU collection) annually exhibited strong disease symptoms. The plants without visible viral disease symptoms were studied using ELISA kit (BIOREBA AG, Switzerland) according to the manufacturer's instruction. The absorption was measured on a Multiskan™ GO Microplate spectrophotometer (Thermo Fisher Scientific, USA). The absorption above 0.1 means a positive reaction (plant sensitivity to viral infection). The tests were carried out twice a year (at the end of June and at the very beginning of August) according to the age-specific requirements for plants of different maturity groups (the International Potato Center, http://www.cipotato.org).

Results. Earlier it has been shown that *S. guerreroense* accession (K-18407) had a marker of the *R3a* gene [24], and *S. neoantipoviczii* (=*S. stoloniferum*) (K-8505) had a marker of the Ry_{sto} gene [24]. Since the aborigine and currently used potato varieties resistant to PVY and GPN (pathotype Ro1) have been involved in hybridization, the markers of the *Ryadg* and *H1* genes were also used in the molecular screening.

The PCR conditions and primers used in the molecular screening of the obtained potato hybrids are given in Table 1.

1. DNA markers associated with *R* genes

1	2	3	4	5	6	7
			Resistant to potato virus Y			
Ry_{sto}	XII	GP122-406/EcoRV	F: CAATTGGCTCCCGACTATCTACAG R: ACAATTGCACCACCTTCTCTTCAG	52	406	[32]
$Ry\text{-}f_{sto}$		YES3-3A	F: TAACTCAAGCGGAATAACCC R: AATTCACCTGTTTACATGCTTCTTGTG	55	341	[16]
Ry_{adg}	XI	RYSC3	F: ATACACTCATCTAAATTTGATGG R: AGGATATACGGCATCATTTTTCCGA	60	321	[33]
			Late blight resistance			
Rpi-blb1	VIII	blb 1	F: AACCTGTATGGCAGTGGCATG R: GTCAGAAAAGGGCACTCGTG	58	821	[34]
Rpi-sto1	VIII	Rpi-sto1	F: ACCAAGGCCACAAGATTCTC R: CCTGCGGTTCGGTTAATACA	65	890	[35]
R 1	V	R1-1250	F: CACTCGTGACATATCCTCACTA R: GTAGTACCTATCTTATTTCTGCAA-GAATTCTTATTTCTGCAAGAAT	65	1205	[36]
R2-like	IV	R2area 1/2	F: AAGATCAAGTGGTAAAGGCTGATG R: ATCTTTCTAGCTTCCAAAGATCACG	60	1137	[27]
R3a	XI	RT-R3a	F: ATCGTTGTCATGCTATGAGATTGTT R: CTTCAAGGTAGTGGGCAGTATGCTT	56	982	[37]
			Golden potato nematode resistance			
H1	V	57 R	F: TGCCTGCCTCTCCGATTTCT R: GGTTCAGCAAAAGCAAGGACGTG	60	452	[38]

Note. 1 — gene, 2 — chromosome, 3 — marker, 4 — primer sequence (5′→3′), 5 — primer annealing temperature, T_m, °C, 6 — diagnostic fragment size, bp, 7 — references.

The cross-combinations many of which are reported for the first time are

described in Table 2.

2. Molecular screening and phenotyping of potato hybrids derived from crossings with polyploid wild Mexican species (in groups depending on the initial maternal form)

Group, variant	Combination	Cytoplasm type (number of studied seedlings)	PF, %	Diagnostic fragments of *R* gene markers									Late blight resistance in 2014-2016, points
				R1	*R2 like*	*R3a*	*blb1*	*Rpi-sto 1*	Ry_{vadg}	Ry_{sto}	$Ry\text{-}f_{sto}$	***H1***	
I	SW93-1015 (♀)		0	–	+	–	–	–	–	+	+	+	8.0; 8.0; nd
1	(SW93-1015 × adg) × Desirée	W/γ (3)	0-1.0	–	+	–	–	–	–	+	+	–	nd; 7.0; 8.0
2	(SW93-1015 × adg) × {[nan × × (mcd × trj)] × (grr × adg)}	W/γ (3)	0-0.3	–	+	–	–	–	–	+	+	+	7.0; 6.0; 6.0
II	*S. neoantipoviczii* (♀)												
3.1	[nan × (mcd × trj)] × (grr × adg)	W/α (1)	69.8	–	+	–	+	+	+	+	–	–	7.0; 8.0;7.0
3.2	[nan × (mcd × trj)] × (grr × adg)	W/α (1)	66.7	–	+	–	+	+	+	+	–	–	8.0; 8.0; 6.0
4	[{nan × (mcd ×tar)} × (grr × × adg)] × SW-0906512	W/α (1)	26.7	–	–	–	–	–	+	+	+	–	6.0; 6.0; 5.5
III	*S. guerreroense* (♀)												
5	grr × adg	W/α (6)	18.0-24.1	–	+	+	–	–	+	–	–	–	8.0; 8.0; 7.0
5.1	grr × Superb	W/α (4)	38.0-65.0	–	+	+			–	–	–	–	8.0; 7.0; 8.0
6	(grr × Superb) × Sarpo Mira	W/α (3)	16.0-16.8	–	+	+	–	–	+	–	–	–	8.0; 7.0; 8.0
7	(grr × Superb) × Desirée	W/α (1)	14.5	–	–	–	–	–	+	–	–	–	6.5; 6.5; 6.9
8.1	(grr × Superb) × NZ2010-10nb	W/γ (1)	0.2	–	–	–	+	+	–	+	+	–	6.5; 7.0; 6.0
8.2	(grr × Superb) × NZ2010-10nb	W/γ (1)	0	–	–	–	+	+	–	+	+	–	7.0; 7.0; 5.5
9	[(grr × Superb) × NZ2010-10nb] × ktz	W/γ (1)	1.0	–	+	+	–	–	+	–	–	–	nd; 6.5; 7.0
10	[(grr × Superb) × NZ2010-10nb] × {[nan × (mcd × tar) × × (grr × adg)] × SW-0906512}	W/γ (4)	0-0.7	–	–	+	+	+	+	+	+	–	nd; 7.0; 6.5
11.1	[(grr × Superb) × NZ2010-10nb] × cv. Campina	W/γ (1)	0	–	+	+	+	+	–	+	+	–	nd; 6.0; 5.0
11.2	[(grr × Superb) × NZ2010-10nb] × cv. Campina	W/γ (1)	0	–	+	+	–	–	–	+	+	+	nd; 7.0; 7.0
11.3	[(grr × Superb) × NZ2010-10nb] × cv. Campina	W/γ (1)	AF	–	–	–	–	–	–	+	+	–	nd; 4.0; 1.0

N o t e. Three crossing series were carried out (the samples were grouped according to the initial maternal forms used). *R* gene marker data is given for individual hybrid genotypes selected from the segregating populations for resistance to pathogens and/or tuber morphology. PF — pollen fertility in the analyzed genotypes (min-max), AF — absence of flowering in the 11.3 hybrid; "+" — marker found, "– " — marker not found, nd — no data (parameter was not studied). Potato species: grr — *Solanum guerreroense*; ktz — *S. kurtzianum;* mcd — *S. microdontum*; trj — *S. tarijense*; nan — *S. neoantipoviczii* (=*S. stoloniferum*); adg — *S. tuberosum* subsp. *andigenum*. The cytoplasm types are given for all analyzed genotypes, of 1 to 6 per combination.

It is known that the potato plants of the same effective ploidy (EBN) can relatively easy cross with each other producing viable hybrid seeds [39]. Crossings of tetraploid potatoes (EBN = 4) with wild Mexican hexaploid *S. guerreroense* (EBN = 4) species closely related to *S. demissum* (both are the members of series Demissa Buk. in classic taxonomy) are good examples of such combinations. Analogously, all the forms involved in the combination nan × (mcd × trj), i.e. *S. neoantipoviczii* (=*S. stoloniferum*), *S. microdontum* and *S. tarijense*, have EBN = 2 (see Table 2). The EBN rule does not apply to more complex combinations, which often occurs in the production of multi-species hybrids [9].

Screening of cytoplasm types and *R* gene using molecular markers. *Hybrids from crossings of the breeding clone SW93-1015.* All studied hybrid clones from the two crossings combinations, derived from the breeding clone SW93-1015 (maternal form), had the W/γ cytoplasm type and fully sterile pollen of anomalous morphology (see Table 2), which indicate on the presence of *S. stoloniferum* in the SW93-1015 pedigree. The presence of *S. stoloniferum* characteristic markers YES3-$3A_{341}$ and GP122-406/$EcoRV_{406}$ linked to genes Ry_{sto} and $Ry\text{-}f_{sto}$, respectively, which are located on chromosome XII is in favor of this assumption. These markers are absent in other parental forms, i.e. *S. tuberosum* subsp. *andigenum* K-8077 and Desirée variety, used in the combination No. 1 [16, 40].

Moreover, the selected hybrid clones had the markers of *R2-like* gene for resistance to late blight and the *H1* gene for GPN resistance (see Table 2). Compared to the breeding clone SW93-1015, the hybrids from the combination No. 1 (SW93-1015 × adg) × Desirée produced more tubers per plant which were more uniform in size.

Hybrids derived from crosses with S. neoantipoviczii (=*S. stoloniferum*). It is shown that of two cytoplasm types, W/α and W/γ, characteristic of *S. stoloniferum* [41], only W/γ is associated with male sterility [16-18, 41]. *S. neoantipoviczii* (=*S. stoloniferum*) K-8505 having W/α cytoplasm was a maternal form of these hybrids. All hybrids of K-8505 also had the W/α cytoplasm and varied in fertility levels (26.7-69.8 %). In further crossing, the hybrid form [nan × (mcd × trj) × (grr × adg)] was a successful pollinator, which indicates functional fertility of its pollen (see Table 2).

All hybrids of group II had the markers of *R* genes for extreme resistance to PVY, and multi-species hybrid No. 4 had markers of three genes, Ry_{adg}, Ry_{sto}, $Ry\text{-}f_{sto}$ (see Table 2). The genotypes with markers of late blight resistance genes *R2 like*, *Rpi-blb1, Rpi-sto1* were selected from multi-species hybrids of this group.

Hybrids derived from S. guerreroense. S. guerreroense species was involved in breeding for the first time. Hybrid plants derived from the combinations (grr × adg) and (grr × Superb) often had violet corolla color typical for *S. guerreroense*, but unlike the wild parent, could form tubers under long light day conditions. The plants of hybrid combination (grr × Superb) had long stolons and an irregular tuber shape. Progenies of (grr × Superb) × Desirée hybrid showed high polymorphism by tuber shape and skin color. The plants of *S. guerreroense* hybrids formed a large number of berries via self-pollination. The percentage of fertile pollen in these hybrids reached 24 % and the plants of the (grr × adg) hybrid were successfully used as effective pollinators for production of multi-species hybrids, which indicated functional fertility of their pollen (see Table 2). Two-species hybrids of three combinations, (grr × adg), (grr × Superb), and [(grr × Superb) × Desirée], had the W/α cytoplasm type. However, the γ mitotype was detected in multi-species hybrids of the group III with *S. guerreroense* as an initial maternal form, at that, all of the multi-species hybrids of the combinations Nos. 8-11 were sterile (see Table 2). The absence of amplification in the intragene spacer *rps10 cob* (mtDNA cytoplasm type W/y) in these multi-species hybrids could be associated with the rearrangement of mtDNA sequences which occurred due to multiple hybridization.

The hybrid clones from group III derived from *S. guerreroense* displayed in various combinations up to 4 markers for late blight resistance genes and 1 to 3 markers for PVY resistance genes (see Table 2). The variety Superb and *S. tuberosum* subsp. *andigenum* K-8077 could be donors of the Ry_{adg} gene in the selected hybrid clones. In multi-species hybrids combinations Nos. 8-11, the donors of the Ry_{sto} and $Ry\text{-}f_{sto}$ genes were either *S. neoantipoviczii* (=*S. stoloniferum*) (combination No. 10), or the PVY-resistant breeding clone NZ2010-10nb, derived from *S. stoloniferum* (combinations Nos. 8, 9, 11) (see Table 2).

We did not revealed genotypes with R1-1250 marker for *R1* gene in any of the combinations studied (see Table 2).

In each of the hybrids of groups II and III, the markers for genes *Rpi-sto1* and *Rpi-blb1* were found simultaneously (see Table 2). It is known that the *Rpi-blb1* and *Rpi-sto1* genes are orthologues related to the same *Rpi-blb1* family which is located on chromosome VIII close to CT88 marker, and their sequences possess high homology [34, 42, 43]. Functional homology of these genes has also been reported [43]. Intragenic markers developed for *Rpi-blb1* gene of diploid Mexican *S. bulbocastanum* species were found in Mexican polyploid species

S. stoloniferum and *S. papita* (=*S. stoloniferum*) [34] and also in hybrids derived from *S. stoloniferum* [12]. Therefore, in our research, both markers, Rpi-sto1 and Rpi-blb1, detect the sequences of the same *Rpi-sto1* gene in the hybrids derived from *S. stoloniferum*.

Assessment of hybrid resistance to late blight and PVY. In 2014-2015, the plants of the Bintje and Desirée varieties were significantly affected by *Ph. infestans* 2.5 weeks after the first symptom manifestation, and in the epiphytotic season of 2016, plants of the same varieties in the same dates were affected completely. Simultaneously, the hybrid clones which were selected earlier from segregating populations were studied. The study in epidemic 2016 season confirmed resistance to late blight of the number of clones derived from crosses with SW93-1015, *S. neoantipoviczii* K-8505 and *S. guerreroense* K-18407 (see Table 2). High field resistance (7-8 point score) was observed in two-species hybrid combinations Nos. 1, 5, 6 (see Table 2). Previously, the hybrids combination (grr × adg) expressed the same hypersensitivity reaction in the lab leaflet test as the parental *S. guerreroense* plants [24]. The leaflet test, in which the inoculums of three *Ph. infestans* isolates, SW058 [26], 88069 [44] and H7, were used at the concentration three times higher than that of the standard [30], allows us to select the genotypes with extremely high late blight resistance from segregating population of the grr × adg hybrid. These data indicate that *S. guerreroense* K-18407 is a valuable source of late blight resistance which effectively transmits this trait to hybrid progenies. Clones of the multi-species hybrid [nan × (mcd × trj) × (grr × adg)] showed high resistance to late blight (see Table 2). In 2017 season with strong *Ph. infestans* invasion, high resistance of hybrid clones expressed in the season of 2016 was confirmed completely (data are not shown).

In some cases, sequential crosses of hybrids with varieties and/or breeding clones led to a decrease in late blight resistance degree though other agronomic characteristics improved. Thus, late blight resistance scores in some clones from the combinations Nos. 7, 8, 9, 10, and 11 were 6.0 to 7.0 points, and in hybrids No. 4 and No. 11.1 the scores averaged 5.5 to 6.0 points (see Table 2). The clone No. 11.3 was selected for good agronomic characteristics (cultivated type tubers) but its plants were totally affected by late blight in 2016. In this clone, none of the markers of genes *R2 like*, *R3a, Rpi-blb1, Rpi-sto1* conferring late blight resistance were detected (see Table 2).

Among plants of groups I, II and III, the molecular screening revealed late blight resistant genotypes with various combinations of markers for broad-spectrum resistance genes *R2 like*, *Rpi-blb1*, *Rpi-sto1*, and the race-specific resistance gene *R3a*. However, the presence of these markers did not always determine phenotypic resistance. E.g. hybrid No. 11.1 had all four markers for late blight resistance genes (see Table 2) bur showed moderate pathogen resistance. Apparently, the markers which we used in the analysis detected in these genotypes the non-functional homologues of *R* genes. At the same time, high field late blight resistance (7-8 points) in the absence of some markers may be due to an introgression of not yet identified genes/QTLs from *S. guerreroense* (e.e. combinations Nos. 5 and 7, see Table 2) or resulted from functioning other *R* genes in these hybrids (e.g. hybrid No. 6 could confer the dominant alleles of *R4*, *R8*, *Rpi-Smira1* genes from the Sarpo Mira variety) [46, 47].

The field PVY resistance was assessed only in two hybrids combinations, No. 3 and No. 5, and in their progeny (10 seedlings per combination). During the entire study, the plants of multi-species hybrid [nan × (mcd × trj)] × (grr × adg)] showed no susceptibility to PVY under high infection pressure and, by ELISA test, were free of the viral infection (absorption value from 0 to 0.001). This hybrid had the markers for two genes, Ry_{sto} and Ry_{adg}, which determine extremely

high resistance to PVY (see Table 2). All studied clones in the hybrids progeny of the combination No. 3 also showed field resistance to PVY during the entire study period. The plants of PVY susceptible variety Magnum Bonum grown in the same field developed strong disease symptoms (the absorption value in ELISA test for PVY infection was from 2.19 to 2.37). RYSC3 marker for Ry_{adg} gene determining extreme PVY resistance was detected in the combination No. 5 (grr × adg). About $^3/_4$ of this hybrid population consisted of plants free from viral infection.

Hence, the results of our study indicate that 17 of 35 hybrid clones evaluated had W/α cytoplasm, and 18 had W/γ cytoplasm. Of 18 hybrid genotypes with the W/γ cytoplasm type, 17 formed fully sterile pollen grains of anomalous morphology and one genotype did not flower. The male sterility and the W/γ cytoplasm type were transferred via the maternal line to multi-species hybrids of various crossing combinations. In 17 hybrids with the W/α cytoplasm the amount of fertile pollen grains varied. Some of these hybrids were used as pollinators. This pattern was characteristic of both the plants of groups I and II which were based on *S. stoloniferum* cytoplasm and the hybrids of group III derived from *S. guerreroense* as maternal forms. In the latter, the γ mitotype might have appeared in multi-species hybridization. Such a linkage between the male sterility and W/γ cytoplasm type in breeding clones and varieties derived from *S. stoloniferum* has been reported earlier [16, 17]. Note, for *S. guerreroense*, this phenomenon was shown for the first time. Our data and the findings obtained by other researchers allows us to assume that searching for the pathogen resistance sources within the populations of *S. stoloniferum*, *S. guerreroense* (and possibly other Mexican species) can be done simultaneously with the selection against the donors of sterilizing cytoplasm type W/γ which is undesirable for the traditional breeding. On the contrary, clones with the W/α cytoplasm would allow for an increase in availability of introgressive male fertile forms. It is highly likely that in the future the selection vector can change. In particular, fixation of genotypes with sterilizing cytoplasm in breeding material may be promising for the development of heterotic hybrid selection as a new direction of potato breeding [48].

To conclude, the combined use of nuclear and cytoplasmic DNA markers for identification of R resistance genes and cytoplasm types will facilitate parental pair selection to reduce the time and the cost for hybridization, and to control crossing when combining genes of desirable traits in one genotype.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 976-985
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 635.657:631.524.02:631.526

doi: 10.15389/agrobiology.2017.5.976rus
doi: 10.15389/agrobiology.2017.5.976eng

CHICKPEA LANDRACES FROM CENTERS OF THE CROP ORIGIN: DIVERSITY AND DIFFERENCES

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The authors declare no conflict of interests
Supported by Russian Science Foundation (project № 16-16-00007)
Received June 11, 2017

Abstract

Chickpea (*Cicer arietinum* L.), a grain legume crop, is considered innovative for the Russian Federation. Over the past fifteen years, its area in our country have increased 20 times and reached 420,300 hectares in 2015. The growing demand of chickpea determines the necessity of breeding new varieties. One of the ways to improve the crop could be the introgression of genes from old landraces, especially those from the regions of species genetic diversity, the centers of its origin (i.e. the primary in Turkey and the secondary in Ethiopia). In this paper the question is raised about the diversity and phenotypic differences of the chickpea gene pool growing in the centers of origin about a century ago and preserved in VIR collection. Here, we first showed the differences in the phenotypic characteristics of the oldest chickpeas from two centers of origin. Fifteen morphological, phenological and agronomic features were studied in 75 local varieties from Turkey and 24 ones from Ethiopia. Both in Turkish and in Ethiopian samples, the most variable signs were the number of seeds per plant (*Cv* 62.6 and 70.4 %, respectively) and the number of beans per plant (*Cv* 62.2 and 63.0 %). Principal component analysis showed that the first five factors determined 78.9 % of the total variability of traits. Factor 2 (22.0 % of the variance) can be called a factor of potential seed production. Correlation analysis revealed a much stronger relationships between all the traits studied in the Ethiopian samples. The correlation between seed production and vegetation period were the strongest ($r \geq 0.9$). We have revealed association of certain traits of chickpea plants with the geographical zones of the sample origins. Landraces from Ethiopia are fairly homogeneous and have small, dark and angular seeds, low attachment of the first bean and low seed productivity, are more early maturated compared with the Turkish ones. Turkish landraces are characterized by a great variety of all the traits studied, revealing all their grades described in the chickpea descriptors. In this region, the landraces typical of the western Mediterranean, as well as for territories bordering Turkey in the east had been grown. The structure of the variability and the strength of the relations of the traits differed in the landraces from the primary and secondary centers. It is obvious that in plants growing in different ecological and geographical environment, there is a specific communications between the traits, reflecting the presence of different blocks of co-adapted genes or another integrated gene complexes that determine adaptation to a particular environment. Useful characters for breeding are found in landraces from both centers of origin and chickpea diversity.

Keywords: chickpeas (*Cicer arietinum* L.), centers of origin, phenotypes, variability, factor analysis, structure of traits relationships, diversity, characters for breeding

Chickpea (*Cicer arietinum* L.) is a grain legume crop that is considered innovative for the Russian Federation. It is very popular in Asian countries (In-

dia, Pakistan, Syria, Turkey, etc.) and North Africa, and is the second in the world in sowing areas and the third in production among grain legumes (excluding soybeans) [1]. The nutritional value of chickpea is determined by the high content of protein in its seeds (17-30 %) and various macro- and micronutrients [2], which determine a healthy diet. The undoubted agronomic merit of this crop is its high drought resistance. However, in our country, chickpeas, which are also known as mutton or turkey peas, are not well known to consumers. For many years in the Russian Federation, the planted areas of chickpea did not exceed 20-25 thousand hectares. However, nowadays its production has sharply increased, and in 2015 the sowing areas reached 420,300 hectares [3]. This is explained by the fact that chickpea is a highly demanded export crop, although, according to experts, it should take a worthy place in the domestic market.

Given the growing demand for this crop, new highly adaptive varieties are required, in particular, those with improved drought resistance and resistance to diseases, especially resistant to *Ascochyta pisi* L. Also, precocity is a trait that is relevant for many regions of chickpea production.

Selection significantly narrowed the historical diversity of cultivated chickpeas [4]. To expand the genetic basis of modern varieties, it is necessary to employ diversified donor parents. The VIR collection preserves populations and old local varieties from the centers of chickpea origin with maximum genetic diversity concentrat. Local varieties are a rich gene pool for the search for highly adaptive genotypes [5, 6]. To date, such varieties in the places of their historical origin are mostly hopelessly lost, as having been almost completely extincted because of replacement by modern commercial varieties, and because of natural disasters, urban, technogenic and other factors that are rapidly changing the modern world.

Introgression of genetic material from local varieties into modern commercial varieties using marker-assisted selection (MAS) can be a radical way to improve them. Therefore, the study of the diversity of local varieties from the centers of origin for a number of selectively significant traits, the identification of the limits of their variability and the search for candidate genes determining variability are relevant for understanding the selection dynamics in the historical perspective and elucidation of its mechanisms.

The center of origin, as well as the place where chickpea was first domesticated, is southeastern Turkey and the adjacent areas of Syria and Iran [7, 8]. N.I. Vavilov [9] considered South-West Asia and the Mediterranean to be the primary center of origin of chickpeas, and Ethiopia was the secondary center. He distinguished four centers of chickpea variety: in the Mediterranean, Central Asia, the Middle East and India [10]. The primary centers of cultivated plant origin, according to N.I. Vavilov, were the ancient centers of civilization, where the primary cultivation of plants took place, and the secondary centers were the territories associated with subsequent periods of the farming culture [9].

We do not know special studies devoted to the differences between the chickpea plants from the primary and secondary centers of origin. In part, these differences are shown by us when studying the phenotypic diversity of local chickpea cultivars from the centers of origin of this crop [11]. In the present article, we first detailed the phenotypic diversity of old local chickpeas from the primary center of origin in Turkey and the secondary one in Ethiopia (Abyssinia) preserved in the collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), for a number of morphological, phonological and agronomical traits and their relationship in both centers.

The aim of the work was to compare manifestations of economically significant traits of chickpea accessions from different centers of origin in the eco-

logical conditions of Syria close to those in the primary and secondary centers of origin of the crop.

Techniques. Old local chickpeas from the VIR collection, including 75 samples from Turkey and 24 samples from Ethiopia (Abyssinia), were studied for 15 morphological, phenological and agronomic traits in the field in 2002-2005 at the International Center for Agricultural Research in the Dry Areas (ICARDA) in Syria. The Turkish samples are expeditionary collections of P.M. Zhukovsky in 1927. The Ethiopian samples were collected by N.I. Vavilov in 1927, as well as by subsequent VIR expeditions in 1962 and 1970. The sowing in Syria was carried out in February, harvesting in August. The samples were sown randomly in 2-fold replication. The row spacing was 60-70 cm; the distance between the plants was 10 cm. Six plants were analyzed in each replication.

Field assessment was conducted in accordance with the international Descriptors for Chickpea (*Cicer arietinum* L.) [12] and the ICRISAT descriptor (International Crops Research Institute for the Semi-Arid Tropics) [13].

Calculations were performed using Statistica 7.0 software (StatSoft, Inc., USA) [14]. Pearson's correlation coefficients were calculated as per common methods [15, 16]. Correlations were considered low at $r < 0.5$, average at $0.7 > r > 0.5$, high at $0.9 > r > 0.7$, and very strong at $r > 0.9$. Correlation pleiades were clustered as described [17]. The variability in the structure of the relationships between traits was assessed using factor analysis. The factor loads were determined by the principal component method. Additionally, the fraction of variance explained by a factor in the total variance and the cumulative fraction of the recoverable factors were calculated. The identification of traits that differentiate the samples by origin was carried out using discriminant analysis [14].

1. Designations of traits studied in chickpeas (*Cicer arietinum* L.) from the collection of the All-Russian Institute of Plant Genetic Resources (VIR) (Syria, 2002-2005)

Trait	Designations
Biomass yield (plant dry weight with seeds and roots), g	byld
Canopy area width per plant, cm	caw
Duration of sprouting-flowering period, days	dflr
Duration of seedling-maturation period, days	dmat
Flowering duration, days	fdu
Flower color, points	fgc
Growth habit (prostrate, spreading, erect), points	grh
Harvest index, %	hi
Height of the lower (first) pod attachment, cm	hlp
Pods per plant, pcs.	ppp
Plant height, cm	ptht
Seed per plant, pcs.	spp
Straw yield (plant dry weight without seeds), g	styld
Seed type, scores	styp
Seed yield per plant, g	syld

Results. The region in which samples were studied for a number of traits (Table 1) is located in comparative proximity to the centers of chickpea origin. One of the main differentiation traits of the chickpea gene pool is the type of seeds (desi and kabuli). Desi seeds are small, angular and have a colored seed coat (color from cream to black). Kabuli seeds are large, roundish, and light-colored. The analysis of mean values and variation of traits (*Cv*) in the Ethiopian samples showed narrower variability limits for most of the traits studied as compared to the Turkish forms (Table 2). The most varying traits in both groups were the number of seeds per plant (*Cv* was 62.6 % for the Turkish samples and 70.4 % for the Abyssinian samples) and the number of pods per plant (*Cv* was 62.2 % for the Turkish samples and 63.0 % for the Ethiopian samples). However, traits such as seed color and type, the duration of the sprouting-flowering and seedling-maturing periods, were less variable in the Ethiopian native varieties.

The Ethiopian samples, except of one, had seeds of desi red, brown and black type, whereas in most of the Turkish samples (57 %) the type of seeds was

classified as kabuli. In the Turkish gene pool, light seeds were found to be characteristic of about half of the samples, fractions with pink and brown seed hulls made up 15 % of the total number; the rest showed all the other seed colors as per the chickpea descriptors except black.

Factor analysis revealed the structure of the relationship between the traits studied in the Turkish and Ethiopian samples (Table 3). The first five factors determined 78.9 % of the total trait variability. Factor 1 (F1 — 30.0 % dispersion) revealed a positive relationship between the dry biomass of plants (with seeds and roots) and straw yield, plant height and lower attachment of the lower pod, color of the flower and type of seed, and a negative relationship with the harvest index. This factor can be interpreted as a characteristic of a plant's ability to gain and accumulate biomass.

2. Descriptive statistics of the studied samples of chickpeas (*Cicer arietinum* L.) of different origin from the VIR collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) (Syria, 2002-2005)

Trait	Mean		Min		Max		Standard Deviation		*Cv*, %	
	Turkey	Ethiopia	Turkey	Ethiopia	Turkey	Ethiopia	Turkey	Ethiopia	Turkey	Ethiopia
byld	53.8	39.4	19.8	18.1	114.6	70.0	15.6	10.4	29.0	21.9
caw	63.5	55.0	43.5	39.0	91.0	72.0	8.7	7.7	15.6	11.0
dflr	106.4	107.9	96.5	100.0	140.0	119.0	9.7	6.1	13.6	13.7
dmat	164.1	157.3	129.0	149.0	183.0	171.0	8.6	5.2	5.3	2.6
fdu	25.9	27.8	9.0	18.0	34.0	31.0	4.0	3.1	9.1	5.9
fgc	5.3	4.1	1.0	4.0	6.5	6.0	1.1	0.4	20.1	11.3
grh	2.7	2.5	1.0	2.0	4.0	3.0	0.5	0.5	19.3	20.5
hi	40.9	45.9	26.0	24.3	72.1	59.6	6.8	8.7	16.6	19.4
hlp	28.8	21.0	20.0	13.0	41.0	30.0	3.8	4.9	13.1	23.1
ppp	33.3	61.1	12.7	18.3	142.0	147.0	20.7	40.6	62.2	63.0
ptht	54.2	46.6	38.0	39.0	70.0	53.0	6.9	5.0	12.7	10.3
spp	34.1	76.6	13.0	18.0	145.0	196.0	21.3	57.1	62.6	70.4
styld	33.5	19.8	5.5	6.2	65.2	32.3	10.7	6.4	31.9	27.1
styp	1.6	1.0	1.0	1.0	2.0	2.0	0.5	0.2	31.6	20.4
syld	20.9	16.8	11.4	5.1	52.1	25.8	7.0	5.4	33.5	28.6

N o t e. For trait abbreviation, see Table 1.

3. Factor loads of 15 traits for 99 Turkish and Ethiopian chickpeas from the VIR collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) (Syria, 2002-2005)

Trait	Factor				
	1	2	3	4	5
Duration of flowering, days	–0.20	–0.24	–0.18	0.77	0.30
Time to 50 % flowering, days	–0.05	–0.73	0.29	–0.42	0.02
Canopy width, cm	0.52	–0.03	–0.11	0.37	0.21
Height of the lower (first) pod attachment, cm	0.76	0.01	-0.29	–0.40	0.17
Plant height, cm	0.67	–0.31	–0.27	–0.20	0.37
Growth habit, point	0.11	–0.26	–0.02	0.22	–0.77
Color of flowers, points	0.66	0.20	0.57	0.13	–0.06
Type of seeds, points	0.64	0.18	0.66	0.17	–0.06
Time before flowering, days	0.47	–0.36	0.55	–0.06	0.21
Pods per plant, pcs.	–0.45	–0.78	0.16	0.12	0.17
Plant dry weight with seeds and roots, g	0.69	–0.55	–0.24	0.08	–0.20
Seeds per plant, g	0.32	–0.77	–0.21	–0.03	–0.20
Straw yield, kg/ha	0.81	–0.32	–0.19	0.19	–0.11
Harvest index, %	–0.56	–0.40	0.07	–0.19	–0.18
Seeds per plant, pcs.	-0.53	–0.72	0.21	0.13	0.18
Fraction of total dispersion, %	30.00	22.00	10.40	8.80	7.50
Cumulative dispersion, %	30.00	52.00	62.40	71.40	78.90

Factor 2 (F2 — 22.0 % of the variance) can be called a factor of potential seed production. It shows a consistent change in pods per plant, the dry weight of a plant with seeds, the seed number and weight per plant, and the duration of the shooting-flowering period. Factor 3 (F3 — 10.4 % dispersion) grouped flower color, sprouting-ripening period, and seed type. It can be condi-

tionally called a factor that reveals the differentiation of samples according to the type of seeds (desi, kabuli). Factor 4 (F4 — 8.8 % of dispersion) combined the flowering period with the shooting-flowering period and the height of attachment of the lower pod with a negative relationship of these traits. In factor 5 (F5 — 7.5 % dispersion), the growth habit (erect or compact plants) and the height (length) of the plant were negatively associated (that is, for compact plant habitus the length of the main shoot is less than that for spreading or prostrate ones).

Figure 1 shows the location of the samples in the space of the first two factors. The samples from Turkey were distributed for the most part of the diagram area, since they are characterized by various combinations of traits. The samples from Ethiopia, except one, were concentrated in the left upper part and in the middle zones. Obviously, the Ethiopian local varieties are much less diverse.

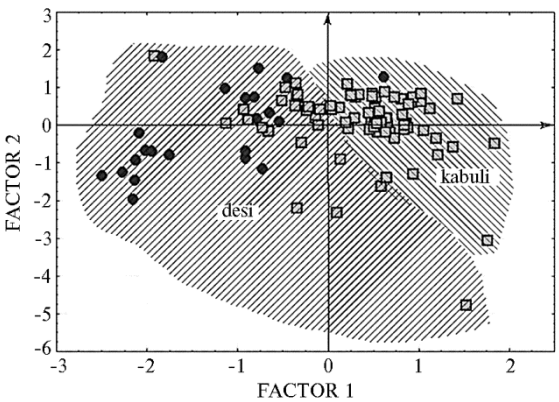

Fig. 1. Distribution of Turkish (•) and Ethiopic (□) chickpea samples from the VIR collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) in the space of the first two factors: desi and kabuli are the types of seeds (Syria, 2002- 2005).

A step-by-step discriminant analysis was performed to identify characteristics that divide local varieties into groups of origin. As a result, four most significant traits were identified: spp (seeds per plant), hlp (the height of the lower pod attachment), syld (seed yield per pant) and grh (growth habit). The distribution of the samples in the space of the canonical axes is shown in the graph (Fig. 2). The arrangement of the samples turned out to be similar to their distribution in the factor space. In the left part of the graph there are early maturing unproductive samples with dark seeds, a larger seed number per plant and a low attachment height of the first pod (predominantly Ethiopian forms), in the right part there are late maturing, productive plants with light seeds, a comparatively low number of seeds per plant and high attachment of the lower pod (mostly Turkish forms).

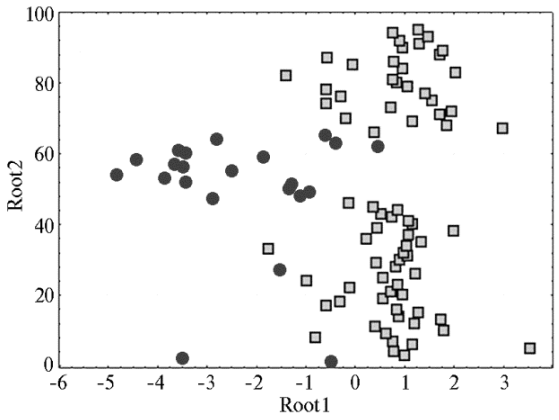

Fig. 2. The distribution of Turkish (•) and Ethiopic (□) chickpea samples from the VIR collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) in the space of the first two factors: in the space of the canonical axes Root1 and Root2 (Syria, 2002-2005).

To expand our understanding of the variability of the relationship between the chickpea traits, correlative pleiades were constructed separately for each of the groups (24 Ethiopian and 75 Turkish samples). In addition, for a more accurate and reliable comparison of the correlation structure in different samples from 75 Turkish samples, a set of 24 samples was randomly selected. Analysis of correlation pleiades revealed a much stronger conjugation between all the investigated

traits in the Ethiopian samples (Fig. 3, A).

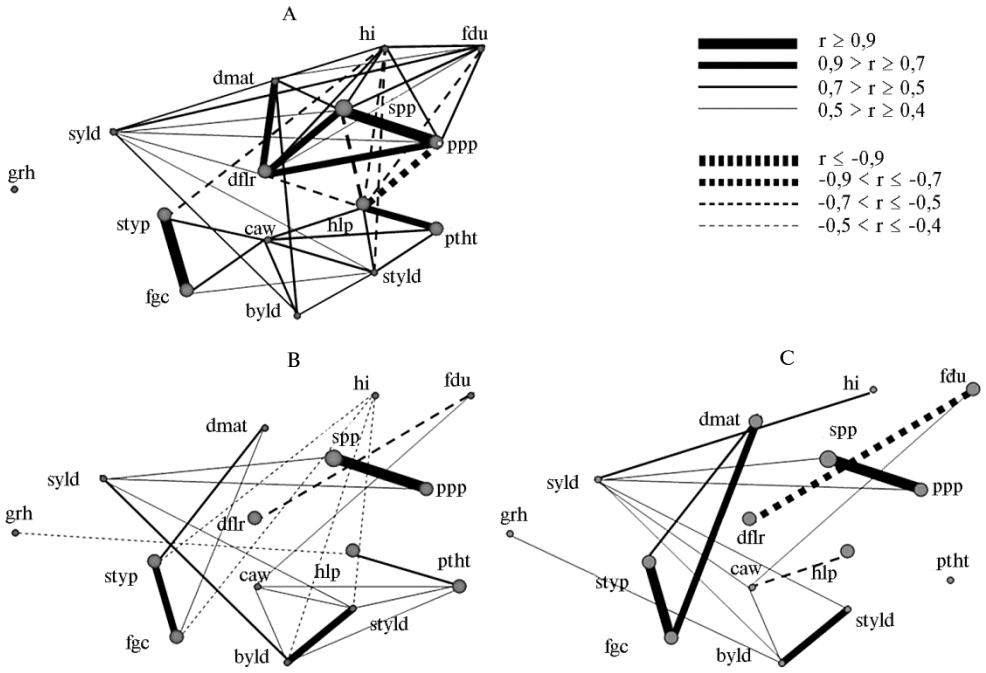

Fig. 3. Correlation structure between the traits of the studied chickpea samples from the VIR collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources): A — Ethiopian (24 samples), B — Turkish (75 samples), C — Turkish (24 samples) (Syria, 2002-2005).

The strongest correlations were found for seed productivity and vegetation period (spp, ppp, dflr, dmat); hi, fdu and syld (harvest index, duration of flowering and seed productivity) were close to these traits. The traits of plant growth character (hlp, ptht, styld) formed the second pleiade, and the separation of these two groups is relatively arbitrary, since they are related by a strong negative correlation through the pod number per plant (ppp). Thus, tall samples with a high pod attachment from Ethiopia differed from the rest because of a small number of pods and seeds and short sprouting-flowering and sprouting-maturing periods. A separate pleiade was formed by the characteristics of the seeds type and the color of flowers (styp, fgc). This pleiade through the width of plant projection (prostration) (caw) was associated with a pleiade of plant growth traits. For example, plants with dark-colored seeds and blue flowers are characterized by a protruding bush shape. An independent trait for the Ethiopian samples was the plant shape type (grh). It should be noted that the harvest index (hi), the duration of the vegetation period (dmat) and a number of other traits were interrelated with different pleiades.

the

In the samples from Turkey, due to their greater diversity, the strength of the correlation between the traits was significantly lower (see Fig. 3, B, C). As can be seen from the figures, a decrease in the number of samples in sets affected the strength of the relationship between the traits and had little effect on the correlation structure of pleiades. For most links with correlation coefficients from 0.4 to 0.5, the *r* values decreased, which confirmed the well-known rule of decreasing the correlation degree between the traits when the sample size reduced [18]. However, the correlations between some traits have intensified (fgc and dmat, dflr and fdu, hlp and ptht, hlp and grh).

The three correlation pleiades found in all sample sets seem to be more

characteristic of the species as a whole and are more stable in the cultivated chickpea in general. These are the pleiades of the pod number and seed number per plant (ppp, spp), the color of generative organs (styp, fgc), the plant weight and seed weight per plant (byld, syld). The correlations structure in the Turkish samples, regardless of their size, turned out to be little similar to that of the samples from Ethiopia. It is obvious that in plants growing in different ecological and geographic conditions, specific interrelation systems are formed between the traits, reflecting the presence of different blocks of co-adapted genes and other integrated gene complexes that determine adaptation to a particular environment.

As we noted earlier [11], both the primary and secondary origin centers have a sufficient degree of geographic traits proximity, which fully corresponds to the N.I. Vavilov's theory [19] about the geographical patterns in the distribution of plant genes. In particular, this refers to the fineness and relatively smaller organ size of plants in the eastern (Indian and adjacent) areas and their larger sizes in the Mediterranean.

Chickpea plants from Ethiopia are low; they have small dark seeds of desi type, that is, posses the traits determined by dominant genes. The same regularities were noted by N.I. Vavilov for other crops from this center of origin, including legumes, *Lens esculentum* Moench, *Pisum sativum* L. and *Lathyrus* sativus L. Meanwhile, west of Ethiopia in the Mediterranean region, plants and their seeds become much larger, flowers and seeds are lighter, that N.I. Vavilov explained by "the loss of dominant genes and the accumulation of recessive forms" [19, p. 415].

The relatively narrow limits of the phenotypic traits variation found in the Ethiopian samples, revealed by us, are also confirmed by modern molecular genetic data [20].

Given that 96 % of the Ethiopian samples had the desi seed type, then the Turkish samples had a ratio of the desi and kabul seed type of 43 %:57 % and were predominantly light. The maturation period in the Turkish samples was about 7 days longer than in the Ethiopian samples (see Table 2). At the same time, the limits of this trait variability in the Turkish varieties were much wider than those of the Ethiopian samples.

In our previous paper [11], we discussed the reasons for the uniformity of Ethiopian samples and explained this by the country's apparent isolation for a long time, its distance from trade routes and the limited international contacts. Moreover, in Ethiopia, even at the beginning of the twentieth century, agriculture had a primitive character [21]. All Ethiopian samples preserved in the VIR collection (except one) are classified as an Abyssinian ecogeographic group, poorly differentiated and absolutely unique and endemic for Ethiopia [22].

The Turkish group of old local varieties exhibits almost the entire range of traits indicated in the chickpea descriptors, and is characterized by medium-sized vegetative organs, large seeds, and high plant productivity. Modern studies with AFLP-markers (amplified fragment length polymorphism) also show a relatively high variety of Turkish chickpeas [23]. Among the samples collected by P.M. Zhukovsky in Turkey, there were representatives of three ecogeographical groups of varieties: actually Turkish, Spanish and Afghan [24]. That is, by the beginning of the 20th century Turkey had varieties inherent in both the western and eastern Mediterranean, including varieties from areas close to Central and Central Asia, namely, from Iran and Afghanistan. In addition, it was noted that already in those days the crops cultivated in Turkey showed visible traits of ancient breeding [25]. The results of our factorial and discriminant analysis showed an obvious tendency to improve agronomic traits in Turkish local varieties com-

pared to the more primitive Ethiopian varieties, which raises the question of the possibility to deem the Ethiopian center as more ancient. This issue has already been discussed by VIR botanists [24, 26] and requires a separate review.

So, old local varieties of chickpeas from their origin centers preserved in the VIR collection have traits that reveal their ecological and geographical differentiation, which is in full accordance with the theory of N.I. Vavilov about the geographical patterns in the distribution of plant genes. Morphological, phenological and agronomic traits of chickpea from the primary (Turkey) and secondary (Ethiopia) origin centers reveal a small variety and primitiveness of Ethiopian samples, while the phenotypes from Turkey are characterized by great diversity and possess traces of agronomic improvement. The structure of the trait relationships in the samples from two origin centers is not very similar. It is obvious that in plants growing in different ecological and geographical conditions, specific likage systems between the traits are formed, reflecting the presence of different blocks of co-adapted genes and other integrated gene complexes that determine adaptation to a particular environment. Currently, local varieties of chickpeas from both centers of origin and diversity may be valuable in breeding for early ripeness (Ethiopian samples), high productivity, large seed size, and high plants (Turkish samples).

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 986-994
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.13:579.26:632.4.01/.08

doi: 10.15389/agrobiology.2017.5.986rus
doi: 10.15389/agrobiology.2017.5.986eng

SYMBIOTIC RELATIONSHIPS BETWEEN AGGRESSIVE *Fusarium* AND *Alternaria* FUNGI COLONIZING OAT GRAIN

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (project № 14-26-00067)
Received June 13, 2017

Abstract

The mycobiota of cereal grain consist of diverse fungal community among which *Fusarium* and *Alternari*a species are the most abundant. In biotic community, the competition and cooperative behavior may impact interspecies interactions. In this paper, we first report significant correlations between abundance of these fungi in oat grain. The symbiotic relationships that were found are of key importance because of mycotoxin production by *Alternaria* and *Fusarium* fungi. qPCR was used to measure *Fusarium* and *Alternaria* DNA in grain of 21 varieties and breeding lines of oats (*Avena sativa* and *A. byzantina*) from VIR World Collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources), and mycotoxin deoxynivalenol (DON) level was evaluated by ELISA test. Gere, Veler, KSI 731/01, KSI 432/08 genotypes appeared to be the most infected by both *F. culmorum* and *Alternaria* fungi. The highest amounts of DON (up to 1179 μg/kg) occurred in the grain of the breeding line KSI 432/08, as well as in Belinda and Konkur varieties. Stipler variety was the most resistant to all studied fungi and did not accumulate DON. Oat genotypes characterized by larger grains were more susceptible to fungal infections, whereas those with high husk proportion contained more DON. A high positive correlation between the amounts of DNA of *F. culmorum* and the *Fusarium* species able to produce trichothecene mycotoxins (Tri-*Fusarium*) was shown ($r = + 0.67$, $p < 0.001$), as well as between *F. culmorum* DNA level and DON accumulation ($r = + 0.57$, $p < 0.01$). When colonizing the same substrate, aggressive *Fusarium* species and relatively weak *Alternaria* pathogens are in symbiotic relationships. In this, we found the significant positive correlations between the amounts of DNA of *Alternaria* fungi and *F. culmorum* ($r = + 0.66$, $p < 0.01$), and between DNA level of *Alternaria* and Tri-*Fusarium* fungi ($r = + 0.86$, $p < 0.001$). In this study, DON produced by *F. culmorum* had no allelopathic effect on *Alternaria* fungi. At the same time, any interspecies interactions between *Alternaria* fungi and *F. poae*, the most abundant species detected in oat grain, were not found. Undoubtedly, the functions of fungal complexes colonizing plants depend on the parameters of the environment and the relationships evolutionarily developed within natural communities.

Keywords: *Avena* L., oat, grain, fungi, *Fusarium*, *Alternaria*, DNA, qPCR, deoxynivalenol, symbiotic interactions

N.I. Vavilov wrote that the immunity of a plant to a disease must be considered as the result of the interaction of many components associated with the habitat [1]. The mutual interaction of pathogens in the mycobiota community of grain undoubtedly influences their vital activity, which ultimately affects the relations with the host plant. Therefore, selection for disease resistance can be successfully carried out only upon considering all environmental factors.

Fungi of *Fusarium* Link genus cause fusariosis, a harmful disease of cereals leading to a decrease in yield and deterioration of its quality. The peculiarity of this disease lies in its specific etiology, i.e. a variety of *Fusarium* genus species

are involved in the infectious process. Thus, 10 to 20 species can coexist in one sample of grain with the explicit dominance of one species or group of species most adapted to the specific environmental conditions. *F. culmorum* (W.G. Smith) Sacc. is an aggressive pathogen of cereal crops in regions with a temperate climate, which can also infect many other plant species. The fungus is most frequently noted in Central and Northern Europe [2, 3], in Russia — in the Central, Central Black Earth and North-West regions [4-6]. *F. culmorum* produces deoxynivalenol (DON), 3-acetate-DON and less often nivalenol [7]. These mycotoxins belong to the most widespread and studied group of trichothecene metabolites, which are a significant threat when using contaminated grains. There are models for assessing the development of fusariosis and the accumulation of mycotoxins, taking into account the influence of the variety, environment, agronomic methods, but actual fungi and mycotoxins contamination of grain does not always corresponds to the forecasted one, which in the opinion of many researchers may be due to the effect of the concomitant mycobiota [8-10]. Indeed, usually fusarium fungi are not the only ones in the grain mycobiota and coexist with its other representatives.

In nature, all fungi inevitably interact directly or indirectly — both in a contact manner in competition for the substrate and allelopathically due to the influence of secondary metabolites accumulated in the substrate as well as volatile organic compounds, the functions of which are still not sufficiently deciphered [11-13]. Fungi of the *Alternaria* Nees genus presented in grain alongside *Fusarium* fungi are isolated at a high frequency [14-16]. About 30 species of the *Alternaria* genus are found in grain crops, the taxonomic position of many of which remains unclear [15, 17]. Small-spore species *A. alternata* (Fr.) Keissl, *A. tenuissima* (Kunze) Wiltshire and *A. infectoria* E.G. Simmons are most often mentioned; these species have similar morphological features [17-18], which often leads to their false identification. Meanwhile, according to some data, these species differ in ability to produce secondary metabolites, for example, alternariol (AOL) [18], and, as a consequence, to affect the rest of mycobiota. It is shown that the presence of *Alternaria* corresponds to various contamination of grain with other fungi. Thus, it was reported that the amount of *Alternaria* spp. DNA in wheat correlated with the content of *F. graminearum* Schwabe DNA negatively in awns and grain [19] and *A. alternata* infestation reduced the accumulation of DON in grain [20]. However, other researchers, despite notable amounts of *Fusarium* and *Alternaria* DNA, did not reveal a reliable relationship between the abundance of these fungi on the grain of barley, rye and wheat [21].

Oat (*Avena* L.) is a hulled crop, the grain of which is largely affected by fungi of varying degrees of pathogenicity [22]; interactions between them during colonization of plant tissue have been studied insufficiently. For grain damage, there are few reports on the mutual influence of typical memers of *Fusarium* and *Alternaria* coexisting on the same substrate.

This paper is the first report on the symbiotic character of the relationship between *Fusarium* and *Alternaria* fungi living on the oat grain, which is of great practical importance, since these fungi produce mycotoxins that are dangerous to humans and animals.

Our aim was to study the interaction between typical members of the natural mycobiota of oat grain, *Alternaria* and *Fusarium* fungi, by assaying DNA of these pathogens in oat grain and mycotoxin contamination.

Techniques. The contamination of grains with *Fusarium* and *Alternaria* fungi was evaluated in breeding varieties and promising lines of *Avena sativa* L. (19 genotypes) and *A. byzantina* C. Koch (varieties Medved and CDC Dancer) of different ecogeographical origin, preserved in the VIR world collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources). The plants of each genotype

were grown in rows of 1 m length in two replicates (experimental station, Pushkin, Leningrad Region, 2015). During earing, plants were inoculated with a suspension of conidia and mycelia (1.3×10^7 CFU/ml, 50 ml/m^2) of four *F. culmorum* strains, MFG 58219, 58562, 58578, 58585 (Collection of microorganisms, Laboratory of Mycology and Pathology, All-Russian Research Institution of Plant Protection). The oats variety Borrus previously characterized as medium-resistant to grain fusariosis [23] were additionally sown as an non-inoculated control. This area was treated with sterile water.

After harvesting and threshing, 1000 grain weight at a 12 % humidity was recorded and the proportion of the flower glume according to the guidelines [24]. For each genotype, 1000 grain weight was determined by weighing two 250-grain portions per sample and doubling the obtained value. The **hull** content was evaluated by weighing 4 portions of grain (50 pieces each), two with flower glumes retained, and two with flower glumes manually removed, calculating the percentage of hull weight from the total grain weight. If the discrepancy between the two measurements did not exceed 5 % of the average, this value was accepted as average weight. With greater differences, a new portion of grains was weighed and the average value between the nearest indicators was again calculated. The grain of each sample (10 g) was homogenized in sterile milling pots in a Tube Mill Control mill (IKA, Germany) at 25,000 rpm for 25 seconds. The milled meal was stored at –20 °C until extraction of DNA and mycotoxins.

DNA was isolated from 200 mg of grain meal by the adapted CTAB method [25]. DNA of *F. culmorum* and *Alternaria alternata* (Fr.) Keiss fungi strains was obtained from actively growing mycelium using a set of Genomic DNA Purification Kit reagents (Thermo Fisher Scientific, USA) in accordance with the attached protocol. The DNA concentration was evaluated in a Qubit 2.0 fluorimeter with a set of Quant-iT dsDNA HS Assay Kit reagents (Thermo Fisher Scientific, USA). Fungal DNA preparations were diluted to a concentration of 10 ng/ml and used to construct a calibration curve (10-fold serial dilutions from 10^{-1} to 10^{-6} ng/ml). DNA isolated from the grain was adjusted to working concentrations of 2-50 ng/ml.

The content of *F. culmorum* DNA in the meal of the grain samples was evaluated by real-time quantitative PCR (qPCR) with SYBR Green [26] stain. The reaction was performed in a volume of 20 μl containing 4 μl of 5× qPCRmix-HS SYBR master mix (Evrogen, Russia), 300 nM of each primer (Evrogen, Russia) and 2 μl of DNA solution. Tri-*Fusarium, Alternaria* and *F. poae* DNA concentrations were determined by the qPCR method with TaqMan fluorescent probes [27-29] in a reaction volume of 20 μl containing 10 μl of 2× TaqAB master mix (AlkorBio, Russia), 300 nM of each primer, 100 nM of fluorescent probe (Eurogen, Russia) and 2 μl of DNA solution, applying the recommended protocols with the authors' modifications. Amplification was performed on a CFX96 Real-Time System thermal cycler (Bio-Rad, USA), with primary data processing using Bio-Rad CFX Manager 1.6 software. The concentration of fungal DNA was expressed as a fraction of the total DNA isolated from the oat meal (ng/ng of total DNA).

DON in the milled grain was determined by competitive enzyme immunoassay (ELISA). Mycotoxin was extracted from 1 g of meal by adding 5 ml of acetonitrile:water (84:16) and allowed for 14-16 hours with continuous stirring (300 rpm) on a S-3M shaker (ELMI, Latvia). ELISA was performed using a test system with a sensitivity limit of 20 μg/kg (VNIIVSGE, Russia). Optical density was measured on a LEDETECT 96 (Biomed, Austria) photometer at λ = 492 nm.

All laboratory tests were performed in at least two replicates. The results were processed using statistical packages of Microsoft Excel 2010 (Microsoft Cor-

poration, USA), Statistica 10.0 (StatSoft, Inc., USA) and 4.PAST [30] software. The table shows the mean (X) with a standard error (±SE); the figure presents the mean (X) with standard deviation (±SD). The relationships between the studied indicators were evaluated by the correlation analysis using the Pearson criterion. The calculated coefficients were considered statistically significant at $p < 0.05$.

Results. We analyzed genotypes of oats, represented by hulled varieties and perspective lines. In these genotypes, the 1000 grain weight waried within the 29.8-46.7 g range, the proportion of flower glumes in the total biomass was 22.8-28.6 % (Table 1).

1. The main parameters of oats (*Avena* L.) varieties and breeding lines from the VIR world collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) used in the study (experimental station, Pushkin, Leningrad Region, 2015)

VIR catalogue No.	Variety, line	Origin	Hull content, % (X±SE)	1000 grain weight, g (X±SE)
k-14648	Argamak	Russia, Kirov Region	24.5±3.2	36.9±5.6
k-11840	Borrus	Germany	28.3±3.7	34.9±5.3
k-15068	Konkur	Russia, Ulyanovsk Region	25.7±3.4	42.5±6.9
k-10841	Binuandorodu	Russia, Sakhalin Region	25.6±3.4	34.4±5.0
k-14329	Kouzan Zairai	Japan	24.5±3.2	42.0±6.4
k-13911	Kambulinskii	Russia, Leningrad Region	25.0±3.3	43.0±6.6
k-14911	Belinda	Sweden	25.5±3.3	42.0±6.4
k-15297	Geszty	Hungary	22.8±2.7	29.8±4.5
k-15301	CDC Dancer	Canada	23.7±3.1	41.7±6.3
k-15506	Fux	Germany	24.6±3.2	39.4±6.0
k-15442	Zalp	Russia, Moscow Region	25.8±3.0	41.5±6.3
k-15496	Stipler	Russia, Ulyanovsk Region	26.4±3.5	44.5±6.8
k-15444	Sapsan	Russia, Kirov Region	24.3±3.2	43.8±6.6
k-15494	Medved	Russia, Kirov Region	27.2±3.8	46.7±7.1
k-15348	Hurdal	Norway	24.5±3.2	42.8±6.5
k-15353	Odal	Norway	23.3±3.1	40.4±6.1
k-15611	Bessin	Norway	25.3±3.3	44.4±6.2
k-15612	Veler	Norway	24.2±3.2	41.2±6.3
k-15347	Gere	Norway	24.0±3.1	37.8±5.7
k-15326	KSI 432/08	Russia, Ulyanovsk Region	28.6±3.7	42.9±6.5
k-15327	KSI 731/01	Russia, Ulyanovsk Region	25.0±3.3	43.1±6.5

2. Primers and probes used and qPCR conditions in the study

Object (reference)	Primers, probes	Nucleotide sequence 5′→3′	Amplification protocol
F. culmorum [27]	FculC561f	CACCGTCATTGGTATGTTGTCACT	2 min at 50 °C, 10 s at 95 °C; 40 cycles: 15 s at 95 °C, 60 s at 62 °C
	FculC614r	CGGGAGCGTCTGATAGTCG	
F. poae [28]	TMpoae,f	GCTGAGGGTAAGCCGTCCTT	15 s at 95 °C; 40 cycles: 15 s at 95 °C, 1 min at 60 °C
	TMpoae,r	TCTGTCCCCCCTACCAAGCT	
	TMpoae,p	TET-ATTTCCCCAACTTCGACTCTCCGAGGA-BHQ1	
Tri-*Fusarium* [29]	TMTRI,f	CAGCAGMTRCTCAAGGTAGACCC	3 min at 95 °C; 40 cycles 15 s at 95 °C, 60 s at 60 °C
	TMTRI,r	AACTGTAYACRACCATGCCAAC	
	TMTri,p	Cy5-AGCTTGGTGTTGGGATCTGTCCTTACCG-BHQ2	
Alternaria [30]	DirITSAlt	TGTCTTTTGCGTACTTCTTGTTTCCT	3 min at 95 °C; 40 cycles: 10 s at 95 °C, 60 s at 60 °C, 3 s at 72 °C
	InvITSAlt	CGACTTGTGCTGCGCTC	
	AltTM	FAM-AACACCAAGCAAAGCTTGAGGGTACAAAT-TAMRA	

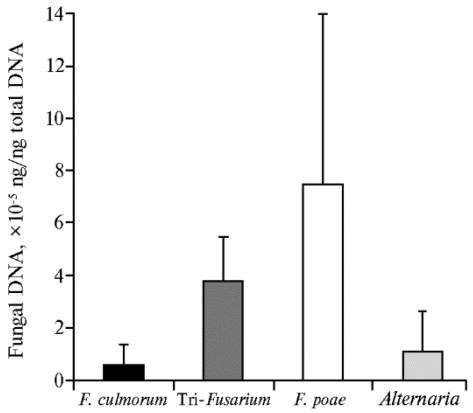
N o t e. Tri-*Fusarium* – all kinds of *Fusarium* fungi, capable of producing trichothecene mycotoxins.

The amplification protocols are summarized in Table 2.

Along with *F. culmorum* in the grain of oats, other *Fusarium* fungi were identified, including those capable to synthesize mycotoxins of the trichothecene group. Therefore, we additionally estimated the DNA amount of *F. poae* which occurs in the oat grain mycobiota with a high frequency [5, 31, 32]. Also, using the group-specific primers, the amount of DNA of all *Fusarium* species capable of producing trichothecene mycotoxins (Tri-*Fusarium*), as well as DNA of *Alternaria* genus fungi were additionally determined.

In Borrus (non-inoculated control), fungal mass per total DNA in grain estimated by qPCR was $(1.45\pm0.24)\times10^{-6}$ ng/ng for *F. culmorum*, $(1.73\pm0.02)\times10^{-5}$

ng/ng for Tri-*Fusarium* group, $(2.23\pm0.33)\times10^{-5}$ ng/ng for *F. poae*, and $(6.24\pm0.31)\times10^{-7}$ ng/ng for *Alternaria*. Under *F. culmorum* inoculation, the abundance of these species varied significantly. Grain level of *F. culmorum* DNA ranged from 7.39×10^{-8} to 3.07×10^{-5} ng/ng total DNA and averaged $(5.3\pm0.42)\times10^{-6}$ ng/ng (Fig.). The total Tri-*Fusarium* DNA ranged from 1.5×10^{-5} to 9.1×10^{-5} and averaged $(5.3\pm0.28)\times10^{-5}$ ng/ng, *F. poae* DNA rang was 1.05×10^{-5}-3.06×10^{-4} with average value of $(7.45\pm0.83)\times10^{-5}$ ng/ng total DNA. For *Alternaria* DNA, these indicators were 3.9×10^{-7} to 5.4×10^{-5} and $(1.07\pm0.6)\times10^{-5}$ ng/ng. Apparently, the *Alternaria* species are well adapted and occupies its niche among mycobiota, regardless of the influence of environmental conditions and the genotype of the host plant. The samples Gere, Veler, KSI 731/01, KSI 432/08 were the most infected by *F. culmorum* and *Alternaria* fungi.



Fungal DNA in grain under oat (*Avena* L.) plant inoculation with a suspension of MFG 58219, 58562, 58578, 58585 *Fusarium culmorum* strains (qPCR, $X\pm$SD, experimental station, Pushkin, Leningrad Region, 2015).

The qPCR method makes it possible to evaluate the DNA content of the target in grain, but has its own limitations. Thus, the primers for quantification of Tri-*Fusarium* and *F. culmorum* species are designed based on the nucleotide sequence of genes that are present in the genome in single copies, *Tri-5* and *EF1*, respectively [26, 27]. In contrast, primers for the quantitative detection of *F. poae* fungus and *Alternaria* species are developed based on multicopy sequences of IGS and ITS regions of ribosomal DNA [28, 33]. The primary differences between the initial quantities of the amplified fragments allow comparing infection by one or another target pathogen among the genotypes themselves, but do not allow comparing the DNA level for different objects with each other in the same sample.

ELISA showed that 100 % of the analyzed oat genotypes after artificial inoculation with *F. culmorum* contained DON (from 25 to 1179 μg/kg, 198±57 μg/kg on average). The greatest quantities of mycotoxin were noted in the grain of the KSI 432/08 breeding line, as well as in the Belinda and Konkur varieties. The Stipler variety, most resistant to infection of all fungi, had 33 μg/kg DON. DON was not found in the grain of non-inoculated control (Borrus variety).

3. Interrelation of indicators (*r*) in oat (*Avena* L.) genotypes under inoculation with *Fusarium culmorum* (experimental station, Pushkin, Leningrad Region, 2015)

Indicator / Indicator	DNA content in grain				DON accumulation
	F. culmorum	*F. poae*	Tri-*Fusarium*	*Alternaria*	
DNA content in grain:					
F. poae	–0.34				
Tri-*Fusarium*	0.67**	–0.25			
Alternaria	0.66**	–0.27	0.86**		
DON accumulation	0.57*	0.19	0.21	0.18	
Hull fraction	0.37	0.08	–0.06	0.01	0.49*
1000 grain weight	0.34	–0.25	0.45*	0.39	0.16

N o t e. DON — deoxynivalenol.
*, ** Correlation coefficients are statistically significant at $p < 0.05$ and $p < 0.001$.

Correlation analysis revealed a high reliable relationship between DNA levels of *F. culmorum* and Tri-*Fusarium* group ($r = +0.67$, $p < 0.001$), which demonstrates the success of the performed inoculation (Table 3). For *F. cul-*

morum, the DON producer, there was also a strong relationship between the DNA amount and DON accumulation ($r = +0.57$, $p < 0.01$).

The 1000 grain weight value significantly correlated with the anount of Tri-*Fusarium* DNA ($r = +0.45$, $p < 0.05$). Genotypes with larger grain were relatively more strongly infected with fungi, which may be due to a greater content of nutrients allowing the fungus to gain biomass. At the same time, an increase in hull fraction led to a grain significant contamination with mycotoxin DON. The flower glume prevents penetration fungi into grain rich in nutrient substrates, and contributes (probably due to structure and chemical composition) to the intensive production of secondary metabolites by *Fusarium* fungi.

A reliable positive relationship was between the DNA content of the *Alternaria* and *F. culmorum* fungi ($r = +0.66$, $p < 0.01$) and between the *Alternaria* DNA level and the entire diversity of *Fusarium* species forming trichothecene mycotoxins ($r = +0.86$, $p < 0.001$). Apparently, during the colonization of the common substrate by aggressive species of *Fusarium* and relatively weak *Alternaria* pathogens, mutually beneficial conditions for the development of both arise, so that their relationships can be characterized as symbiotic. Even a significant DON level in grain did not adversely affect growth of *Alternaria* fungi. At the same time, we did not observe any relationship between *Alternaria* fungi and *F. poae* pathogen, a relatively weak and the most abundant member of *Fusarium* genus on the grain. Consequently, no competition arises between these fungi for the nutrient substrate during oat grain colonization and their relationship can be characterized as commensalism.

It is known that some mycotoxins produced by fungi can play an important role in the relationship between fungi and host plants [2, 10, 14]. In our study, we did not find the allelopathic effect of *F. culmorum* mycotoxins on *Alternaria* fungi, although some researchers noted various types of interaction between representatives of these genus. In particular, the slowing of *A. alternata* growth under the influence of *F. graminearum* has been described in laboratory experiments with co-cultivation [34]. In such a mixed culture, DON production by *F. graminearum* was lower, and the production of zearalenone (ZEN) was higher than in the pure culture of this pathogen. Another group of researchers reports that the rate of colonization of wheat grains by *A. tenuissima* fungus and the amount of secondary metabolites generated, for example AOL, increased significantly if the grain was pre-treated with DON or XEN mycotoxins [35]. Grain treatment with AOL had a negligible effect on *F. culmorum*, but led to an increase in *F. graminearum* growth. At the same time, the presence of *Fusarium* fungi on ears did not affect the amount of AOL.

Thus, we have identified a symbiotic relationship between *Alternaria* and *Fusarium* fungi, colonizing oat grain. Statistical analysis confirmed that there is a significant positive relationship between the abundance of these fungi in grain, which is especially important when combining *Alternaria* fungi and the highly aggressive *F. culmorum* species. During oat grain colonization, there is no competition for the nutrient substrate between *F. poae*, which is considered a relatively weak pathogen, and *Alternaria* fungi, hence their relationship can be characterized as commensalism. The interaction of fungi and plants is evolutionary conditioned, and the knowledge of its mechanisms in complex ecosystems is necessary for their control.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 995-1003
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Symbiogenetics

UDC 631.461.52:575.1:577.257.065

doi: 10.15389/agrobiology.2017.5.995rus
doi: 10.15389/agrobiology.2017.5.995eng

COMPARATIVE PHYLOGENETIC ANALYSIS OF SYMBIOTIC GENES OF DIFFERENT NODULE BACTERIA GROUPS USING THE METATREES METHOD

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The authors declare no conflict of interests
Acknowledgements:
The experiments were carried out using equipment of the ARRIAM Center of Genomic Technologies, Proteomics and Cell Biology.
Supported by Russian Science Foundation, project № 14-26-00094П
Received December 12, 2016

Abstract

We applied modified phylogenetic analysis method of building meta-trees to study the evolutional patterns of various groups of symbiotic genes (*nod*-genes control the formation of nodules and *nif/fix*-genes control symbiotic nitrogen fixation). The method consists in the pairwise comparison of topologies and construction combined dendrograms («meta-trees»), where the relative position of the two trees is a measure of corresponding gene phylogenies congruence. Homologues of 18 symbiotic genes (*nodABCDIJN*, *nifABDEHKN*, *fixABC*, *fdxB*) are present in each test organism (9 strains belong to the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* and *Neorhizobium*). These genes were selected for the implementation of this method, as well as the gene 16S rRNA as a traditional taxonomic chromosome marker. We constructed and compared phylogenetic trees for all these genes and then calculated the pairwise similarity coefficients for their topologies. According to the obtained data we built a meta-tree, and there were two statistically distinct gene clusters identified within. Cluster 1 includes mainly *nif*- and *fix*-genes and cluster 2 — mostly *nod*-genes, that is related to the data of separate localization of these gene groups in the rhizobial genomes. The exception is the arrangement of *nifB* and *fixC* genes with *nodA* in cluster 2, as well as co-localization of *nodI* and *nifD* in cluster 1. During the identified clusters structure analysis we found strong relationship between the relative gene position and the characteristics of their genome localization in nodule bacteria. Importantly, the differences between clusters 1 and 2 are not expressed less clearly than the differences between *nod*- and *nif/fix*-gene groups. It is obvious that clusters 1 and 2 of our meta-tree reflect primarily different mechanisms of nodulation evolution and symbiotic nitrogen fixation associated with the independent origin of the relevant gene groups, and possibly, with their separate horizontal transfer between different groups of rhizobia. Further study of the symbiotic gene evolution in nodule bacteria requires improvements used phylogenetic analysis techniques, including separate analysis meta-tree for rhizobia, representing different stages of symbiosis evolution.

Keywords: phylogenetic analysis, meta-trees, nodule bacteria, symbiotic genes

Soil bacteria of the *Rhizobiales* order are characterized by the ability to enter into symbiosis with leguminous plants, providing them with products for atmospheric nitrogen fixation, which is accompanied by the formation of nodules on the roots, which provide conditions for effective nitrogen fixation and assimilation of ammonium formed as a result of nitrogenase reaction. However, this group of organisms is of interest not only for microbiology, but also for evolutionary genetics of symbiosis. The analyzed model makes it possible to study the mechanisms of evolution of bacterial genes, which control interactions with different groups of eukaryotes [1].

The formation of nodules and symbiotic nitrogen fixation are complex multi-stage processes that are controlled by the genes of both partners. The symbiotic genes of rhizobia are divided into two main groups: virulence genes (*nod* genes), responsible for signal dialogue with the host plant, as a result of which nodules [2] are formed on its roots, and the genes necessary for symbiotic fixation of atmospheric nitrogen — the *nif* genes (control the synthesis and regulation of nitrogenase activity) and *fix* genes (a heterogeneous group of genes involved in the work of the nitrogenase complex, primarily in its supply with electrons and energy) [3].

In most nodule bacteria, *nod* and *nif/fix* genes are assembled into groups located in the genome as separate clusters, the structure of which varies in different species. For example, in nodule bacteria of alfalfa (*Sinorhizobium meliloti* and *S. medicae*), pea (*Rhizobium leguminosarum* bv. *viciae*), clover (*R. leguminosarum* bv. *trifolii*), goat's-rue (*Neorhizobium galegae*) and soybean (*Bradyrhizobium japonicum*), the main *nod* genes are closely linked, while the *nodABC* genes are organized into one operon (Fig. 1). In many rhizobia, the structural genes of *nifHDK* nitrogenase are one operon and are closely linked to the *nifEN* genes. Such organization of nitrogen fixation genes is characteristic for representatives of *Sinorhizobium*, *R. leguminosarum*, *N. galegae* and *Mesorhizobium loti.* However, in *B. japonicum*, an additional copy of the *nifH* gene is located outside the *nifHDK* operon, and the *nifA* gene is located outside the *nif/fix* cluster together with the *nod* genes.

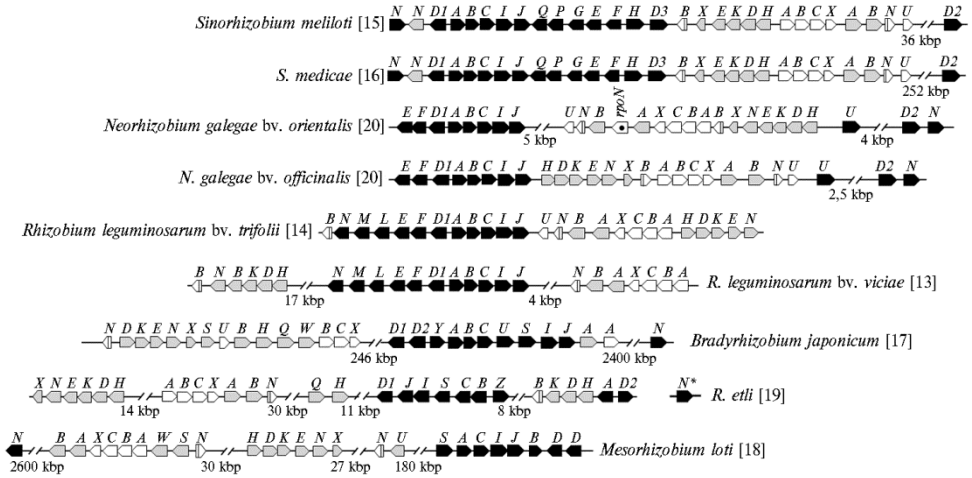

Fig. 1. 1. Location of symbiotic genes in various species of nodule bacteria: — *nod* genes, — *nif* genes, — *fix* genes, — *fdx* genes, — non-symbiotic genes; the *nodN* gene in the genome of *R. etli* (marked with an asterisk) has a chromosomal localization. The schemes are based on the data of genome sequencing of the studied rhizobial strains [4-11].

In evolutionarily advanced species of nodule bacteria (for example, *R. leguminosarum*), symbiotic genes are located on high-molecular plasmids [12]. At the same time, their chromosomal localization is characteristic for the ancestral form — *B. japonicum,* and also for *M. loti* [13-14]. There are rhizobia, in which only a few symbiotic genes can be located on the chromosome. This is typical, for example, for *R. etli:* most symbiotic genes are located on the plasmid, and the *nodN* gene is located on the chromosome [15] (see Fig. 1).

For a long time, the molecular mechanisms of the evolution of the legumes-rhizobia symbiosis, numbering 60-70 million years [16], remained unclear. Only as a result of the methods of high-performance sequencing, when the rapid accumulation of data on the composition and structure of the genomes of vari-

ous bacterial species began, the pangenome and metagenomic analysis managed to reconstruct the long path, which micro- and macrosymbionts passed from recruitment into bacterial cells of the nitrogen fixation genes (*nif* genes) to the development of modern highly specialized legumes-rhizobia symbiotic systems. This process included several stages, in which the groups of genes under consideration were obtained from different sources: *nif* genes and most *fix* genes were obtained by ancestral slow-growing rhizobia *(Bradyrhizobium)* from their free-living ancestors (*Rhodopseudomonas*) through vertical inheritance, while the genes for synthesizing lipo-chitooligosaccharide Nod factors responsible for the formation of nodules (*nod* genes) were obtained from fungi or from gram-positive bacteria during horizontal transfer [17]. The later rapidly growing rhizobia (*Rhizobium, Sinorhizobium, Neorhizobium*) obtained *nod* and *nif/fix* genes from ancestral rhizobia during horizontal gene transfer, which led to the formation of special plasmids (*pSym*) containing both groups of *sym* genes.

The connection of the phylogeny of *sym* genes with the mechanisms of their origin and evolution, as well as with localization in the rhizobia genomes, has not been adequately studied. For its study, we used a new method of phylogenetic analysis with the construction of "meta-trees" — combined dendrograms, in which the relative position of two trees serves as a measure of the congruence of phylogeny of individual genes. Previously, this method was developed and used to analyze alternative phylogeny obtained in the study of functionally different genes in various groups of eukaryotes (yeasts, fish), in which horizontal gene transfer is limited [18].

In our study, this method was applied to the phylogenetic analysis of two functionally different groups of genes (*nod* and *nif/fix*), which are of unequal origin and distributed among taxonomically unrelated groups of rhizobia through both vertical inheritance and horizontal gene transfer. The identified features of the phylogeny topologies constructed for the nodule formation genes and symbiotic nitrogen fixation make it possible to significantly supplement the earlier concept of the directions and mechanisms of the evolution of the gene symbiotic systems in nodule bacteria.

The purpose of the work was to use meta-trees to analyze the contribution of horizontal transfer to the process of evolutionary assembly of the symbiotic gene cluster in rhizobia.

Techniques. Nucleotide sequences of symbiotic genes from the full genomic sequences of 9 representatives of nodule bacteria were used for the analysis: *R. leguminosarum* bv. *viciae* 3841 (GenBank GI:115259115) [4], *R. leguminosarum* bv. *trifolii* WSM2304 (GI:209537694) [5], *S. meliloti* 1021 (GI:25168258) [6], *S. medicae* WSM419 (GI:150031715) [7], *B. japonicum* USDA110 (GI:47118316) [8], *M. loti* MAFF303099 (GI:47118328) [9], *R. etli* CFN42 (GI:89213252) [10], as well as *N. galegae* bv. *officinalis* HA-MBI1141 (GI:659665307) and *N. galegae* bv. *orientalis* HAMBI540 (GI:659657635) [11].

Cluster analysis was performed in the computer program MEGA 5.1 (http://www.megasoftware.net/). The alignment of nucleotide sequences was performed using the ClustalW algorithm; the mathematical model when composing the tree for each gene under study was p-distance, the clustering mechanism — Neighbor-Joining. The numerical expression of similarities of phylogenetic tree topologies was calculated using statistical approaches (implemented using the program presented at http://www.mas.ncl.ac.uk/~ntmwn/compare2trees/index.html) [19]. To construct the meta-tree and analyze the reliability of the clustering, the dissimilarity factors were used, which were calculated as the difference of unit (100%, minuend) and the corresponding similarity factor (subtrahend).

Results. At the initial stage of the work, phylogenetic trees were con-

structed for each of the following 18 genes: virulence genes *nodA*, *nodB*, *nodC* (responsible for the synthesis of the core part of the Nod factor) [20-22], *nodD* (in the presence of multiple copies of *nodD1*, it is a constitutively expressed gene of the flavonoid-sensitive transcriptional activator of *nod* genes) [23], *nodI*, *nodJ* (genes of membrane transporters of the Nod factor) [24], *nodN* (encodes the dehydratase enzyme) [25]; genes of symbiotic nitrogen fixation *fixA*, *fixB*, *fixC* (components of the electron transport chain of nitrogenase) [26], *nifA* (gene of the transcriptional regulator of *nif* genes), *nifB*, *nifN*, *nifE* (responsible for the synthesis of the Fe-Mo cofactor of nitrogenase), *nifD*, *nifH*, *nifK* (structural components of nitrogenase) [27], *fdxN* (ferredoxin). The choice was due to the fact that homologs of these genes are present in all representatives of nodule bacteria selected for analysis. In addition, a traditional chromosomal taxonomic marker, the 16S rRNA gene, was included in the study due to the conservation of its nucleotide sequence and low frequency of horizontal transfer.

The methodology for comparing topologies is based on the idea that horizontal transfer of genes from one species of rhizobia to another during evolution introduces significant differences in the topology of the phylogeny of these genes. In the case when the topology of the trees of two genes does not differ, it is assumed that they either do not participate in the horizontal transfer at all, or are transferred together (Fig. 2, A).

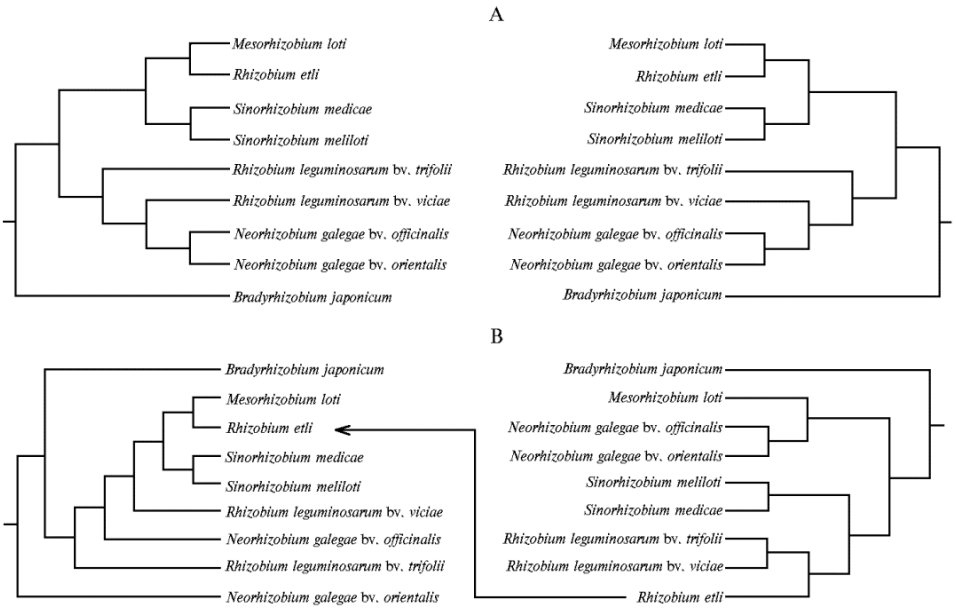

Fig. 2. Comparison of the topology of phylogenetic trees for the *nifH* (left) **and *nifN*** (right) **genes with a similarity of 100 %** (A) **and for the genes *fdxN*** (left) **and 16S rRNA** (right) **with a similarity of 50.5 %** (B)**.** The arrow shows the most noticeable difference in topology: the transfer of *Rhizobium etli* from the *R. leguminosarum* cluster on the 16S rRNA gene tree to the *M. loti* cluster on the *fdxN* tree.

At the same time, the discrepancy in the tree topology based on the analysis of the sequences of the same set of strains is a result of the horizontal transfer of symbiotic genes (see Fig. 2, B). According to these differences in the topology, we can assume how the horizontal gene transfer occurred during evolution. For example, it can be seen that in the tree for the 16S-rRNA gene in one cluster there are two biovars *R. leguminosarum* and *R. etli*. Since the 16S rRNA gene is a chromosomal marker, it can be said that clustered together representatives are evolutionarily similar. At the same time, on the tree of the plas-

mid marker *fdxN*, rhizobia *R. etli* grouped in the same cluster with *M. loti*, although *R. etli* and *M. Loti* belong to different families – *Rhizobiaceae* and *Phyllobacteriaceae*. This fact allows assuming that there was a horizontal transfer of the *fdxN* gene between the nodule bacteria *M. loti* and *R. etli*, or these species obtained this gene from a single source during the horizontal transfer.

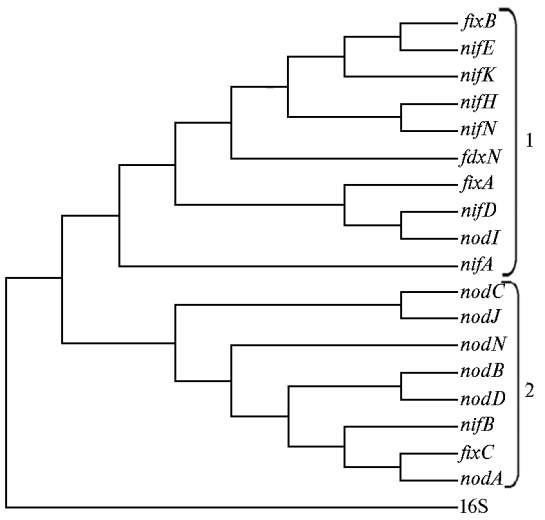

Fig. 3. The main clusters of the meta-tree according to the matrix of similarities of topologies in phylogenetic trees constructed on the basis of the data on the nucleotide polymorphism of symbiotic genes: 1 — *nif/fix* cluster, 2 — *nod* cluster.

Then we compared all the 19 phylogenetic trees obtained in order to identify differences in their topologies. The bioinformatic analysis of the obtained data made it possible to calculate the coefficients of pairwise similarity of topologies for all trees, ranging from 1 (complete congruence of two phylogenies) to 0 (complete absence of congruence). Then the constructed matrix of similarities was converted into a matrix of differences by subtracting the corresponding similarity factors from unit. According to this matrix, a meta-tree was constructed (Fig. 3).

It can be seen that on the meta-tree two clusters were reliably identified: the group of *nif/fix* genes was predominantly concentrated in cluster 1, the group of *nod* genes — in cluster 2, while the chromosomal marker (16S rRNA gene) did not enter any of the clusters (Table).

Evaluation of the reliability of the meta-tree clustering in accordance with the average distance inside and between clusters/groups of genes using Student's *t*-test

Comparison group	TD	SD	SE	Reliability of differences according to the Student's *t*-test at the significance level p
Inside the cluster 1	0.197	0.094	0.013	< 0.001
Inside the cluster 2	0.216	0.104	0.020	< 0.01
Between the clusters	0.269	0.107	0.012	
Inside the *nif-fix* group	0.194	0.084	0.011	< 0.001
Inside the *nod* group	0.201	0.082	0.018	< 0.01
Between the groups of symbiotic genes	0.270	0.099	0.011	

N o t e. TD — average difference of topologies, SD — standard deviation, SE — standard error. Comparison of the average distance between tree topologies within one cluster/group, as well as between the trees of one cluster/group and each tree of another cluster/group was performed. The structure of the clusters is shown in Fig. 3.

Such clustering may indicate that in the evolutionary history of the group of genes, *nif/fix* genes are not related to each other and to chromosomal genes. It is determined by their independent origin and separate horizontal transfer. Indeed, the difference between the groups of *nod* and *nif/fix* genes is expressed not less clearly than the dissimilarity between clusters 1 and 2. However, exceptions were found: the *nodI* gene, which is located in the *nif/fix* cluster 1 and is transferred along with the *nifD* gene, as well as the *nifB* and *fixC* genes that enter the *nod* cluster 2 together with *nodA*.

As noted above, in the genomes of many nodule bacteria, symbiotic genes are grouped into clusters. In this regard, the joint horizontal transfer of groups of *nod* and *nif/fix* genes may be due to the characteristics of their cluster arrangement in the genome (see Fig. 1). However, it is not possible to explain

the obtained picture only by the specificity of the arrangement of genes on genetic maps. So, in cluster 1, all the structural genes of nitrogenase were identified, with the genes *nifH*, *nifK*, *nifE* and *nifN* being grouped together. Such clustering of genes corresponds to their location in the genomes, since in all studied organisms the *nifHDK* genes constitute one operon, to which the genes *nifE* and *nifN* are adjacent. The only exception is the localization of the *nifN* gene outside the *nif/fix* zone in nodule bacteria of alfalfa (*Sinorhizobium meliloti*). Together with the same *nod* genes, *nodN* was detected in cluster 2, but it was located separately inside the cluster. It should be noted that the localization of *nodN* in the genome may be different. In some representatives, it is located in the same cluster with the *nodABC* genes *(S. meliloti, S. medicae, R. leguminosarum)*; in others, it is located separately from the common *nod* genes. For example, in *R. ga-legae*, the *nodN* is located in another *nod* cluster, and in *B. japonicum, M. loti* and *R. etli* — outside the cluster of symbiotic genes. In addition, in *R. galegae*, the *nodN* gene has a chromosomal localization.

An interesting result of the analysis was the entry of the *nifB* and *fixC* genes into cluster 2 and their grouping together with the *nodA* gene. It is now known that the genes responsible for the synthesis of the core Nod factor (*nodABC*) are of a non-rhizobia origin. Thus, Hirch *et al.* [28] analyzed the sequences of bacterial and fungal genes encoding enzymes with catalytic centers similar to those of the *nodABC* gene products. It was suggested that the *nodB* and *nodC* genes could be obtained by rhizobia during the horizontal transfer from gram positive bacteria in which they participate in the biogenesis of the murine cell wall. In addition, these genes could be obtained by nodule bacteria from fungi actively synthesizing chitin-like substances. It is known that many fungi enter into close symbiotic relationships with bacteria, in case of which horizontal gene transfer is quite possible. However, for the *nodA* gene, homologous sequences in other organisms were not found. Thus, the *nodA* gene has a different origin than *nodB* and *nodC* [28]. In this regard, its clustering, together with the *nifB* and *fixC* genes, can indicate their initially simultaneous horizontal transfer from unidentified organisms.

It is important to note that the specifics of genes clustering on a meta-tree should be influenced not only by their location in modern forms of rhizobia, but also by evolutionary events that took place before, including independent rearrangements of genomes (see Fig. 1), what is more, both separate genes and genes containing parts of the genome could change their localization. The example of such rearrangements are the inversion of the *nif/fix* zone in *N. galegae* bv. *orientalis* [18] and the translocation of *nifHDKEN* genes in *R. leguminosarum* bv. *viciae* [11]. There is no doubt that genomic rearrangements have a significant influence on the order of clustering of genes, but the degree of their influence within the framework of the applied model is not clear enough. A significant role in the occurrence of differences in the topologies of the two genes is probably played by the unequal rate of their evolution.

The results obtained in our study do not agree with the data obtained by the methods of molecular phylogeny. Previously, it was shown that the polymorphism of *nod* genes was formed independently of the divergence of the core elements of the bacterial genome, but correlates with the taxonomy of hosts, whereas the polymorphism of the *nif* genes correlates with the divergence of the core part of the bacterial genome, but is not associated with the divergence of the host [29]. In our study, the meta-tree method showed the independence of the evolutionary history of both *nod* and *nif/fix* genes from the chromosomal marker (16S rRNA gene). Perhaps the method used by us is more sensitive to the influence of evolutionary factors that are not related to the horizontal gene

transfer and not taken into account in traditional phylogenetic approaches.

Thus, the results of phylogenetic analysis of *nod* and *nif/fix* genes, which we carried out using the method of constructing and analyzing meta-trees, confirm the previously identified differences in the evolutionary histories of these genes. The detection of two clusters, one of which consists primarily of *nif/fix* genes (cluster 1), and the other of *nod* genes (cluster 2), may indicate the fact that the compared groups of genes originated at different stages in the evolution of rhizobia (in connection with which they occupy different sites on chromosomes or plasmids of rhizobia), as well as the expression of these genes at different stages of symbiosis development (infection of root hairs and release into plant cytoplasm for *nod* genes, symbiotic fixation of nitrogen — for *nif/fix* genes). The presence of some *nod* genes in cluster 1 and some *nif/fix* genes in cluster 2 may be due to the fact that in the late stages of the evolution of rhizobia, the whole complex of *sym* genes was transferred as a whole in the composition of *Sym* plasmids or genomic islands. We cannot exclude the fact that the absence of correlation between the structures of clusters identified in the analysis of the meta-tree and the location of the studied genes on genetic maps is a consequence of intensive intragenomic rearrangements typical for many rhizobia and different rates of evolution of the primary structure in the studied genes.

Further analysis of the role of these evolutionary mechanisms requires a modification of the approach we used, in particular, of the separate analysis of the structures of the meta-trees constructed using samples of strains that occurred at early stages of rhizobia evolution (when horizontal gene transfer was limited due to the chromosomal localization of *sym* genes in *Bradyrhizobium*) or at later stages (when horizontal gene transfer was most intense and most likely captured the entire complex of *sym* genes located on the *Rhizobium* and *Sinorhizobium* plasmids). An important purpose of improving the method used is also to develop statistical and bioinformational criteria for supporting clusters that are detected on meta-trees, since in the first stage of the work we used only standard biometric approaches to solve this problem.

Thus, the phylogenetic analysis of *nod* and *nif/fix* genes, carried out using the construction and comparison of meta-trees, confirmed the previously identified differences in the evolutionary histories of these genes. The presence of two clusters in the meta-tree, one of which combines mainly *nif/fix* genes, and the other — *nod* genes, may be due to the fact that the stages of evolution of the rhizobia, at which the compared groups occurred, did not coincide, and also due to the expression of these genes at different stages of symbiosis development. The absence of correlation between the structure of clusters in the meta-tree and the results of genetic mapping may be due to intra-genomic rearrangements and the unequal rate of evolution of nucleotide sequences of genes. To improve the method used, it is necessary to develop statistical and bioinformational criteria for supporting clusters that are detected on meta-trees.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1004-1011
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 582.736:579.26:577.21

doi: 10.15389/agrobiology.2017.5.1004rus
doi: 10.15389/agrobiology.2017.5.1004eng

STUDY OF THE GENETIC DIVERSITY OF MICROSYMBIONTS ISOLATED FROM *Hedysarum gmelinii* subsp. *setigerum*, GROWING IN THE BAIKAL LAKE REGION

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The authors declare no conflict of interests
Acknowledgements:
We thank A.V. Verkhozina (SIPPB SB RAS) for organizing the expedition to the Baikal Lake region.
Supported by the Federal Agency of Scientific Organizations (Program for the development and inventory of bioresource collections, № ISGZ 0664-2016-0018). ITS-sequencing of the isolates was supported by the Russian Science Foundation (grant 16-16-00080).
Received March 11, 2017

Abstract

One of the urgent problems of modern microbiology and biotechnology is the study of the mechanisms of interaction between leguminous plants and root nodule bacteria (rhizobia), which are an extensive group of microorganisms capable to form nitrogen-fixing symbiosis with a host plant. Knowledge of these mechanisms is necessary for carrying out scientifically based selection of highly effective rhizobia-legume symbiotic systems. To understand the evolution of the specificity of plant-microbe interactions, symbiotic systems with the participation of relic leguminous plants, which are an intermediate link between the extinct and modern species, are of particular importance. One of these unique objects is the pleistocene relict *Hedysarum gmelinii* Ledeb. subsp. *setigerum* (Turcz. ex Fischer et Mey.) Kurbatsky. The aim of this study was to isolate and identify the world's first collection of microsymbionts of this plant species growing in the Lake Baikal region. The study of taxonomic positions of 19 isolates from root nodules of *H. gmelini* subsp. *setigerum* plants was conducted by the methods of ITS-RFLP and 16S rRNA gene (*rrs*) sequencing. Phylogenetic analysis revealed the considerable genetic diversity among microsymbionts of the plant species studied. Fourteen rhizobial isolates belonged to 3 genera: *Rhizobium* (family *Rhizobiaceae*), *Phyllobacterium* (family *Phyllobacteriaceae*) and *Bosea* (family *Bradyrhizobiaceae*). It was noted the presence in the root nodules of non-symbiotic rhizobial species that are not able to form symbiosis with leguminous plants (*Phyllobacterium endophyticum*, *Ph. loti* and *Bosea* sp.). In addition, five non-rhizobial isolates belonging to the genera *Acinetobacter, Stenotrophomonas, Sphingomonas* и *Agromyces* were obtained. The obtained data may indicate that the relic rhizobia-legume symbioses, formed in particular by the *H. gmelini* subsp. *setigerum* plants, are prototypes of modern symbiotic systems and reflect the evolutionary pathways in the direction of recruiting symbiotic genes of different microorganisms and increasing the specificity of plant-microbe interactions. It is possible that strains of non-symbiotic rhizobial species are present in nodules as a source of genes that do not participate directly in the formation of symbiosis, but affect its activity. Such strains, after appropriate genetic and phenotypic study, can be used for the production of biopreparations with increased efficacy.

Keywords: leguminous plants of the Baikal region, *Hedysarum gmelinii* subsp. *setigerum*, ribosomal RNA genes sequences, *Rhizobiaceae* taxonomy

Rhizobia, a large genetically diverse group of Gram-negative soil microorganisms, are capable of establishing intracellular symbiosis with leguminous plants and performing fixation of atmospheric nitrogen by forming symbiotic nodules on the roots of host plants, due to which these microorganisms are also known as nodule bacteria. Scientific selection of highly effective plant-microbial systems necessitates understanding the molecular mechanisms that ensure the interaction of leguminous plants with rhizobia, so these studies are among most

urgent in modern microbiology and biotechnology [1]. Symbiotic systems of relict leguminous plants, which are an intermediate link between extinct and existing species, are of particular importance for understanding evolution of specific plant-microbial interactions. These unique objects include the sweetvetch *Hedysarum gmelinii* Ledeb. subsp. *setigerum* (Turcz. ex Fischer et Mey.) Kurbatsky which grows in the Baikal region [2]. Botanical and geographical analysis showed the belonging of this species to Pleistocene petrophytic steppe relics of South Siberian origin [3, 4].

The evidence was reported that bacteria *Pantoea agglomerans, Enterobacter kobei*, *Enterobacter cloacae*, *Leclercia adecarboxylata*, *Escherichia vulneris* and *Pseudomonas* sp. (class *Gammaproteobacteria*) were isolated from the nodules of *Hedysarum spinosissimum* subsp. *capitatum* and some other species of this genus (*H. pallidum, H. carnosum*) growing in the Mediterranean region [5]. *H. coronarium* is nodulated by *Rhizobium sullae* strains [6]. According to Chinese researchers [7], *Rhizobium* strains were isolated from the nodules of *H. scoparium* and *H. polybotrys* which grow in the Chinese northwest. In 2011, it was shown that the plants of the alpine sweet vetch *H. alpinum* are nodulated by representatives of *Mesorhizobium* genus [8].

The microsymbionts of the *H. gmelinii* Ledeb. subsp. *setigerum* have never been isolated before. In the present work, we have created and described the world's first collection of rhizobial microsymbionts of this relict leguminous plant growing in the Baikal region.

The purpose of our study was to isolate the *Hedysarum gmelinii* subsp. *setigerum* microsymbionts and determine the taxonomic position of the strains using the ITS-RFLP method and 16S rDNA sequencing.

Techniques. The objects of the study were 19 strains isolated using the traditional method [9] from the root nodules of *H. gmelinii* subsp. *setigerum* growing on the Zunduk Cape (mainland coast of the Baikal strait Maloye More, coordinates 53.383333, 107.41666753°23′00″ N. 107°25′00″ E). Microorganisms were grown on modified mannitol yeast agar YMSA with addition of 0.5 % succinic acid [10] at 28 °C. All isolates are deposited in the Departmental Collection of Useful Agricultural Microorganisms and placed at the Station for Low-Temperature Automated Storage of Biological Samples (Liconic Instruments, Liechtenstein) [11]. The information on the strains is available in the Internet database of the Departmental Collection of Useful Agricultural Microorganisms [12].

In the initial assessment of the intra-species diversity of strains, RFLP (restriction fragment length polymorphism) analysis of sequences between the genes 16S and 23S rRNA (ITS-RFLP method) was performed. For this, the amplified DNA fragment was cleaved with MspI restriction endonuclease (Promega, USA) and the restricted DNA fragments were separated electrophoretically in the standard mode [13]. For species identification of the strains, the nucleotide sequence of the 16S rRNA (*rrs*) gene was used.

For amplification of 800 bp ITS region, the primers FGPS1490-72 (5′-TGCGGCTGGATCCCCTCCTT-3′) and FGPL-132 (5′-CCGGGTTTCCCC-ATTCGG-3′) were used, for amplification of 16S rDNA of about 1500 bp, the primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAG-GTGATCCAGCC-3′) were used. The resultant PCR product was isolated from the gel and purified [14] for RFLP analysis or sequencing on a genetic analyzer ABI PRISM 3500xl (Applied Biosystems, USA). Detection of homologous sequences was performed using the NCBI GenBank database and the BLAST program [15]. A neighbor-joining tree was constructed by using the program MEGA5 [16] were used. Differences in the number of nucleotides which differed between the obtained sequences were revealed by a pairwise com-

parison. To assess the levels of clusters support, a bootstrap analysis was performed based on 1000 replicates. The resulting sequences were deposited in the GenBank database (accession numbers KY290459-KY290467, KY290469, KY290470 and KY290472-KY290474).

Results. Isolates of nodule bacteria are presented in Tables 1 and 2.

1. 16S rRNA gene sequence homology (%) in fast-growing isolates from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* (Baikal region) and type strains of *Phyllobacterium* and *Rhizobium*

Type strain	Isolate										
	Rhizobium sp.	*Ph. loti*			*Ph. endophyticum*		*Phyllobacterium* sp.				
	Hse-26	Hse-9	Hse-19	Hse-30	Hse-10	Hse-24	Hse-13	Hse-14	Hse-17	Hse-20	Hse-29
1	95.7	98.3	98.3	98.2	97.4	97.1	99.9	96.9	98.6	98.3	98.7
2	94.5	99.6	99.6	99.5	97.9	97.7	99.6	97.4	98.9	99.7	98.9
3	94.9	99.1	99.1	99.0	97.1	96.8	99.9	96.6	98.1	99.2	98.1
4	94.6	98.6	98.6	98.6	96.6	97.1	99.2	96.1	97.7	98.6	97.7
5	94.3	98.6	98.1	98.6	99.9	99.6	99.4	99.3	98.8	98.6	98.9
6	95.1	98.5	98.6	98.4	98.5	98.2	99.6	98.0	99.3	98.6	99.3
7	95.1	99.8	99.8	99.8	98.5	98.3	99.6	98.0	99.0	99.8	99.1
8	98.9	94.4	94.4	94.3	93.5	93.3	95.0	93.1	94.8	94.4	94.8
9	96.4	93.5	93.5	93.5	92.5	92.3	93.4	92.2	93.8	93.6	93.8

N o t e. 1 — *Ph. myrsinacearum* STM 948T, 2 — *Ph. trifolii* PETP02T, 3 — *Ph. ifriqiyense* STM 370T, 4 — *Ph. catacumbae* CSC19T, 5 — *Ph. endophyticum* PEPV15T, 6 — *Ph. brassicacearum* STM 196T, 7 — *Ph. loti* S658T, 8 — *Rh. giardinii* NBRC 107135, 9 — *Rh. alamii* GBV016T.

2. 16S rRNA gene sequence homology (%) in slow-growing isolates from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* (Baikal region) and type *Bosea* strains

Type strain	Isolate *Bosea sp.*		
	Hse-21	Hse-22	Hse-32
B. vaviloviae Vaf-18T	98,5	98,0	98,5
B. massiliensis 63287T	98,4	97,5	98,4
B. eneae 34614T	98,6	97,7	98,6
B. vestrisii 34635T	98,6	97,7	98,6

All the studied isolates were divided into two groups based on the growth rate: three strains formed colonies on YMSA on days 4-5, and in the remaining strains, visible growth occurred on day 3. Since each isolate generated a unique ITS-RFLP pattern (data not shown), all the strains studied were identified by *rrs* gene sequencing.

Sequence analysis showed that 11 fast-growing strains belong to the genera *Phyllobacterium* and *Rhizobium* and form 3 statistically reliably different clusters with a support level of 100 % (Fig. 1). The first cluster included the strains Hse-14, Hse-24 and Hse-10, and also the type strain *Ph. endophyticum* PEPV15T. Hse-24 and Hse-10 strains which were identified as *Ph. endophyticum* showed high homology of *rrs* gene with that of the type strain PEPV15T (99.6 % and 99.9 %, respectively). The Hse-14 strain has been identified as *Phyllobacterium* sp. (Table 1). The second cluster combined the strains Hse-29, Hse-17, Hse-20, Hse-30, Hse-19, Hse-9, Hse-13 and the type strains *Ph. sophorae* CCBAU03422T, *Ph. bourgognense* STM201T, *Ph. brassicacearum* STM 196T, *Ph. loti* S658T, *Ph. trifolii* PETP02T, *Ph. catacumbae* CSC19T, *Ph. myrsinacearum* STM 948T and *Ph. ifriqiyense* STM 370T (Fig. 1). The Hse-20 strain was identified as *Phyllobacterium* sp., since being simultaneously the closest to two strains, *Ph. trifolii* PETP02T and *Ph. loti* S658T, by the *rrs* gene (the homology was 99.7 % and 99.8 %, respectively). Similarity of *rrs* gene in the strains Hse-9, Hse-19 and Hse-30 and the type strain *Ph. loti* S658T reached 99.8 %. Hence, these strains were assigned to *Ph. loti* (see Table 1). The closest to the isolates *Phyllobacterium* sp. Hse-17 and Hse-29 was the type strain *Ph. brassicacearum* STM 196T (99.3 % homology of *rrs* gene).

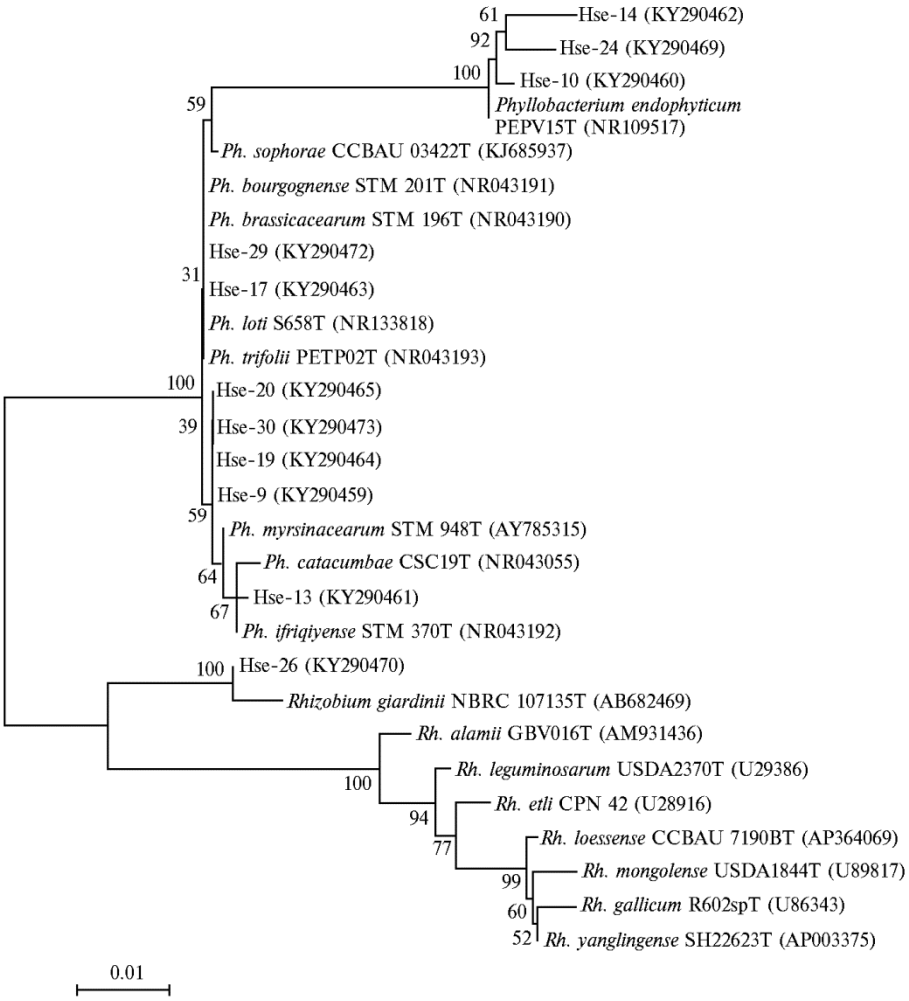

Fig. 1. *rrs*-Phylogram of the fast-growing strains isolated from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* in Baikal region and the representatives of related species *Phyllobacterium* and *Rhizobium*. The obtained isolates are designated as Hse, type strains are marked with the letter "T".

The isolate Hse-13 and two type strains, *Ph. myrsinacearum* STM 948T and *Ph. ifriqiyense* STM 370T, showed 99.9 % similarity; therefore, Hse-13 was identified only to the genus *Phyllobacterium* sp. (see Table 1). The third cluster was formed by the strain Hse-26 and the type strain *Rhizobium giardinii* NBRC 107135. Based on *rrs* gene sequencing (see Table 1), the Hse-26 isolate was identified as *Rhizobium* sp. with 98.9 % similarity to the type strain *Rhizobium giardinii* NBRC 107135.

Fig. 2 shows the *rrs*-dendrogram reflecting the taxonomic position of three slow-growing rhizobial isolates within the *Bradyrhizobiaceae* genera. The strains Hse-21 and Hse-32 showed 98.5 % *rrs* gene homology with the type strain *Bosea vaviloviae* Vaf-18T and 98.6 % homology with the type strains *B. eneae* 34614T and *B. vestrisii* 34635T (see Table 2). The similarity of *rrs* genes in the Hse-22 isolate and the closest type strain *vaviloviae* Vaf-18T was 98.0 % (see Table 2). On this basis, the strains Hse-21, Hse-22 and Hse-32 were identified as *Bosea* sp. It should be noted that *B. vaviloviae* was described quite recently, in 2015, when three microsymbiont strains were discovered in the relict legume plant *Vavilovia formosa* which grows in North Ossetia [10]. In addition, the microorganisms of the *Bosea* and *Phyllobacterium* genera were not isolated

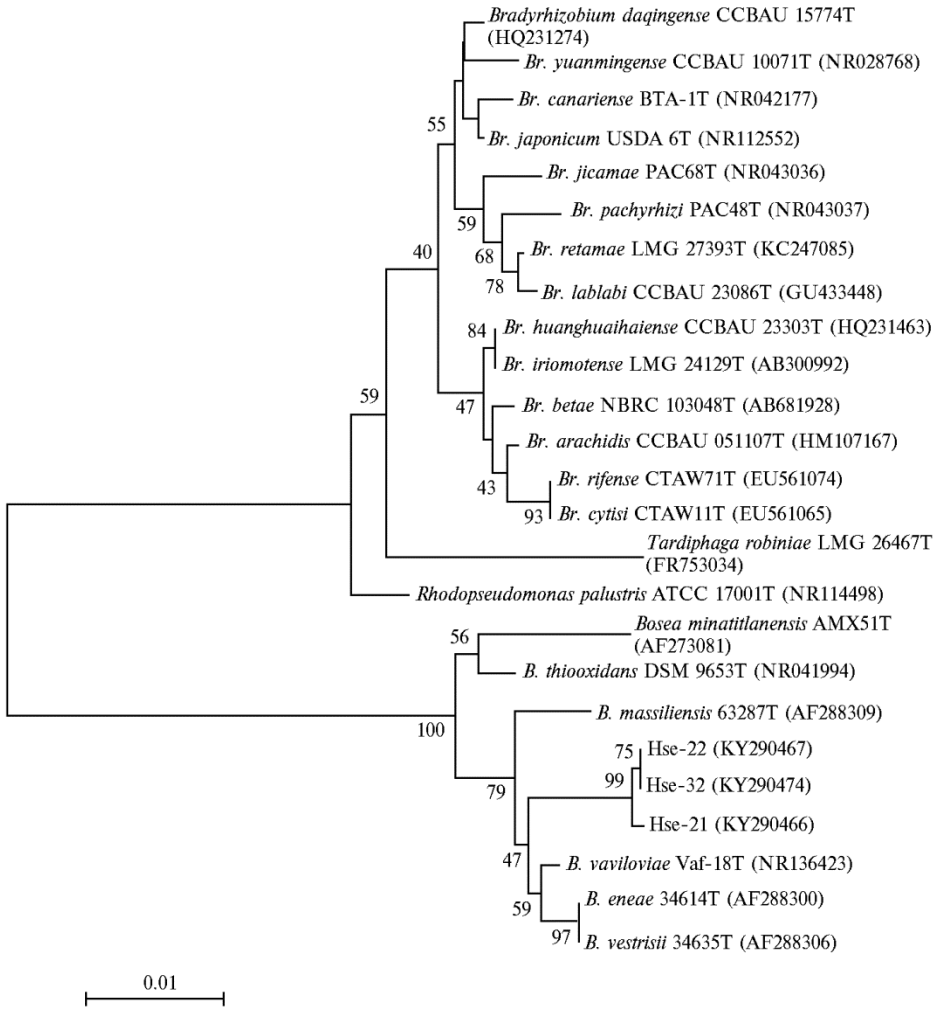

Fig. 2. *rrs*-Phylogram of the slow-growing strains isolated from nodules of sweetvetch *Hedysarum gmelinii* subsp. *setigerum* in Baikal region and the representatives of related species *Bosea*. The obtained isolates are designated as Hse, type strains are marked with the letter "T".

from the nodules of other sweetvetch species that grow in the Mediterranean, northwestern regions of China and central Russia [5-8].

The ability of any members of *Bosea* genera to form a nitrogen-fixing symbiosis has not been confirmed before, although strains of four species, *B. lupini*, *B. lathyri, B. robiniae* and *B. vaviloviae*, were isolated from the nodules of *Lupinus*, *Lathyrus, Robinia* and *Vavilovia* leguminous plants, respectively [10, 17]. However, at least two *Phyllobacterium* species, *Ph. sophorae* and *Ph. trifolii*, were described as effective microsymbionts of *Sophora flavescens* [18], *Trifolium repens* and *Lupinus albus* [19]. Therefore Baikal isolates of *Bosea* genera, which show high similarity to *B. vaviloviae* species, and *Phyllobacterium* bacteria are of great interest for further study.

Based on *rrs* gene sequences, five non-rhizobial isolates from sweetvetch *Hedysarum gmelinii* subsp. *setigerum* nodules were attributed to genera *Acinetobacter*, *Stenotrophomonas*, *Sphingomonas* and *Agromyces* (data not shown). Bacteria of *Stenotrophomonas* genera were isolated from other leguminous plants of the Baikal region [20]. According to data reported, the members of these genera can inhabit the nodules of legumes and also the rhizosphere and phyllosphere of various plant species [21-25].

Thus, in the present paper, for the first time, we have obtained a collection of strains isolated from the relict leguminous plant, the sweetvetch *Hedysarum gmelinii* Ledeb. subsp. *setigerum*, which grows in the Baikal region. Our study showed that among the microsymbionts of this plant there are strains of symbiotic species of nodule bacteria (*Rhizobium* sp.), as well as atypical species, which representatives do not form symbiosis (*Phyllobacterium endophyticum, Ph. loti* and *Bosea* sp.). Strains of non-symbiotic rhizobial species may be present in nodules as carriers of genes that do not directly participate in the formation of symbiosis, but can affect its effectiveness. Further phenotypic and genetic study of isolated microorganisms can make a significant contribution to understanding the ways of evolution and development of plant-microbial interactions in the legume-rhizobial system.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1012-1020
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.358:631.461.5:57.052:577.112

doi: 10.15389/agrobiology.2017.5.1012rus
doi: 10.15389/agrobiology.2017.5.1012eng

FEATURES OF PROTEIN ISOLATION FOR PEA *Pisum sativum* L. ROOT PROTEOME ANALYSIS DURING SYMBIOSIS WITH RHIZOBIA

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The authors declare no conflict of interests
Acknowledgements:
Supported by Russian Science Foundation (grant № 16-16-10043)
Received November 30, 2016

Abstract

Pea *Pisum sativum* L. is a convenient model to study the molecular-genetic mechanisms of nitrogen-fixing symbiosis establishment with rhizobia, because a representative collection of mutants, blocked at different stages of symbiosis development was obtained. A comparative analysis of the proteomes of the wild type cultivars and lines of peas and mutants can be a useful approach for carrying out studies aimed on at identification and further analysis of regulators controlling the formation of nitrogen-fixing nodules. However as the review of modern literary data shows, studies of differential proteome changes in pea roots during symbiosis are almost not performed. Sample preparation is a key stage in proteomic studies. The quality of gels obtained after 2-D electrophoresis and the opportunity of following analysis depend on protein isolation efficiency from the tissues and purification from accompanying substances. Our work is aimed on finding the most effective method of protein isolation from *Pisum sativum* roots inoculated with rhizobia, which might be applied for carrying the 2-D electrophoresis. Special requirements aimed at separation stages minimization important for protein stability, as well as the efficient removal of contaminants which can negatively affect the quality of separation and the subsequent evaluation of qualitative and quantitative changes in the protein synthesis are necessary for proteomics. Analysis of data revealed a number of possible methods for the protein isolation from plant tissues. A comparison of three methods of the proteins isolation using the commercial protocol from Bio-Rad; the method based on treatment with phenol and ammonium acetate as well as the trichloroacetic acid application. Pea plants of cv. Frisson were used in our work, the strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 was used for inoculation. After protein isolation from the wild-type cv. Frisson roots of pea seedlings inoculated with rhizobia (1 day after inoculation) using three methods and consequent 2-D electrophoresis, it was shown that the best results are achieved using the method with phenol following by ammonium acetate precipitation. The gels were analyzed for trace presence that made it difficult to search for different proteins, the efficiency of total protein isolation and possible degradation products. Using this selected method, the differential 2-D electrophoresis of extracted proteins was carried out with fluorescent Cy2 and Cy5 labels based on isoelectric focusing of proteins using strips with a pH range of 3-10 and subsequent separation in a polyacrylamide (PAGE) gel. The analysis showed that when proteins were isolated using phenol and ammonium acetate, it was possible to obtain rather representative proteomes of the roots of pea seedlings. The differential 2-D electrophoresis allowed to see the differences between the control samples (non-inoculated roots) and the samples inoculated with rhizobia (inoculated roots). This method may be recommended for further proteomic studies in pea roots.

Keywords: *Pisum sativum* L., pea, legume rhizobium symbiosis, proteomics analysis, receptors, Nod factors, legumes, rhizobia

In the formation of a symbiosis between leguminous plants and nodule bacteria (rhizobia), the plant undergoes significant changes in metabolic processes, hormonal status, and structural rearrangements associated with the reorganization of the cytoskeleton [1]. These changes are aimed at the formation of new organs on the roots — symbiotic nodules, in which nitrogen fixation is carried out. In recent years, with the spread of complex analysis methods that allow

studying changes in the transcription activity of plant genes under the influence of various factors (transcriptomics), it has become possible to identify regulators involved in controlling the development of symbiosis. However, such studies are conducted mainly on model species of *Medicago truncatula Gaertn.* and *Lotus japonicus L.*, the genomes of which are deciphered due to relatively small sizes (~ 470-500 MB) [2, 3]. It is shown that during the development of symbiosis in the roots of leguminous plants, the transcription activity of several thousand genes changes [4-6]. However, the functional significance of such changes in gene expression remains quite far from understanding.

Peas (*Pisum sativum* L.) has a very large genome (about 4300 MB), which has not yet been deciphered. Studies to identify genes that are differentially expressed in response to inoculation are a fairly complex task. In addition to the above, unique collections of mutants for *sym* genes, which control the development of symbiosis on the part of the plant, determine the interest in using this object. Proteomics serves as a complex approach for studying changes in the composition of proteins under the influence of biotic and abiotic factors. It allows to carry out comparative studies on peas and its mutants and to identify previously unknown regulators necessary for the formation of symbiosis. However, proteomic studies of pea proteins, which change in response to the treatment with rhizobia, were almost never carried out. The peribacteroid space of symbiosome in the development of symbiosis of peas with nitrogen-fixing bacteria was studied [7]. Pea proteomes at antagonistic interactions with crenate broomrape (*Orobanche crenata*) and mycosphaerella (*Mycosphaerella pinode*) [8, 9], at the development of vegetative organs and seeds [10-12], and the formation of resistance to temperature stress were analyzed [13]. The influence of salicylic and jasmonic acids on the protein composition of roots and leaves of peas was studied [14-16].

Sample preparation is a key step in proteomic studies using 2-D electrophoresis [17]. It is especially difficult when working with plants, the tissues of which are rich in proteases and substances that interfere with proteome analysis, i.e. polysaccharides, lipids and phenolic compounds [18]. Such compounds interfere with the separation of proteins and their analysis due to the occurrence of horizontal and vertical bands on the gels, blurs, reduced number of bright spots. In addition, plant tissues contain less protein than animal tissues and microorganisms, so effective protein extraction is important for obtaining successful results of 2-D electrophoresis [17, 18]. Proteomics allows qualitative and quantitative comparison of proteins in different samples, so the protein losses during analysis are crucial. When extracting proteins from the sample, it is necessary to preserve their quality and quantity, for which the chosen method should contain as few steps as possible in order to minimize losses [19, 20].

In this paper, we for the first time compared the effectiveness of three methods for isolating the total protein from the roots of the field pea. The method for isolating proteins using phenol and ammonium acetate proved to be most preferable for differential 2-D electrophoresis.

The purpose of the study was to optimize method for isolating the total protein pool from the roots of the field pea inoculated with rhizobia for obtaining better results during 2-D electrophoresis.

Techniques. Pea seeds (*Pisum sativum* L., variety Frisson) were sterilized for 5 min in sulfuric acid, washed 3 times with distilled water and germinated on 1 % aqueous agar in the dark for 4 days. Seedlings were transferred to the pots with sterile vermiculite impregnated with Jensen liquid medium [21]. The inoculation was carried out usinf strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 (OD_{600} ≈ 0.5), 1 ml per seedling. Noninoculated seedlings were used for the control. The plants were grown in the phytotron Sanyo MLR-351H (Japan) at 21 °C, 16-hour

light day, 60 % humidity. For the analysis, fragments of the major roots corresponding to the zone of susceptibility to the rhizobia infection were selected 24 hours after inoculation with rhizobia. Samples were frozen in liquid nitrogen and stored at –80 °C. For isolating proteins, the roots (100 mg for each procedure) were crushed in liquid nitrogen in cooled mortars.

Protein extraction according to the standard procedure of Bio-Rad Laboratories (USA) included homogenization of the material in the buffer for isoelectric focusing (IEF). The samples was transferred to eppendorfs and a buffer for the IEF was added, containing 25 mM Tris-HCl (pH 8.0), 9 M urea, 4 % 3-(3-cholamido-1-propyl-dimethylammonio)-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.2 % ampholytes (Bio-Rad Laboratories, USA). The mixture was incubated for 20 min on ice, and then centrifuged for 15 min at 12,000 g, +4 °C. The supernatant containing proteins (150 μl) was applied to the rehydration strips.

To isolate proteins with phenol and ammonium acetate, the extraction buffer cooled up to +4 °C was added, containing 0.1 M Tris-HCl (pH 8.0), 30 % sucrose, 10 mM of DTT, 2 % sodium dodecyl sulfate (SDS), and a cocktail of protease inhibitors (Sigma, USA). The mixture was centrifuged (Hettich 320R, Germany) for 15 min at 12,000 g and +4 °C. The supernatant was mixed in a ratio of 1:1 with phenol (pH 8.0) (Invitrogen, USA), shaken for 30 seconds (Vortex Genius, Germany) and centrifuged at 10,000 g for 5 min at +4 °C. The upper phase was transferred to a new eppendorf; the proteins were precipitated with 5 volumes of cooled 100 mM ammonium acetate in methanol for 30 min at –20 °C, and then centrifuged for 5 min at 10,000 g. The precipitate was washed twice with 100 mM ammonium acetate in methanol and twice with 80 % acetone. The protein precipitates were air dried and dissolved in isofocusing buffer containing 25 mM Tris-HCl (pH 8.0), 9 M urea, 4 % CHAPS, 50 mM DTT, and 0.2 % ampholytes (Bio-Rad Laboratories, USA).

For isolation of total proteins with trichloroacetic acid (TCA), its 10 % solution and 0.07 % β-mercaptoethanol, prepared with acetone, were added to the crushed roots. The resultant mixture was ultrasonically treated 3 times for 20 seconds at 10 μm amplitude (Soniprep 150 Plus, MSE, UK). The suspension was incubated for 1 hour at –20 °C, with stirring every 15 min in a shaker, and then centrifuged for 20 min at 9,000 g and +4 °C. The resulting precipitate was washed twice with 0.07 % β-mercaptoethanol in acetone, then dried in a vacuum evaporator (Concentrator Plus, Eppendorf, USA), dissolved in a buffer containing 25 mM Tris-HCl (pH 8.0), 9 M urea, 4 % CHAPS, 50 mM DTT, and 0.2 % ampholytes (Bio-Rad Laboratories, USA), incubated for 20 min on ice and centrifuged for 10 min at 12,000 g and +4 °C. The resulting supernatant containing proteins (150 μl) was applied to the rehydration strips.

Isoelectric focusing (IEF) was carried out using 7-cm-long strips with a pH gradient of 3-10 and a Protean IEF Cell (Bio-Rad Laboratories, USA). Preliminarily, rehydration of strips and the loading of the obtained protein samples (150 μl per strip) were performed for 12-14 hours. The IEF was carried out at a temperature of +20 °C, the samples were desalted at 250 V for 15 min, then the voltage was linearly increased up to 4,000 V for 2 hours, then the IEF was carried out (up to 10,000 V/h with a current limit of 35 mA per gel). After the IEF procedure, the strips were frozen and stored at –80 °C or immediately separated in the second direction.

Before electrophoresis of the proteins in the polyacrylamide gel (PAAG), the strips were incubated for 10 min in a buffer containing 0.375 M Tris-HCl (pH 8.8), 6 M urea, 2 % SDS, 2 % DTT, 20 % glycerol, then alkylated for 10 min in buffer with 0.375 M Tris-HCl (pH 8.8), 6 M urea, 2 % SDS, 20 %

glycerol, 2.5 % iodoacetamide. Then the strips were laid on a concentrating gel, and melted 0.5 % agarose (25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS) with bromophenol blue (BFS) were layered down on top to control the separation. Electrophoresis was performed in 15 % PAAG using 4 % gel (35 mA per gel until the BFS was completely released from the gel) and Tris-glycine buffer (25 mM of Tris-HCl, pH 8.3, 192 mM glycine, 0.1 % SDS). After the electrophoresis completed, the gels were washed with deionized water and stained in SimpleBlue™ (Invitrogen, USA) according to the manufacturer's protocol. The gels washed from the dye were photographed on G:BOX-CHEMI-XX9 (Syngene, Great Britain).

To perform differential 2-D electrophoresis (D-D fluorescence difference gel electrophoresis, DIGE), the total protein obtained after isolation with phenol and ammonium acetate was dissolved in 200 μl of IEF buffer without DTT and ampholytes. The protein samples were then conjugated with the fluorophores Cy2 and (or) Cy5 (Lumiprobe, Russia) in different combinations by incubation of protein extract (50 mg) with 400 pM of dye dissolved in dimethylformamide for 30 min on ice in the dark. The labeling reaction was stopped with 10 mM of L-lysine (Sigma-Aldrich, USA) for 10 min on ice. Control and test samples labeled with different dyes were mixed and, after adding DTT and ampholytes, used for rehydration of strips. After separation of the proteins, the gels were visualized with a laser scanner Typhoon FLA 9500 (GE Healthcare, Germany). To analyze photos of gels, the program ImageJ (https://imagej.nih.gov/ij/) was used.

Results. In the last decade, proteomics has established itself as a method that makes it possible to rather effectively estimate the changes occurring in a plant under the influence of various biotic, abiotic and anthropogenic factors. The success of the method depends on the preparation of samples and obtaining the original extract of proteins [22]. This is especially important for differential proteomics, which is related to the study of the differences between the control samples and the samples obtained after the treatments. The choice of the optimal method for extraction of the total protein pool is a key factor for obtaining reliable experimental results [23]. The biochemical properties of proteins, such as solubility, overall charge, as well as localization features (e.g., membrane proteins) and a low amount of protein in the origin material, can negatively affect the study of full protein spectrum. In addition, plant cells contain significant amounts of non-protein substances — polysaccharides, lipids and organic acids [24], and the cell wall consists of a large amount of fiber and pectin. These substances have a significant effect on the quality of protein extracts and, consequently, on the results of 2D-electrophoresis [25, 26]. The optimal method of sample preparation is necessary in order to effectively remove non-protein substances from the sample.

We tested three methods for the total protein extraction from pea roots after 24 hours of incubation with rhizobia. In all cases, the destruction of tissue was carried out in liquid nitrogen, which made it possible to efficiently homogenize the material and destroy the cell wall. After additional washing (when phenol and ammonium acetate or TCA were used), all samples were dissolved in a buffer containing urea, detergent CHAPS, reducing agent DTT and a cocktail of protease inhibitors. These substances allow avoiding degradation, modification, loss and precipitation of proteins [27].

The preparation of the extract of pea proteins using the standard method was the least suitable for analysis. 2D-electrophoresis of samples detected a small amount of isolated protein in the control and processed samples (Fig. 1, A, B). The gel also showed traces of contamination, i.e. bands and background noise, which prevented the detection of differences in the spectrum of the separated proteins.

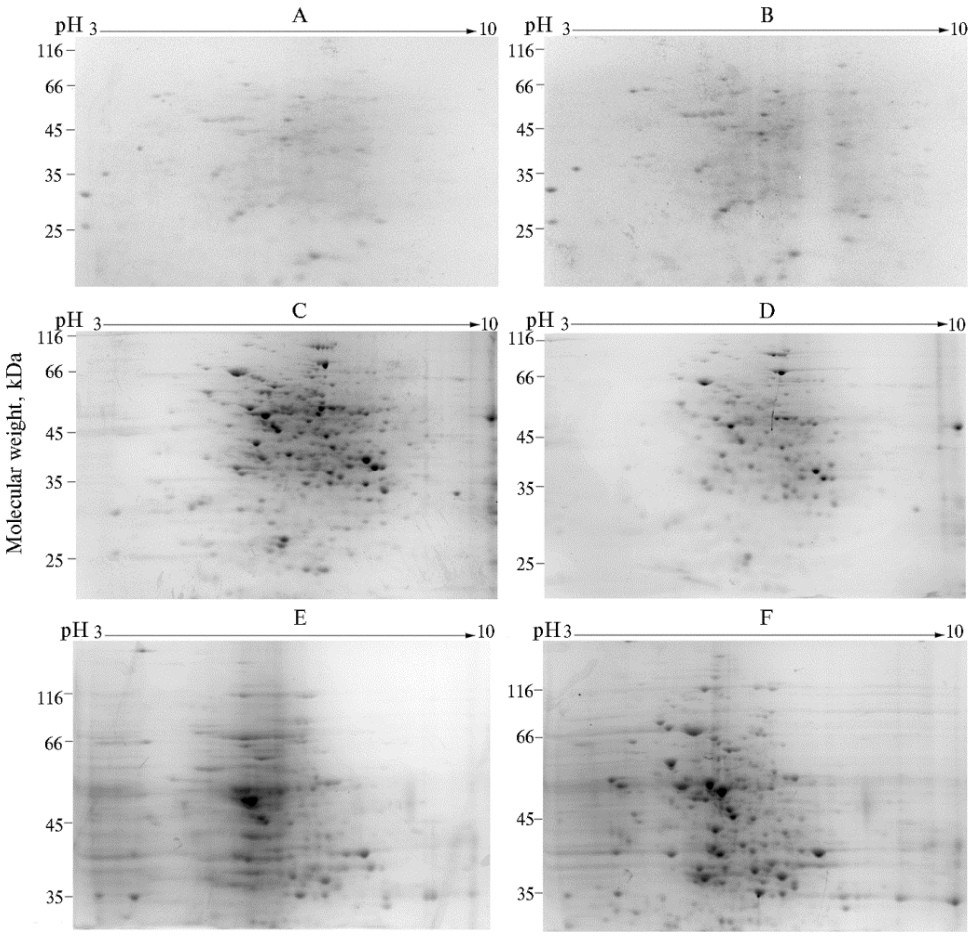

Fig. 1. 2-D electrophoregrams of proteins isolated from roots of pea (*Pisum sativum* L.) Frisson variety according to the standard procedure of Bio-Rad Laboratories (USA) (A, B), **using phenol and ammonium acetate** (C, D)**, using trichloroacetic acid** (E, F)**:** on the left — control samples, on the right — samples from roots inoculated with rhizobia (1 day after inoculation). Strips with pH 3-10 were used. Separation in the second direction was carried out in 12 % PAAG.

Isolation of proteins with TCA proved to be more effective. We were able to extract greater amount of protein compared to the standard method, due to a more saturated spectrum of spots in PAAG. However, 2-D electrophoresis of samples obtained using TCA (see Fig. 1 E, F) showed the impossibility of removing background noise (horizontal bands), the occurrence of which is associated with the residues of nucleic acids, polysaccharides and phenolic compounds [28, 29]. Polysaccharides were also the cause of protein aggregation in the sample. Such protein complexes can block the PAAG pores and make it impossible for the peptides to pass through the gel and to focus in the desired zone [30]. According to reported findings, the disadvantages of this method include small number of extractable proteins, which was noted earlier in experiments on obtaining a protein extract from cells of sugar beet *Beta vulgaris* L., cactus *Mammillaria gracilis* Pfeiff. and Jupiter's-beards *Sempervivum tectorum* L. Such a disadvantage can be eliminated using a larger amount of the origin material, but this, in turn, will increase the amount of polluting agents.

A method using phenol and ammonium acetate was first proposed by W.J. Hurkman and C.K. Tanaka for proteomic analysis [19]. Phenolic extraction was used to isolate proteins from potatoes, rape, apples, banana and olive leaves,

tomatoes, alfalfa, avocado and bananas [17, 18, 24, 31-33]. When studying plant-microbial interactions, it was used to analyze the *Lotus japonicus* proteome in the late stages of symbiosis with *Mesorhizobium loti* [34, 35]. The method is more time-consuming than others, but in our case it turned out to be most effective for obtaining high-quality 2-D electrophoregrams. There were no clearly expressed horizontal bands on the gel that are typical of polysaccharide contamination (see Fig. 1, C, D). The high quality of the gel was achieved due to the large amount of isolated protein, which makes possible a more reliable comparative analysis of the control sample and the sample after treatment. The advantages of the method include efficient extraction of membrane proteins from the sample [19].

Based on the results obtained, this method was selected for isolation of proteins when performing differential 2-D electrophoresis (DIGE). The obtained protein samples were incubated with fluorescent dyes; IEF and separation in PAGE were performed (Fig. 2).

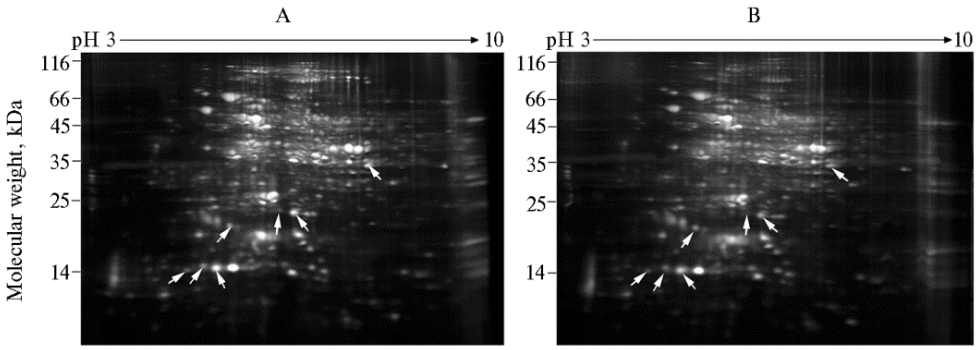

Fig. 2. 2-D differential electrophoregrams of proteins isolated from roots of pea (*Pisum sat*ivum L.) Frisson variety using phenol and ammonium acetate: A — control sample (Cy2, blue fluorescent label) and a sample after treatment with rhizobia (Cy5, yellow fluorescent label); B — control sample (Cy5, yellow fluorescent label) and sample after treatment with rhizobia (Cy2, blue fluorescent label). Strips with pH 3-10 were used. Separation in the second direction was carried out in 12 % PAAG. White spots indicate the absence, color indicate the presence of a difference in the level of protein synthesis. Arrows indicate proteins, the synthesis of which is enhanced after treatment.

Thus, extraction of the total protein pool of pea roots inoculated with rhizobia using phenol and ammonium acetate proved to be the most effective and qualitative in preparing samples for carrying out both standard electrophoresis in PAAG followed by SimpleBlue™ staining and for differential 2-D electrophoresis. This method made it possible to isolate a sufficient amount of protein and to eliminate impurities that interfere with qualitative electrophoresis, to perform effective labeling and to obtain a picture of high resolution, which is necessary for determining the difference in the spectrum of synthesized proteins. In the future, the method will be used to study the differences in the spectrum of synthesized proteins at successive stages of development of symbiosis between the field pea and *Rhizobium leguminosarum* bv. *viciae* CIAM1026.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1021-1029
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Biofortification

UDC 635.649:581.19

doi: 10.15389/agrobiology.2017.5.1021rus
doi: 10.15389/agrobiology.2017.5.1021eng

ANTIOXIDANT CONTENTS OF PEPPER *Capsicum* spp. FOR USE IN BIOFORTIFICATION

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The authors declare no conflict of interests
Acknowlegdgements:
Supported in part by Russian Science Foundation (grant № 16-16-10022)
Received July 17, 2017

A b s t r a c t

Natural potential of bioactive compounds accumulated by plants is often not taken into account in particular breeding. Biofortification, the enrichment of basic food crops with essential vitamins and minerals using breeding, is one of the most notable recent innovations in agriculture. In this paper, we studied pigment composition with regard to the amount of carotenoids and their contribution to fruit coloration in 20 accessions of different species of genus *Capsicum*. Red and yellow pigment levels and their ratios detected in the study were indicative of the carotenoid composition in fruits of each species. Also the varieties differed significantly in the carotenoid accumulation. In temperate climate of Moscow region the highest amount of pigments was found in the sweet variety Shokoladnyi (*C. annuum*; 0.536 mg/g), and in the spice varieties Purpurnyi tigr (*C. annuum*; 0.708 mg/g), Kitaiskii fonarik (*C. baccatum*; 0,685 mg/g), Ideya (*C. annuum*; 0.629 mg/g) and Chudo Podmoskov'ya (*C. annuum*; 0.628mg/g). The highest level of ascorbic acid was accumulated by chili pepper Ideya (*C. annuum*; 414 mg%), Rozhdestvenskii buket (*C. annuum* × *C. frutescens*; 370 mg%), Yubileinyi VNIISSOK (*C. annuum*; 326 mg%), and Ognennaya deva (*C. chinense*; 301 mg%). The ascorbic acid content did not depend on fruit color and plant species. Among the sweet pepper varieties high total antioxidant content (TAC) was characteristic of hybrid F_1 Oranzhevoe naslazhdenie (*C.* annuum). The maximum total antioxidant amounts, as milligram equivalents (MME) of gallic acid per g, were 2.82 for Rozhdestvenskii buket (*C. annuum* × *C. frutescens*), 2.65 for Ognennaya deva (*C. chinense*), 2.57 for Idea (*C. annuum*), and 2.19 for Kitaiskii fonarik (*C. annuum*). In assessment of thermostable antioxidants extracted with 80 % ethanol at 60 °C it was shown that the unstable antioxidants, mainly ascorbic acid, averaged 16 % of the total antioxidants. Rozhdestvensciy buket, Kitaiskii fonarik, Purpurnyi tigr, Ognennaya deva plants, additionally to antioxidants, can accumulate one of the strongest natural antioxidant, the capsaicin, which determines their hot taste. The capsaicin content of the studied chili peppers varied from 1.36 to 9.57 mg/g of dry weight. High contents of carotenoids, ascorbic acid and TAC combined with capsaicin at 8 to 9 score points increase the total antioxidant capacity of these samples.

Keywords: *Capsicum annuum*, *Capsicum baccatum*, *Capsicum pubescens*, *Capsicum chinense*, *Capsicum frutescens*, pigments, carotenoids, antioxidants, ascorbic acid, biofortification

The global burden of disability is estimated by DALY's (disability adjusted life years), which in medicine means the number of years lost, adjusted for the duration of disability. In 2000, 136 million years of healthy life was lost in Europe, including over 56 million years due to nutritional factors. Analysis using DALY's shows that in Europe the cause of almost 60% of diseases is high blood pressure, smoking, excessive alcohol consumption, high cholesterol, overweight, insufficient intake of fruits and vegetables and a sedentary lifestyle [1-4]. Four of

these risk factors are closely related to nutrition. According to some researchers, the state of health of modern man is largely determined by the nature, completeness and structure of the diet [5], which must be balanced by the complex of nutrients necessary for the body to level out the increasing effect of stressors.

High grade food of plant origin, including vegetables, is deemed effective way to reducing the risk of many diseases associated with metabolic disorders. Biofortification, which is the enrichment of foods with essential vitamins and minerals through selection, has become one of the most notable innovations in plant breeding. Until now, there is practically no production of vegetables for functional purposes in Russia, and breeders often do not pay due attention to the natural potential of plant accumulation of bioactive compounds during breeding for qualitative traits.

Pepper, like many other vegetable crops, is the source of the most important biological components such as macro- and microelements, antioxidants and vitamin C. Peppers are rich in carotenoids, various acids, sugars, polyphenols, especially flavonoids, quercetin and luteolin. Pepper can be included into the dietary products category [6-9]. Capsaicinoids, another biologically active substance specific for the *Capsicum* genus, in small concentrations acts as a gastroprotective agent, and also has a local analgesic effect [10, 11]. Among the fat-soluble compounds present in peppers, carotenoids occupy a special place with their important role in the prevention of gastric ulcers, age-related macular degeneration and cataract, and stimulate the immune system [8]. All these compounds are included in the antioxidant pool. Consumption of pepper fruits reduces the risk of inflammation [12], cancer [13-15] and chronic non-infectious diseases, including cardiovascular, diabetes [16-18] and obesity [13, 14].

Coloring is one of the most important indicative parameters of quality for pepper fruits and products based on them. Four genes (*y*, c_1, c_2, *cl*) with epistatic interaction control and about 20 carotenoids (yellow-red pigments) form the color of mature *Capsicum* genus fruits, but the way of carotenoid synthesis inheritance as well as genetics of color intensity is not fully understood. Capsanthin, capsorubin and cryptoxanthin are found only in the *Capsicum* genus and serve as effective free radical stabilizers. Red color of pepper fruits is determined by capsanthin, capsanthin-5,6-epoxide and capsorubin, yellow-orange color is due to zeaxanthin, β-carotene, β-cryptoxanthin, violaxanthin, antheraxanthin and cucurbitaxanthin A [19]. Capsanthin in mature red fruits amounts to more than 60 % of the total carotenoids.

Genotype, environmental conditions (light, temperature, mineral nutrition, atmospheric composition) and agrotechnologies (e.g., ripeness at harvesting, irrigation system) affect the antioxidant status of the fruit [20]. There are conflicting data on the effect of heat treatment of vegetables in cooking in the literature 22], while other works evidence for its increase or preservation [23, 24].

In this paper, the comparative studies of the antioxidant status of fruits in varieties and species of both sweet and hot peppers were first associated with *Capsicum* spp. biofortification in a temperate climate conditions. It is established that the color of fruits and its intensity in some genotypes is determined by the sum of carotenoids, while in others is due to the presence of only one group of pigments. No direct relationship is observed between the total content of carotenoids, capsaicinoids and the antioxidant status of pepper fruits.

The purpose of our work is to reveal the peculiarities of the antioxidants accumulation in the organs of different pepper varieties and types in case of insufficient heat supply, to evaluate the contribution of the thermostable antioxidants to their total quantity and to identify forms promising for breeding.

Techniques. Four samples of sweet *Capsicum annuum* pepper and 16

samples of hot peppers of different ecogeographical origin, belonging to *C. annuum, C. baccatum, C. pubescens, C. chinense* and *C. frutescens* species, were studied in unheated film greenhouses in the temperate climate zone (Moscow Region, 2015-2016). Plants were grown from May to October (the agrotechnology corresponded to the one that is generally accepted for film greenhouses). The experiments were arranged in four replications; the plots (5 m^2 each) were randomized; a sample size per estimation was 20 plants. In order to minimize the influence of weather conditions, layering and other factors, the comparative evaluation was performed in one year, on the same plants, on the fruits of the same tier, at the same time.

In biochemical analyzes, plant material was milled in a homogenizer with the extractant. The total antioxidant capacity (TAC) was determined amperometric method [25], adapted for the determination of hydrophilic and lipophilic fractions. The result was expressed via gallic acid equivalents (mg-equivalent GA/g). Ethyl alcohol of 80 % [26, 27] or a mixture of acetone and ethyl alcohol (1:1 v/v) was used [29] when extracting. In case of using ethyl alcohol, the analysis was carried out in two ways. In the first one, the plant material milled in the solution was placed in test tubes with lids and heated at 60 °C in a water bath for 60 min [26]. In the second one, the heating stage was passed [27], as well as for the extraction with a mixture of acetone and ethyl alcohol. The homogenate was then centrifuged for 15 min at 10,000 g and 4 °C. An aliquot of the supernatant was used to determine antioxidant concentrations, diluting, if necessary. The measurements were carried out on a Tsvet-Yauza-01-AA device (NPO Khimavtomatika, Russia) in a constant current mode.

The pigment content was determined spectrophotometrically. The samples were homogenized in acetone and left for 1 hour at 5 °C in the dark. The homogenate was centrifuged and the absorbance of the supernatant was measured. The approach applied had been proposed by Hornero-Mendez *et al.* [19]. It is based on the properties of chromophores of carotenoids, which allow grouping them into two isochromatic families – yellow and red pigments. The content of each isochromatic fraction (mg/ml) was calculated by the formulas:

$$\text{Red pigments} = \frac{2{,}144A_{508} - 0{,}4033A_{472}}{270{,}9},$$

$$\text{Yellow pigments} = \frac{1{,}7243A_{472} - 2{,}4501A_{508}}{270{,}9}.$$

Concentration of deoxidized ascorbic acid was determined iodometrically by titration of the extracts with potassium iodate in the presence of potassium iodide and starch in an acidic solution [29]. The amount of dry matter was evaluated after drying in an oven to a constant absolutely dry mass for 2 days at a temperature of 85 °C.

Statistical data processing was carried out with Origin Pro 9.0 software (http://www.originlab.com/Origin). Mean (X) and standard errors of mean (±SE) were calculated.

Results. It is known that all representatives of the *Capsicum* genus accumulate the largest amount of capsanthin, capsorubin and cryptoxanthin in their fruits [19]. The analysis of the content of red and yellow pigments in the fruit testified to the originality of the quantitative content of carotenoids in each species. There were significant varietal differences in the accumulation of carotenoids. The content of red and yellow pigments determines not only the color of the fruit, but also the color intensity. In a hybrid of sweet pepper F_1 Oranzhevoe naslazhdenie with a rich orange color of the fruit, the yellow pigment level was 6.20 times higher than in red-colored, with the 0.16 ratio of red and yellow pigments; in the Zheltyi buket variety with a yellow color of the fruit, the excess was 1.34-fold with a quantitative pigment ratio of 0.75 (Table 1). It should be

noted that the yellow or orange color of the fruit and intensity of coloration is determined not by the total amount of yellow pigments, but by the ratio of zeaxanthin, β-cryptoxanthin and β-carotene.

1. Fruit color, content of pigments and their ratio in pepper (*Capsicum* spp.) (*X*±SE, Moscow Province, 2015-2016)

Sample	Species	Color during the stage of technological/biological ripeness	Carotenoids, mg/g			Yellow to red pigments ratio
			yellow pigments	red pigments	∑ red and yellow pigments	
Sweet pepper						
F_1 Oranzhevoe naslazhdenie	*C. annuum*	Green/orange	0.169±0.008	0.027±0.001	0.196±0.010	0.16±0.01
F_1 Sibiryak	*C. annuum*	Dark green/red	0.052±0.003	0.081±0.004	0.133±0.007	1.56±0.08
Zheltyi buket	*C. annuum*	Dark green/yellow	0.063±0.003	0.047±0.002	0.110±0.006	0.75±0.04
Shokoladnyi	*C. annuum*	Dark green /brown	0.205±0.010	0.331±0.017	0.536±0.027	1.61±0.08
Hot pepper						
Rozhdestvenskii buket	*C. annuum* × *C. frutescens*	Green/red	0.212±0.011	0.235±0.012	0.447±0.022	1.11±0.06
Samotsvet	*C. annuum* × *C. frutescens*	Violet/red	0.178±0.009	0.240±0.012	0.418±0.021	1.35±0.07
Ideya	*C. annuum*	Light-green/yellow	0.625±0.031	<0.004	0.629±0.031	< 0.01
Rocoto	*C. pubescens*	Dark green/red	0.263±0.013	0.308±0.015	0.571±0.029	1.17±0.06
Kitaiskii fonarik	*C.baccatum*	Light green/red	0.240±0.012	0.445±0.022	0.685±0.034	1.85±0.09
Kolokolchik	*C. chinense*	Dark green/yellow	0.318±0.016	<0.001	0.319±0.016	< 0.01
Trinidad Dglahou	*C. chinense*	Black/red	0.119±0.006	0.077±0.004	0.196±0.010	0.65±0.03
Trinidad Scorpion Chocolate	*C. chinense*	Black/brown	0.088±0.004	0.179±0.009	0.267±0.013	2.03±0.10
Tsyganenok	*C. annuum*	Black/black and pink	0.012±0.001	0.051±0.003	0.063±0.003	4.25±0.21
Bhyt jolokia	*C. chinense* × *C. frutescens*	Light green/yellow	0.266±0.013	<0.003	0.269±0.013	< 0.01
Purpurnyi tigr	*C. annuum*	Dark violet/red	0.267±0.013	0.441±0.022	0.708±0.035	1.65±0.08
Mech	*C. annuum*	Red	0.127±0.006	0.196±0.010	0.323±0.016	1.54±0.08
Mech	*C. annuum*	Light green/yellow	0.470±0.024	<0.003	0.473±0.024	< 0.01
Chudo Podmoskovya	*C. annuum*	Light green/red	0.295±0.015	0.333±0.017	0.628±0.031	1.13±0.06
Yubileinyi VNIISSOK	*C. annuum*	Green/red	0.093±0.005	0.312±0.016	0.405±0.020	3.35±0.17
Ognennaya deva	*C. chinense*	Green/red	0.277±0.011	0.354±0.018	0.581±0.029	1.56±0.08

During the phase of biological ripeness of fruits in most studied varieties, β-carotene amounted about 10 % of total carotenoids. The bright red color of the F_1 Sibiryak hybrid was due to higher content of red pigments. The highest quantity of carotenoids among sweet pepper varieties was noted in Shokoladnyi with a brown skin and burgundy-red pericarp at biological ripeness (with yellow and red pigments 0.205 and 0.331 mg/g, respectively). The ratio of red and yellow pigments in the Shokoladnyi variety was 1.61. F_1 Sibiryak with a bright red color of the fruit showed 1.56. Apparently, the presence of a significant amount of yellow and red pigments in the fruit of Shokoladnyi variety in combination with proteins could cause the formation of a brown color. According to A.S.H. Ong et al. [30], pigments provide color from yellow to dark red, and in combination with proteins can give green and blue color.

Red pigments prevailed in all samples of hot pepper with red fruits at biological ripeness. The same trend was observed in Trinidad Scorpion Chocolate hot pepper: the ratio of red and yellow pigments was 2.03, but the color of the fruits at biological ripeness was brown. In Trinidad Dglahou with a red color of the fruit, the amount of yellow pigments was 1.5 times greater than that of the red ones, 0.119 and 0.077 mg/g, respectively. In both forms, the fruits were in a dark violet almost black color at technical ripeness phase.

We found red pigments in sweet peppers F_1 Oranzhevoe naslazhdenie (orange fruits) and Zheltyi buket (yellow fruits), 13.7 and 42.7 %, respectively. This trend was not observed in the hot peppers. In all the hot peppers with yel-

low fruit coloring (Ideya, Kolokolchik, Bhyt jolokia, Mech), only yellow pigments and traces of red pigments were found. The largest accumulation of pigments occurred in sweet paper Shokoladnyi (0.536 mg/g) and in hot papers Purpurnyi tigr (0.708 mg/g), Kitaiskii fonarik (0.685 mg/g), Ideya (0.629 mg/g), and Chudo Podmoskovya (0.628 mg/g).

2. Antioxidant accumulation in fruits of various peppers *Capsicum* spp. (X±SE, Moscow Region, 2015-2016)

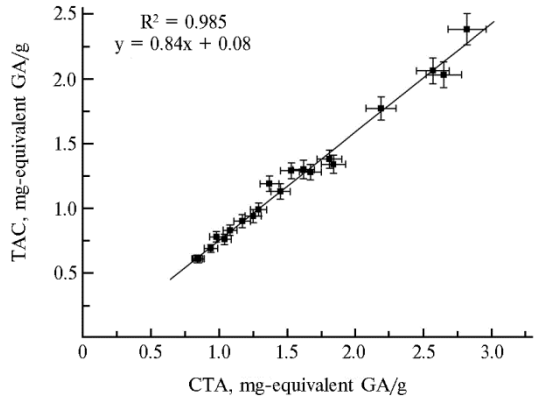
Sample	Pungency, points.	CCA, mg-equivalent GA/g			Ascorbic acid, mg%	Dry matter, %
		acetone:ethanol, 1:1 v/v	ethanol, 80 %	ethanol 80 %, 60 °C, 60 min		
Sweet pepper						
F_1 Oranzhevoe naslazhdenie	0	1.27±0.06	1.25±0.06	0.94±0.05	246±17	10.6±0.5
F_1 Sibiryak	0	1.15±0.06	0.98±0.05	0.78±0.04	225±16	9.2±0.5
Zheltyi buket	0	0.82±0.04	0.85±0.04	0.61±0.03	225±16	7.8±0.4
Shokoladnyi	0	1.18±0.06	0.94±0.05	0.69±0.03	238±17	9.5±0.5
Hot pepper						
Rozhdestvenskii buket	7-8	1.66±0.08	2.82±0.14	2.38±0.12	370±26	16.4±0.9
Samotsvet	8	0.88±0.04	1.84±0.09	1.34±0.07	150±10	17.3±0.9
Ideya	2-3	2.17±0.11	2.57±0.12	2.06±0.10	414±29	15.5±0.8
Rocoto	10	0.90±0.05	1.04±0.05	0.76±0.04	79±6	14.2±0.7
Kitaiskii fonarik	2	0.81±0.04	0.84±0.04	0.61±0.03	158±11	19.9±0.9
Kolokolchik	9	1.44±0.07	1.37±0.07	1.19±0.06	202±14	13.1±0.7
Trinidad Dglahou	10	1.26±0.06	1.17±0.06	0.90±0.05	176±12	12.8±0.6
Trinidad Scorpion Chocolate	10	1.01±0.05	1.08±0.05	0.83±0.04	178±12	12.2±0.6
Tsyganenok	2	0.96±0.05	1.53±0.08	1.29±0.06	192±13	12.0±0.6
Bhyt jolokia	10	0.87±0.04	1.29±0.06	0.99±0.05	158±11	12.0±0.6
Purpurnyi tigr	9	2.17±0.11	2.19±0.11	1.77±0.09	216±15	18.7±0.9
Mech	0,5	1.20±0.06	1.45±0.07	1.13±0.06	220±15	10.2±0.5
Mech	1	1.35±0.07	1.81±0.09	1.38±0.07	299±21	12.0±0.6
Chudo Podmoskovya	1,5	1.48±0.07	1.67±0.08	1.28±0.06	299±21	11.6±0.6
Yubileinyi VNIISSOK	3	1.58±0.09	1.62±0.08	1.30±0.07	326±23	17.8±0.9
Ognennaya deva	8	1.85±0.09	2.65±0.13	2.03±0.10	301±21	16.3±0.8

Note. CCA — total antioxidant capacity.

Vitamin C is an important intracellular antioxidant along with carotenoids, protecting the human body from both free radicals and peroxides [31]. Peppers are among the leaders of vegetable crops for vitamin C level. The highest amount of ascorbic acid was found in hot peppers Ideya (414 mg%), Rozhdestvenskii buket (370 mg%), Yubileinyi VNIISSOK (326 mg%), and Ognennaya deva (301 mg%). Note that the vitamin C level did not depend on species or fruit color. Among the members of different species there were varieties with both red and yellow fruits (Table 2). There were no significant differences in the indices depending on the extractant used. Only four hot pepper varieties, Rozhdestvenskii buket, Samotsvet, Tsyganenok, Ognennaya deva, showed the differences of 40-70 % in SSA depending on the extractant.

Among sweet peppers, F_1 Oranzhevoe naslazhdenie plants had high antioxidant values. The maximum sum of antioxidants among the hot peppers was in Rozhdestvenskii buket (2.82 mg-equivalent GA/g), Ognennaya deva (2.65 mg-equivalent GA/g), Ideya (2.57 mg-equivalent GA/g) and Purpurnyi tigr (2.19 mg-equivalent GA/g). The value of TAC varied significantly, depending on the variety and species. The estimation of total antioxidant whole, given their cooperative action, allows isolating the genotypes rich in antioxidants to involve them breeding as a parental forms. Rozhdestvenskii buket, Kitaiskii fonarik, Purpurnyi tigr, and Ognennaya deva varieties accumulated more capsaicin, another strongest natural antioxidant which determined the burning taste of pepper fruit, than other samples. The level of capsaicin in the studied hot pepper samples varied between 1.36-9.57 mg/g dry weight [32]. High content of carotenoids, ascorbic

acid, total antioxidants and capsaicinoids (8-9 point pungency) further strengthened the synergetic antioxidant effect.



Contribution of thermostable antioxidants (CTA) to total antioxidant content (TAC) in pepper (*Capsicum* spp.) fruits of the studied samples (Moscow Province, 2015-2016).

The high content of dry matter (over 10 %) in fresh hot pepper fruits at biological ripeness (see Table 2) determines their use for multivitamin products with high antioxidant activity. Samotsvet, Kitaiskii fonarik, Purpurnyi tigr and Yubileinyi VNIISSOK peppers contain more than 17 % dry matter, which allows quick drying fruits used for paprika powder and guarantees high quality of sauces and pasta.

Assessment of thermostable antioxidants to total antioxidant level when extracted with 80 % ethanol and heated at 60 °C for 60 min showed that the contribution of the antioxidants unstable to thermal action, primarily ascorbic acid, was 16 % on average (Fig.).

Thus, the obtained results allow us to conclude that the Shokoladnyi sweet pepper variety with brown fruits and the hot pepper varieties Ideya, Rocoto, Kitaiskii fonarik, Purpurnyi tigr, Chudo Podmoskovya, and Ognennaya deva are the leaders in pigments accumulation. In all the varieties, except Ideya, red and yellow pigments comparably contribute to pigment accumulation, while for Ideya variety there was a high pigment content solely due to the yellow fraction. Ideya, Chudo Podmoskovya, and Ognennaya deva varieties were characterized by an increased amount of deoxidized ascorbic acid. Ideya, Purpurnyi tigr, and Ognennaya deva varieties were characterized by an increased sum of low molecular weight antioxidants. So, Ideya and Ognennaya deva genotypes may serve as genetic sources of high antioxidant accumulation. The amount of red and yellow pigments, their total content, as well as the accumulation of ascorbic acid and capsaicinoids determine high antioxidant status of these samples, which can be the basis of biofortified foodstuff used both for functional products and in breeding programs.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1030-1040
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 582.663.2:581.19

doi: 10.15389/agrobiology.2017.5.1030rus
doi: 10.15389/agrobiology.2017.5.1030eng

METABOLITES WITH ANTIOXIDANT AND PROTECTIVE FUNCTIONS FROM LEAVES OF VEGETABLE AMARANTH (*Amaranthus tricolor* L.)

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The authors declare no conflict of interests
Acknowledgements:
The research was carried out under the theme № 0575-2017-0001 supported by the program of Federal Agency of Scientific Organizations for bioresource collections
Received July 8, 2017

Abstract

Antioxidant metabolites of plant origin are able to regulate many physiological functions of the body and reduce the risk of developing chronic diseases caused by free radical oxidation. Vegetable plants are the most affordable source of essential antioxidant metabolites lack of which leads to a sharp decrease in resistance to environmental stresses. Amaranth (*Amaranthus tricolor* L.) is a promising food and medicinal plant. Variety Valentina (originated by V.K. Gins, P.F. Kononkov, M.S. Gins, All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops) was successfully introduced and grown in several Russian regions. Our objective was to study the composition and content of low-molecular biologically active antioxidant metabolites that determine the nutritional and pharmacological value of amaranth leaves, and to assess the main antioxidant accumulation in plant organs under the conditions of the Moscow Region. For analysis, fresh and dried leaves (juvenile, those with a formed blade, and old ones), inflorescences, stems, veins, petioles and roots were used. Amaranthine, reduced ascorbic acid, and total antioxidant content was measured in water and ethanol extracts from fresh and dry leaves and plant organs. Also, simple phenols and oxybenzoic acids, flavonoids, condensed and polymeric polyphenols were assayed. Chlorogenic, gallic, ferulic acids and arbutin content was determined in aqueous extract by high performance liquid chromatography (HPLC). The metabolites were analyzed by gas chromatography-mass spectrometry (GC/MS). It was shown that actively photosynthesizing leaves with a fully formed blade predominantly accumulated ascorbic acid, while in the aging leaves its amount decreased. Veins, petioles and stems contained substantially less metabolites with antioxidant activity compared to leaves. In aqueous extracts, the main betacyanins were amaranthine and iso-amarantine. Chromatography of aqueous extracts from amaranth leaves showed the presence of highly active antioxidants, e.g. arbutin-glucoside hydroquinone and oxycinnamic acids including ferulic, chlorogenic, oxybenzoic (gallic) acids. In the tests, gallic acid concentration was 1.51 μg/100 ml, chlorogenic acid concentration was 2.05 μg/100 ml, ferulic acid concentration was 0.01 μg/100 ml, and arbutin concentration was 472.51 μg/100 ml. Water-extracted squalene ($C_{30}H_{50}$), a powerful antioxidant usually isolated from amaranth seeds only, was first discovered in amaranth leaves. Ethanol extraction revealed a greater number of the colored components in the spectral range of the 350-700 nm, in addition, gallic, chlorogenic and ferulic acids were found. A total of 37 low-molecular metabolites were identified by gas chromatography-mass spectrometry. Our findings indicate that vegetable amaranth, as a promising reproducible source of antioxidants, can be used in functional foods and phytobiologicals.

Keywords: amaranth, *Amaranthus tricolor* L., Valentina variety, low-molecular antioxidant metabolites, amaranthine, ascorbic acid, phenolic compounds

Amaranth (*Amaranthus* genus) is used in many countries as a grain, vegetable and medicinal crop. In Africa, the leaves of vegetable amaranth are used as an additional source of native protein which contains balanced essential

amino acids [1]. Leaves and young plants of vegetable amaranth in Indonesia, China, India and South-East Asia are the main product that enriches foods with essential amino acids, biologically active substances and antioxidants [2, 3]. Preparations made of amaranth plants are widely used in phytomedicine to perform regulator and protective functions and to reduce the risk of chronic diseases caused by free radical oxidation [4].

In Russia, the use of vegetable species of amaranth with a high content of gluten-free protein and low-molecular antioxidants (e.g., vitamin C, phenolic compounds, betalain pigments, and squalene) is relevant and necessary for compensation of essential nutrient deficiency. A detailed study of amaranth metabolites with antioxidant properties and a wide range of biological activity is necessary for the development and manufacture functional foods and beverages from amaranth leaves [5]. Vegetable amaranth species are also a valuable medicinal crop due to the high content of betacyanins, ascorbic acid, phenolic compounds and antioxidants of a different nature [6]. Antidiabetic [6-8], hepatoprotective [9, 10], gastroprotective [11], antitumor [12], and antimicrobial [13, 14] properties of the extracts from the leaves of *Amaranthus tricolor* L. have been proved.

Modern analytical methods such as mass spectrometry and high performance liquid chromatography (HPLC) allow identifying components that were not previously detected in assessing nutritional and pharmacological value of plant raw material, in order to develop innovative and biologically active plant preparations and products [15, 16].

This paper is the first report of identification of 37 metabolites with antioxidant properties form different classes of compounds (i.e., organic acids, sugars and their derivatives, phenols, triterpenes, amino acids) in the leaves of the Valentina variety vegetable amaranth. This widens using amaranth leaves for functional products with antistress activity.

The purpose of our study was to assess the qualitative composition of metabolites in aqueous and ethanolic amaranth extracts and to assess accumulation of the main antioxidant in plant organs under the conditions of the Moscow Region.

Techniqques. For study, we used fresh and dried leaves, inflorescences, stems, veins, petioles and roots of vegetable amaranth (*Amaranthus tricolor* L.) Valentina variety (originated by All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, VNIISSOK, Moscow Province), and also the biologically active additive Amarantil phytotea made of leaves of Valentina variety plants (VNIISSOK, Russia). Plants were grown in open experimental fields of VNIISSOC in 2015-2017. Laves of different ages (juvenile, those with a formed blade, and old ones) were used. Fresh material was placed in a homogenizer; samples were prepared in accordance with the methods intended for each special study. The dried samples were crushed in a mill up to 1-2 mm size. Aqueous and ethanolic extracts of fresh and dried plant material were used.

The amaranthine in aqueous extracts was quantified using molar extinction coefficient 5.66×10^4 $1/mole^{-1}\cdot cm^{-1}$ and a molar weight of 726.6 $g/mole^{-1}$ [17]. The concentration of reduced ascorbic acid (AA) was evaluated by iodometric method based on the AA titration in extracts stained with potassium iodate under acidic conditions in the presence of potassium iodide and starch [18]. The total antioxidants (TA) in aqueous and ethanolic extracts were determined amperometrically [19]; the result was expressed via gallic acid equivalents (mg-equivalent GA/g).

The samples were crushed in a homogenizer in the presence of extracting liquids (bidistilled water or 96 % ethyl alcohol) at 20-25 °C. The homogenates were centrifuged for 15 minutes at 10,000 g and 4 °C. An aliquot of the superna-

tant was used to determine the antioxidant content after dilution, if necessary. The measurements were carried out on a Tsvet-Yauza 01-AA (NPO Khimavtomatika, Russia) in a constant current mode.

The content of simple phenols and oxybenzoic acids, oxycinnamic acids and their esters, flavonoids, condensed and polymeric polyphenols was determined in the fractions of dry inflorescences, leaves and stems [20].

Spectral characteristics were measured using a Helios Gamma spectrophotometer (Thermo Electron Corporation, USA) at λ = 200-600 nm. The amount of chlorogenic, gallic, ferulic acids and arbutin in the aqueous extract from Amarantil phytotea was determined by high performance liquid chromatography (HPLC) with a liquid chromatograph KNAUER (KNAUER Wissenschaftliche Gerдte GmbH, Germany). A 150 mm column with a reversed phase was filled with sorbent Diasfer 110-S18 (Russia) with a grain size of 5 μm, the mixture of 0.03 % trifluoroacetic acid:acetonitrile (70:30) served as the eluent. Peaks were identified by the retention time; the content of individual substances was determined using calibration solutions. The qualitative analysis of metabolites was performed by gas chromatography mass-spectrometry (GC/MS) with a chromatograph JMS-Q1050GC (JEOL Ltd., Japan). A capillary column DB-5HT was used (Agilent, US; 30 m in length, inner diameter of 0.25 mm, film thickness 0.52 μm, with helium as a carrier gas). The temperature gradient ranged from 40 to 300 °C, with the injector and interface temperature of 280 °C, and the of ion source temperature of 200 °C; the gas flow rate in the column was 2.0 ml/min. The time for analysis was 45 min ubder the flow division input mode and a 1 μl sample introduced. To determine the substances, derivatization with silylation agent N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) [21] was performed. The substance identification was based on the retention time and mass spectra indicated in the NIST-5 library of the National Institute of Standards and Technology (USA). The scanning range was 33-900 m/z.

All measurements were carried out in three replications; the tables show the mean values (X) and their standard deviations ($\pm x$).

Results. A specific feature of the leaves of *A. tricolor* L. Valentina plants is the red coloration of different intensity which depends on a eaf age and is determined by the amount of the amaranthine pigment found in all the studied plant organs (leaves, roots, petioles, stems, inflorescences). The aqueous extracts from leaves of different ages differed in the amount of the main metabolites with antioxidant properties, i.e., amaranthine, ascorbic acid, and carotenoids (Table 1). Juvenile leaves accumulated the maximum amount of amaranthine the content of which decreased with the growth and aging of the leaf blade. Ascorbic acid was predominantly accumulated in actively photosynthetic leaves with a fully formed blade; in the aging leaves its amount decreased. In juvenile leaves, there was also a tendency towards an increase in the content of ascorbic acid.

Since the organs of amaranth plants differ in antioxidant levels, the total content of antioxidants [19] can be used as an integral parameter to analyze the pattern of their combined change. In the leaves with an unformed blade, the content was lower than that in the leaves with a fully formed blade. At the same time, veins, petioles, and stems accumulated significantly less metabolites with antioxidant activity compared to the leaves.

Among the secondary metabolites synthesized in vegetable plants a group of aromatic phenolic compounds [13] was specified by the biological activity. We have found that simple phenols such as oxybenzoic and oxycinnamic acids and their esters, flavonoids, polymeric and condensed polyphenols are widespread in the leaves and fruits of vegetable and spicy plants [20, 21]. The role of secondary metabolites in plants is still poorly understood. It is known that they determine the

medicinal and toxic properties, participate in protection from biotic stressors, and also in the interactions of plants with each other and with other organisms, are involved in reproduction via color, smell of flowers and fruits [22]. In the leaves of amaranth plant, a significant proportion of phenolic compounds were flavonoids (Table 2).

1. Content of antioxidant metabolites in the organs of amaranth (*Amaranthus tricolor* L.) Valentina plants ($X \pm x$, All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Region, 2015)

Organ	Leaf number (from the bottom upwards)	Содержание		Total antioxidants, mg-equivalent GA/g wet weight
		ascorbic acid, mg%	amaranthine, mg/g wet weight	
Old leaves	1	161±15.0	0.26±0.02	2.32±0.11
	2	186±15.0	0.29±0.02	2.30±0.11
	3	176±15.0	0.44±0.05	2.25±0.11
Leaves with a formed blade	4	230±15.0	0.48±0.05	2.26±0.11
	5	202±15.0	0.51±0.05	2.20±0.11
	6	216±15.0	0.57±0.05	2.21±0.11
Leaves with an unformed	7	149±15.0	0.55±0.05	1.81±0.10
blade and juvenile	8	133±15.0	0.72±0.05	2.00±0.11
	9			
	10	167±15.0	0.94±0.05	2.11±0.11
	11			
Leaf vein			0.31±0.02	0.70±0.04
Leaf petioles		17±1.5	0.79±0.05	0.69±0.04
Stem		10±1.5	0.21±0.02	0.43±0.03
Inflorescence		130±15.0	0.75±0.05	1.18±0.05
Roots		10±1.5	0.11±0.02	0.45±0.03

N o t e. GA is gallic acid. Gaps represent the absence of data.

2. Phenolic compounds (PhC, % per absolute dry weight) **in inflorescences, leaves and stems of the amaranth (*Amaranthus tricolor* L.) Valentina plants** ($X \pm x$, All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2015)

Organ	PhC sum	Simple phenolic compounds and hydroxybenzoic acids	Hydroxycinnamic acids and their ethers	Flavonoids	Condensed and polymeric PhC
Inflorescence	5.52	0.55±0.05	0.09±0.01	3.94±0.11	0.94±0.11
Leaves	5.47	0.43±0.05	0.09±0.01	4.21±0.11	0.74±0.11
Stem	3.23	0.32±0.05	0.05±0.01	0.52±0.11	2.34±0.11

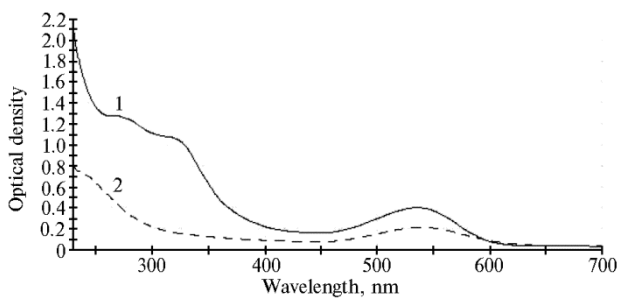

Fig. 1. Absorption of aqueous extracts from the leaves of amaranth (*Amaranthus tricolor* L.) Valentina variety: 1 — Amarantil phytotea, 2 — amaranthine (All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2016).

The content of flavonoids in the amaranth leaves is comparable with their quantity in medicinal plants [22]. In the inflorescences, less flavonoids and oxycinnamic acids were found than in the leaves. Stem extracts had a minimum content of flavonoids, but showed higher values of condensed and polymeric phenols compared to the inflorescence and leaves. In extracts from fresh amaranth leaves, we found the following physiologically active substances with antioxidative activity: amaranthine, ascorbic acid, flavonoids, simple phenols and phenolcarbonic acids.

Spectrum of the extract from the biologically active food Amarantil phytotea exhibited three absorption maxima at 250-270, 325 and 540 nm (Fig. 1),

which indicates the presence of components of phenolic nature. One of the maxima (λ = 540 nm) coincided with the maxima for the amaranthine pigment extracted from the leaves.

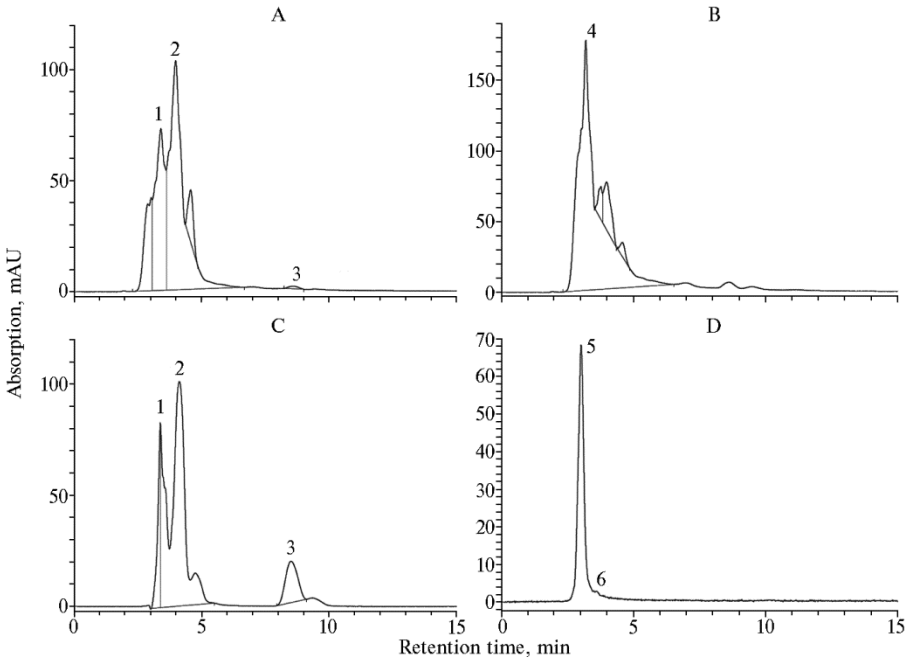

Fig. 2. Chromatographic profiles of water extract from amaranth (*Amaranthus tricolor* L.) Valentina leaves (Amarantil" phytotea) (λ = 325 nm) (A)**, gallic acid standard (λ = 268 nm)** (B)**, standard mixture of arbutin, chlorogenic and ferulic acids (λ = 325 nm)** (C)**, amaranthine pigment (λ = 540 nm)** (D)**:** 1 — arbutin, 2 — chlorogenic acid, 3 — ferulic acid, 4 — gallic acid, 5 — amaranthine, 6 — isoamaranthine (All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2017). Liquid chromatograph KNAUER (Germany).

In chromatographic analysis, phenolic compounds in an aqueous extract of amaranth leaves (Amarantil phytotea) were detected at 268 and 325 nm. In the fresh amaranth leaves, chlorogenic, ferulic, gallic acid and arbutin were found (2.05±0.10, 0.01, 1.51±0.04 and 473±20 g/ml, respectively), as well as unidentified compounds with retention time 4-8 min (Fig. 2, A, C). V.B. Gopal et al. [23] found gallic and ferulic acids in an amount of 0.083 and 0.001 μg/ml in an aqueous extract of amaranth leaves. Gallic acid was analyzed at λ = 268 nm, and red pigment was analyzed at λ = 540 nm. For the red pigment absorption, two components were determined, amaranthine and its isomer isoamaranthine (Fig. 2, D). The spectra of amaranthine detected by the HPLC method and the pigment standard completely coincided.

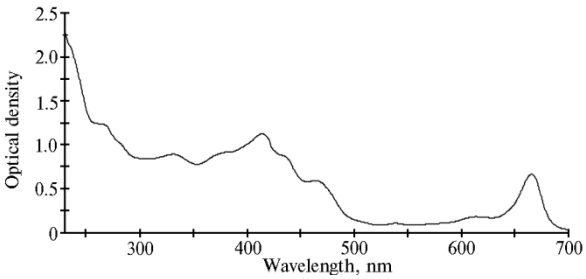

Fig. 3. Absorption ethanolic extract of amaranth (*Amaranthus tricolor* L.) Valentina leaves (All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2017).

The ethanolic extract of amaranth leaves had an intense green color due to the extracted chlorophyll. Its spectrum was significantly different from the spectrum of the water extract, especially at 350-500 nm and 600-700 nm (Fig. 3). In the range of 250-300 nm, the water and ethanolic extract spectra were similar.

Consequently, more colored components were extracted from the leaves by the ethanol. The chromatographic analysis of the ethanolic extract of amaranth leaves at 268 and 325 nm was also carried out with gallic, chlorogenic and ferulic acids detected (Fig. 4). With a minimum of absorption (see Fig. 3, $\lambda = 540$ nm), only the solvent peak was detected.

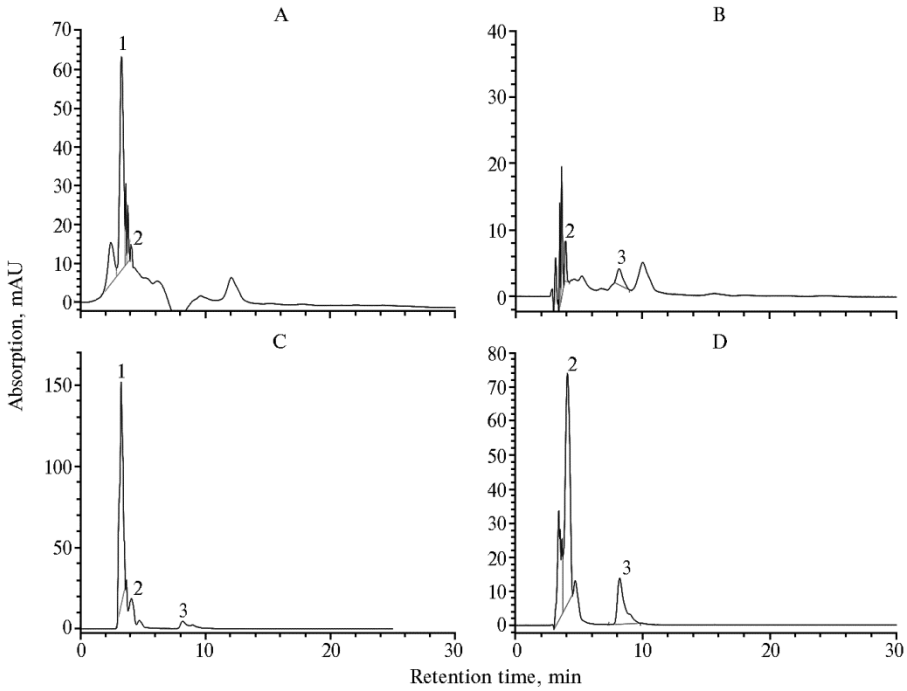

Fig. 4. Chromatographic profiles of the extract of amaranth (*Amaranthus tricolor* L.) Valentina leaves for the analyzed sample (A, B) **and standard mixture** (B, D) **at λ = 268 nm** (A, C) **and λ = 325 nm** (B, D)**:** 1 — gallic acid, 2 — chlorogenic acid, 3 — ferulic acid (All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2016) Liquid chromatograph KNAUER (Germany).

Our data on phenolic compounds in the *A. tricolor* leaves broaden the idea about nutritive and medicinal low-molecular metabolites in amaranth plants and can supplement the information available in the scientific literature. All metabolites identified by HPLC are biologically active substances that exhibit antioxidant activity. Phenolic acids and betacyanin (amaranthine) are characterized by antibacterial [13, 14], antimycotic, anti-inflammatory, and wound-healing properties [23]. Ferulic acid possesses radioprotective properties [24], glycosylated hydroquinone arbutin exhibits antioxidant activity [25].

The results of qualitative GC/MS analysis of water and ethanolic extracts from amaranth leaves are illustrated in Fig. 5.

The chromatograms clearly show the qualitative differences between the extracts in composition. More than 30 individual substances (15 in aqueous extract and 19 in ethanolic extract) representing different classes of chemical compounds (Table 1) were identified based on the library mass spectra (Table 3).

In the extracts of *A. tricolor* leaves, six organic acids were found: valerian and cinnamic acids in aqueous extract, amber, propene, methylmalonic and ribonic acids in ethanolic extract. Five saturated fatty acids were found: myristic ($C_{13:0}$), palmitic ($C_{15:0}$), margarine ($C_{15:0}$) and stearic ($C_{17:0}$) for the aqueous extract, and palmitic ($C_{15:0}$) and lignoceric ($C_{23:0}$) for ethanolic in the aqueous extract. Fatty acids have bactericidal, antiviral and fungicidal activity leading to suppression of pathogenic microflora and yeast fungi. Among unsaponifiable li-

pids, there are bioactive phytosterols. It is known that squalene ($C_{30}H_{50}$) is a unique phytosterol of amaranth seed, which participates in the synthesis of cholesterol as its precursor [26] and also in the synthesis of an active antioxidant with anticarcinogenic and wound-healing effect. An adequate amount of squalene consumption is 400 mg per day. Squalene is a precursor of a number of valuable pharmacological substances: cardiac glycosides, glycoalkaloids, and saponins. Squalene, first discovered by us in the aqueous extract of amaranth leaves of the Valentina variety, improves their pharmacological value.

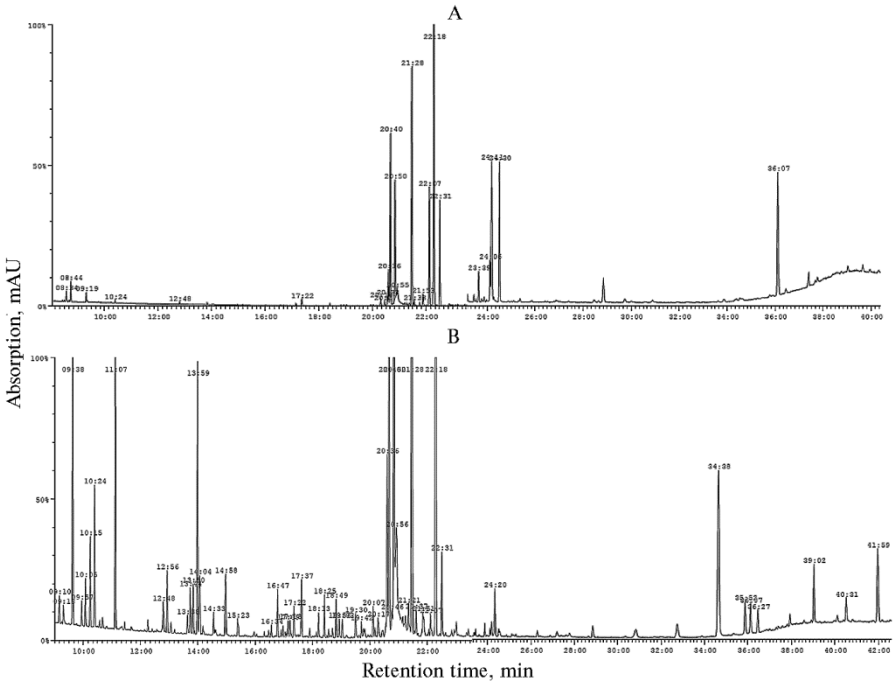

Fig. 5. Fragments of the mass spectra of water (A) **and ethanol** (B) **extracts of amaranth (*Amaranthus tricolor* L.) Valentina leaves** (All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2017). Gas chromatography mass spectrometer JMS-Q1050GC (JEOL Ltd., Japan).

In the aqueous fraction of extracts we identified five carbohydrates: monosaccharides of galactose and mannose, cyclic forms (galactopyranose and fructofuranose) and ketohexose tagatose. In the ethanolic extract, we identified seven carbohydrates and their cyclic forms, a sugar derivative (gluconic acid) and two hexatomic alcohols (inositol and mannitol). Polyatomic alcohols exhibit cryo- and osmoprotective properties in the cell.

3. Metabolites discovered in aqueous and ethanol extracts of amaranth (*Amaranthus tricolor* L.) Valentina leaves by gas chromatography mass-spectrometry (All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops, Moscow Province, 2017)

Rt, min	Compound	Extract
	Organic acids	
22:07	Propenoic acid, Acrylic acid, $C_3H_4O_2$	C
14:04	Succinic acid, $C_4H_4O_4$	C
16:34	Monoamidoethyl malonic acid, $C_4H_6O_4$	C
20:47	Pentanoic acid, CH_3 $(CH_2)COOH$	B
22:41	Ribonic acid, $C_5H_{10}O_5$	C
22:51	Cinnamic acid (Isoferulic acid), $C_9H_8O_2$	B
	Fatty acids	
17:51	Octadecanoic (Stearic) acid, $C_{17}H_{35}COOH$	B
19:07	Myristic acid, $C_{13}H_{27}COOH$	B
23:11	Margaric acid, Heptadecanoic acid, $C_{16}H_{33}COOH$	B
21:15	Palmitic acid, Hexadecanoic acid, $C_{15}H_{31}COOH$	B, C

Continued Table 3

36:27	Lignoceric acid, Tetracosanoic acid, $C_{23}H_{47}COOH$	C
	Sugars	
18:36	Ribose, D-(-)-Ribofuranose, $C_5H_{10}O_5$	C
19:03	Galactopyranose, β-L-Galactopyranose, $C_6H_{13}O_8$	B
19:26	Galactose, D-Galactose, $C_6H_{16}O_6$	B, C
20:11	Tagatose, D-(-)Tagatofuranose, $C_6H_{12}O_6$	B
20:36	Fructose, D-(-)-Fructofuranose, $C_5H_8O_6$	C
20:51	Fructofuranose, D-(-)-Fructopyranose, $C_5H_8O_6$	C
20:55	Glucofuranose, $C_6H_6O_6$	B
20:56	Talose, D-(+)-Talofuranose, $C_5H_{12}O_6$	C
21:28	Mannose, D-Mannopyranose, $C_6H_{12}O_6$	B, C
22:17	Glucopyranose, β-D-Glucopyranose, $C_6H_6O_6$	C
	Sugar derivatives	
19:43	Gluconic acid γ-lacton, $C_6H_{12}O_7$	C
	Alcohols	
21:51	Inositol, $C_6H_{12}O_6$	C
22:07	Mannitol, D-Mannitol, $C_6H_{12}O_6$	C
22:08	Sorbitol, D-Sorbitol, $C_6H_{14}O_6$	B
	Other compounds	
36:06	Squalene, unsaturated triterpene, antioxidant, $C_{30}H_{50}$	B
16:48	Proline, L-Proline, $C_3H_9NO_2$	C
19:30	D-(+)-Ribono-1,4-lactone	B
23:06	Dry Betaine, Methanaminium (Betaine) Glycine	B
39:57	Ribothymidine, 5-Methyluridine, $C_{10}H_{14}N_2O_6$	C
40:31	Vitamin E, (+)-α-Tocopherol	C

Note. Rt — retention time, B — aqueous extract, C — ethanolic extract.

In the literature, there is evidence that simple sugars and their derivatives (alcohols, lactones), when accumulating in cells, are capable of performing protective functions under the action of stress factors. Glucose, fructose, mannose, mannit, inositol and galactose exhibit antioxidant properties in cells at low temperatures. The protective effect of simple sugars is due to their ability to bind free radicals non-specifically, to prevent the development of lipid peroxidation and to inactivate reactive oxygen species [27-30].

In our experiments, the ethanolic extract of leaves of the *A. tricolor* Valentina variety contained vitamin E, which is the universal protector of cell membranes from oxidative damage, shows neuroprotective, antioxidant properties and reduces the risk of cancer. Micromolar amounts of vitamin E reduce the activity of 3-hydroxy-3-methylglutaryl-CoA-reductase, responsible for the synthesis of cholesterol, thus reducing cholesterol level in the body [31].

Thus, a comprehensive analysis of the metabolites in the leaves of amaranth (*Amaranthus tricolor* L.) Valentina variety showed the presence of powerful antioxidants — squalene, which was previously found in seeds only, ascorbic acid, the content of which is comparable to that of pepper, gallic, ferulic and chlorogenic acids, sugars and their derivatives, triterpenes, betacyanins with antioxidant activity. The pharmacological value of the identified metabolites is due to their ability to reduce the risk of developing pathologies with a free-radical component (cardiovascular, oncological, diabetes, etc.), which makes it possible to use these natural compounds in antioxidant therapy as anti-stressors. The high content of such metabolites in Valentina variety, their nutritive and pharmacological value, makes this variety promising for use in food industry and medicinal technologies to develop non-toxic antioxidant food additives, functional products (teas, natural pigments, non-alcoholic vitamin drinks, confectionery fillings) and phytobiologicals using leaves and leaf extracts.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1041-1048
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Genetic and physiological basis of crop breeding

UDC 633.14:631.524.6

doi: 10.15389/agrobiology.2017.5.1041rus
doi: 10.15389/agrobiology.2017.5.1041eng

PENTOSAN CONTENT GENOTYPIC VARIABILITY IN WINTER RYE GRAIN

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The authors declare no conflict of interests
Received February 6, 2017

Abstract

Varieties intended for diverse use are modern priority in winter rye (*Secale cereale* L.) breeding. Composition and content of pentosans are indicators to diversify rye grain use. The aim of this work was estimation of variability in total arabinoxylans and soluble arabinoxylans in rye grain. Pentosans content was determined by high performance liquid chromatography in the HPLC-RI system (JASCO Deutschland GmbH, Germany), by chemical micro method with use of orcin-chloride, and indirectly by determining the viscosity of water extract (VWE). As a result, the samples with low and high pentosan content were identified at the linear, population and hybrid level in domestic and foreign gene pools. It was shown that Russian population varieties and high-pentosan lines selected at Tatar Research Institute of Agriculture stood out due to high rates of general pentosan level and extracted viscosity. The relationship between VWE and the content of water-soluble pentosans in the studied quantitative limits with a high probability (95 %) has a rectilinear character. In low pentosans lines originated from Tatar Research Institute of Agriculture VWE amounted to 6.40-of 6.45 centistokes (sSt), in the domestic population varieties VWE ranged from 15.40 to 34.50 sSt, and in hybrid varieties from Germany VWE reached 47.50 sSt. So we have a gene pool sufficient for baking rye breeding. In high-pentosan forms, we found the high significant positive correlation between the total content of pentosans, viscosity of water extracts and water-soluble fraction. An indirect estimate of pentosans fraction through determination of the water extract viscosity of rye meal allows to start selection in the early steps of breeding and to analyze a large number of samples in a relatively short time. Further search is necessary to select donor lines with low total level of arabinoxylans and water-soluble fraction. It is difficult to phenotypically evaluate low-pentosans plants based on an indirect indicator of viscosity only. Low pentosan lines had a significant correlation between VWE and the water-soluble fraction ($r = 0.745$, $P = 0.05$). Heritability of water extract viscosity of grain meal was rather high ($H^2 = 0.71$), and genotypic variation coefficient reached 32.53 %, indicating advisability of VWE improving by breeding techniques. Heritability index of water-soluble pentosan content was 0.50, and genotypic coefficient of variation was 13.02 %, so the impact of breeding on these indicators should be low. The presented genotypic variability parameters are applicable only to the genotypes used in our experiment. The smallest amounts of water-soluble pentosans in flour and meal were characteristic of the Russian varieties Marusenka, Ogonek, Chulpan 7. We revealed a low content of water-soluble fraction in the bran in variety Ogonek. To distinguish rye genotypes more precisely, it is necessary to develop effective tests which will allow to assess water absorption, viscosity and solubility of pentosans (high-molecular arabinoxylans) in addition to their quantitation in grain grind products.

Keywords: winter rye, pentosans, fractions, arabinoxylan, viscosity of water extract, meal, variety, lines, heritability, genotypic variability

Rye is superior to wheat in minerals and lysine and dietary fiber (cellulose) [1, 2]. In addition to starch, amounting 65 % of dry matter, the richest components in whole grain rye are non-starch polysaccharides (NSPs) (17 %).

These macromolecules are the main elements of cell walls. The main non-starch polysaccharides are arabinoxylans (AXs) which make about 8 % of ray grain and are synthetized from two sugars, L-arabinose and D-xylose [3-5].

The content and qualitative composition of pentosans, as well as their state (water absorption, viscosity, solubility in water), determine the diversification of rye grain use [6-8]. Good organoleptic and baking quality, freshness and dietary properties of rye bread are due to starch-arabinoxylan complexes [9-12]. At the same time, pentosans, more specifically, high-molecular arabinoxylans, are the main factor limiting using rye when for feeding animals, especially monogastric [13, 14].

The molecular structure and structural organization of arabinoxylans determine their physical properties. Long molecules of soluble pentosans can form a network, possessing high absorption capacity, and sticky gel-like solutions. The anti-nutritional properties of pentosans are due to the ability to bind water, the amount of which can be 10 times greater than their own weight. In the digestive tract of the most sensitive groups of farm animals (poultry and swine), when eating food made of rye grain, a highly viscous suspension is formed that envelops the granules of starch and proteins, which limits the absorption of already digested protein, starch, fat and other nutrients. As a result, indigestion, weakening and a decrease in productivity occur. A decrease in the amount of pentosans, primarily the water-soluble group, contributes to the improvement of forage properties of rye grain [15]. According to D. Boros et al. [16], the number of pentosans varies depending on the genotype from 35 to 88 mg/g, which allows selection of forms with a high and low content of the water-soluble fraction. As per H.-U. Jürgens et al. [17], the total number of pentosans varied from 89 to 103 mg/g, and the extractable viscosity ranged from 2.6 to 5.1 centistokes (cSt).

Many researchers suggest an indirect method of measuring amount of pentosans by viscosity of grain meal water extract (VWE) [18-20]. The viscosity of the water-protein meal suspension can serve as an integral indicator of the quality of breeding material, delimiting rye lines for bakery and fodder [22]. The heritability of this property differs in divergent selection and depends on the genotype [15, 22, 23]. In this case, the VWE value depends both on genotypic [24, 25] and environmental factors [26, 27]. The greatest differentiating ability is manifested in the years with optimum and average arid weather conditions during the filling period, which are most favorable for selection for this feature [28)]. In connection with the foregoing, it is important to identify the economic value of each variety of winter rye as a raw material.

In the present study, we identified rye genotypes, contrasting in pentosans, by high-performance liquid chromatography and extracted viscosity and obtained mapping hybrid populations to be used in marker-assisted and traditional selection.

The aim of this paper was to estimate the variability of total pentosans and the content of water-soluble fraction of arabinoxylans in Russian and foreign populations, hybrids and lines of winter rye.

Techniques. Studies of winter rye (*Secale cereale* L.) grain were carried out in 2010-2016 at the Tatar Scientific Research Institute of Agriculture (TatNIISH). Population varieties of Russian and own selection, samples from the EU collection, low pentosans and high pentosans lines, hybrids of the second generation, obtained at TatNIISH by crossing contrasting lines, and hybrid varieties of KWS Lochow GmBH (Germany) were studied. A total of 110 samples were analyzed. Plants were grown annually in competitive variety testing, in collection nursery and in hybrid nurseries. Two weeks after harvesting, the grain was ground using a Laboratory Mill 3100 (Perten Instruments, Germany), flour and meal were sampled as per State

Standard GOST 13586.5-85. Pentosans were quantified by high-performance liquid chromatography in a HPLC-RI system (JASCO Deutschland GmbH, Germany) using a RF-10AXL fluorescent detector and 465 autosampler (Kontron Instruments, Germany) [17]. The qualitative composition of pentosans was evaluated according to the total number of arabinoxylans (total arabinoxylans, TAX) and the fraction of water-soluble arabinoxylans (soluble arabinoxylans, SAX).

The extraction of the samples to determine the content of water-soluble pentosans (WSP) and the viscosity of water extract (VWE) was carried out according to the procedure described by D. Boros et al. [16]. Viscosity of water extracts was measured using a capillary viscometer VPZH-1 (Labtech, Russia) with a capillary diameter of 1.52 mm [19]. The ratio of meal and water was 1:5. The kinematic viscosity of the water extract was calculated in centistokes according to the formula $V = g/9.807 \cdot T \cdot K$, where K is the constant of the viscometer, mm^2/s^2; V is the kinematic viscosity, mm^2/s; T is the time of flow of the liquid, s; g is the acceleration of gravity at the place of measurement, m/s^2. The content of water-soluble pentosans was determined by the micro-method with orcine chloride [29], as modified for rye grain [30].

Phenotypic and genotypic variance, as well as coefficient of variation calculated based on a two-factor dispersion analysis with the Duncan multiple rank test [31]. To evaluate the genotypic variability and calculate the coefficient of heritability, the corresponding formulas [31, 32] were applied:

$$\sigma_p^2 = \sigma_g^2 + \sigma_e^2,\ \sigma_g^2 = \frac{M_g - M_e}{r},$$

$$PCV = \frac{\sqrt{\sigma_p^2}}{\bar{x}} \times 100\ \%,\ GCV = \frac{\sqrt{\sigma_g^2}}{\bar{x}} \times 100\ \%,\ H^2 = \frac{\sigma_g^2}{\sigma_p^2},$$

where σ_p^2 and σ_g^2 are the phenotypic and genotypic dispersion, respectively; σ_e^2 is dispersion of the residue, M_g is the mean square for the variants, M_e is the mean square for the residue, r is the number of replicates, PCV is the phenotypic coefficient of variation, %, GCV is the genotypic coefficient of variation, %, $\bar{x}$ is the mean value of the characteristic, H^2 is coefficient of heritability. The statistical processing was performed using the Excel 7.0 software package. The tables show the mean values (*X*) and mean errors (±Sx).

Results. Among the samples studied, a significant number of bakery varieties with high level of SAX and WSP, which have a beneficial effect on the formation of rye dough, stability of its form and the quality of bread as a whole, were identified (Table 1). Recombination breeding using European varieties and samples from the VIR collection, as well as hybrid varieties, will allow creating forms suitable for bakery. Domestic population varieties and high-pentosan selection

1. Viscosity of water extracts (VWE) and the pentosan fractions in winter rye (*Secale cereale* L.) grain (Tatar Agricultural Research Institute, 2010-2016)

Origin of samples	Number of samples	SAX, %	TAX, %	VWE, cSt	WSP, %
Population varieties of Russian selection	7	3.82±0.21	12.04±0.41	23.6±2.2	4.55±0.13
		3.12-4.66	10.53-13.72	15.4-34.5	4.03-4.97
Samples from the collections of the European Union	7	5.02±0.07	11.86±0.43	–	–
		4.74-5.35	10.86-13.8		
Low pentosan lines of the Tatar Agricultural Research Institute	10	3.76±0.13	11.04±0.18	9.5±1.2	3.33±0.39
		3.03-4.43	10.19-11.74	6.4-18.2	1.90-4.41
High pentosan lines of the Tatar Agricultural Research Institute	5	3.96±0.16	12.30±0.19	28.1±2.5	5.63±0.59
		3.65-4.42	11.68-12.82	23.5-34.7	4.59-7.14
F_2 hybrids of Tatar Scientific Research Institute	10	4.30±0.10	11.11±0.15	12.5±0.8	1.78±0.04
		3.70-4.90	10.60-12.07	9.9-18.5	1.63-1.97
Hybrid varieties of German selection	18	4.76±0.13	11.65±0.21	22.6±4.8	1.84±0.10
		3.93-5.48	10.60-12.85	8.0-47.5	1.48-2.41

N o t e. SAX — water-soluble arabinoxylans, TAX — totsl arabinoxylans, VWE — viscosity of water extract (cSt, centistokes), WSP — water-soluble pentosans. The mean values and the error of the mean (*X*±Sx) are above the line, the minimum and maximum values (min-max) are below the line. Dashes mean the absence of data.

lines of TatNIISH were distinguished by high indices of the total pentosan content (TAX) and extractable viscosity (VWE).

It was found that VWE of grain meal has a wider range of variability than WSP does. Thus, in several low pentosan lines of our selection, the VWE was 6.4-6.45 cSt. In F_2 hybrids, an intermediate inheritance of the characteristic was identified. Russian population varieties had a VWE in the range of 15.4-34.5 cSt, while in German hybrid varieties it reached 47.5 cSt.

2. Parameters of genotypic variability and heritability of water extract viscosity and the content of water-soluble pentosans in winter rye (*Secale cereale* L.) grain meal (Tatar Agricultural Research Institute, 2010-2016)

Trait	GCV, %	PCV, %	σ_p^2	σ_g^2	H^2
Viscosity of water extracts	32.53	38.58	67.068	94.336	0.71
Content of water-soluble pentosans	13.02	18.39	0.076	0.152	0.50

N o t e. PCV — phenotypic coefficient of variation, GCV — genotypic coefficient of variation, σ_p^2 — phenotypic dispersion, σ_g^2 — genotypic dispersion, H^2 — coefficient of heritability.

Variance analysis showed significant genotypic differences ($P \leq 0.05$) both for VWE ($F_{fact.} = 15.76 > F_{theor.} = 2.18$) and WSP ($F_{fact.} = 7.03 > F_{theor.} = 2.42$) for the samples under study (Table 2). Using the heritability coefficient, we attempted to identify the proportion of the observed variation in the studied features, which depends on the genotypic differences, in the overall phenotypic variability. For the viscosity of the water extract of the grain meal $H^2 = 0.71$, which indicates the expediency of improving this feature using selection methods (see Table 2). It is also shown that 50 % of phenotypic variability in the amount of water-soluble pentosans is due to hereditary characteristics, that is, the effectiveness of selection for this trait will be low. It should be noted that heritability as a measure of a relative contribution of genetic and environmental differences to phenotypic variability has a number of limitations. In particular, heritability does not serve as an attribute of the feature, but depends on the composition of the genotypes of the population under study [33]. In another population with a different composition of genotypes, the heritability of the same feature may be different. The relative values of GCV and PCV give an idea of the magnitude of variability in the studied gene pool (see Table 2). The genotypic coefficient of variation for VWE was high (32.53 %), which influences the choice of the selection method. The differences observed in the genotypic and phenotypic coefficient of variation were practically equal for both indices.

By correlation analysis, a highly significant positive relationship between TAX and VWE ($r = 0.736$, $P = 0.05$), TAX and WSP ($r = 0.639$, $P = 0.05$) was established in a group of Russian population varieties. In high pentosan lines, created at the Tatar Agricultural Research Institute, $r = 0.790$ ($P = 0.01$) for the first pair of features, and $r = 0.812$ ($P = 0.01$) for the second one. The correlation coefficients for VWE and WSP were close to 1, that is, the content of water-soluble pentosans (arabinoxylans) in selecting forms for use in bakery can be estimated by VWE value of grain meal.

Low pentosan forms which may be used in breeding are the rarest. During their 10-year selection and study at TatNIISKH, it was shown that the average value of SAX in such samples was 3.76 % with a range from 3.03 to 4.43 % (see Table 1), which was significantly lower than that in the hybrids of German selection and in thhe collection forms. The differences in viscosity were even more pronounced. It should be noted that for now we can not specify the limiting parameters for the studied indicators for feed and bakery varieties. The smallest amount of pentosans was in flour and meal of the Marusenka, Ogonyok, Chulpan 7 grain, and a low amount of soluble arabinoxylans was detected also in bran of Ogonyok variety (Fig. 1). In all the studied varieties, the content of pentosans was the greatest in bran. This can be explained by the fact that the hull of rye

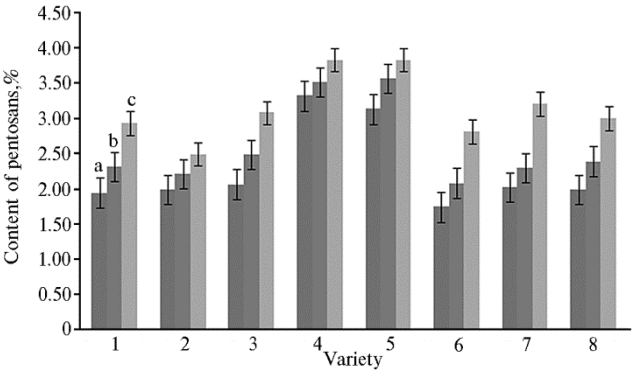

Fig. 1. The content of pentosans in flour (a), meal (b) **and bran** (c) **in rye (*Secale cereale* L.) population varieties:** 1 — Tatarskaya 1, 2 — Ogonyok, 3 — Tantana, 4 — Pamyati Kunakbayeva, 5 — Roksana, 6 — Marusenka, 7 — Tatyana, 8 — Chulpan 7 (Tatar Scientific Research Institute of Agriculture, 2013-2014).

grain and the aleurone layer contain up to 50 % of pentosans, while the germ and the starchy part of the endosperm, from which the flour is produced, contain no more than 15 %. The varieties Pamyati Kunakbayeva and Roksana had a higher content of pentosans in all grinding products.

Wide screening of the breeding material involves the use of a low-cost, but objective methodology that makes it possible to quickly differentiate the created varieties for their further use. An indirect evaluation of the pentosan fraction through by viscosity of the water extracts of rye meal provides early selection and analysis of a significant number of samples during relatively short time.

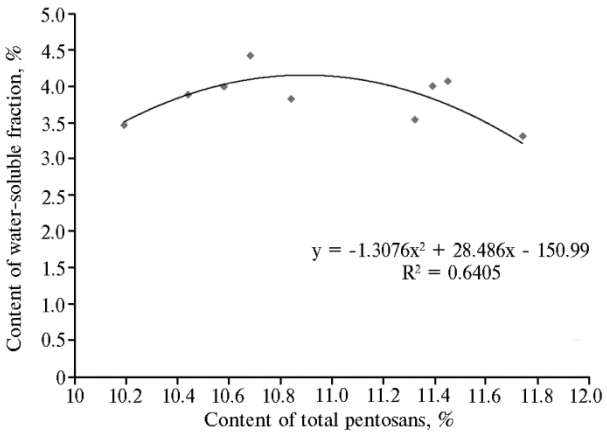

Fig. 2. Interrelation of the content of total pentosans and their water-soluble fraction in grain meal in low pentosan winter rye (*Secale cereale* L.) (Tatar Scientific Research Institute of Agriculture, 2013-2014).

The relationship between VWE and the content of water-soluble pentosans in the studied quantitative limits with high probability (95 %) was of a straightforward nature and was described by the following regression equation: $y = 0.0335x + 1.5401$, where y is the content of pentosans in the grain, %, x is viscosity of the grain meal extract, cSt.

In low pentosan lines, a significant correlation between VWE and WSP was found ($r = 0.754$, $P = 0.05$). Regression analysis showed a non-linear relationship between the amount of total arabinoxylans and their water-soluble fraction (Fig. 2). The F_2 hybrids we obtained between the contrast genotypes from Russian and foreign gene pools are of great interest. They had comparatively low VWE and WSP. Consequently, a small number of sources of low content of pentosans have been identified, the phenotypic assessment of which by VWE indirect index is difficult.

Thus, our studies have shown a sufficient gene pool for selection of bakery rye. The Russian varieties Marusenka, Ogonek, Chulpan 7 with the least amount of water-soluble pentosans in flour and meal were distinguished. A highly significant positive relationship is established between the total content of pentosans, the viscosity of the water extract and the fraction of water-soluble arabinoxylans. It is shown that the grain meal VWE value may serve as an indicator of the content of water-soluble pentosans (arabinoxylans). The phenotypic evaluation of the low-pentosan forms, based on the indirect VWE index only, is difficult. The inheritance of the viscosity of the grain meal water extract in our

experiments was $H^2 = 0.71$, the genotypic coefficient of variation was 32.53 %. According to the content of water-soluble pentosans, $H^2 = 0.50$, the genotypic coefficient of variation was 3.02 %; therefore, the selection efficiency for this property will be low. The presented indexes of genotypic variability are applicable only in respect to the genotypes used in our experiment. In addition to the quantitative evaluation of pentosans (high molecular arabinoxylans) in the products of grain grinding, low cost methods to analyze their propertied (water absorption, viscosity and solubility) are required, which will make it possible to differentiate the samples more clearly.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1049-1055
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 635.64:631.52:631.527.5

doi: 10.15389/agrobiology.2017.5.1049rus
doi: 10.15389/agrobiology.2017.5.1049eng

ANALYSIS OF HYBRIDIZATION EFFECT BY THE APPEARANCE OF TARGET TOMATO TRAITS IN F_2, F_3 PROGENIES IN BREEDING FOR MULTI CIRCLE HYDROPONICS

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Received March 17, 2017

A b s t r a c t

Classic genetic methods remain actual in practice and study of inheritance and heritability of the main commercial crop traits. *Solanum lycopersicum* L. genetics is well developed, but the special approach is necessary to solve special breeding tasks. Heritability analysis of the main traits in F_1 tomato progeny, which we have been carried out in 2009-2011, revealed some regularity to be further used in breeding practice. We first found that the main fruit yield parameters of *Solanum lycopersicum* L., the average fruit weight ($h^2 = 0.99$) and the average fruit number per plant ($h^2 = 0.96$), are inherited on the maternal side, and dwarfism ($h^2 = 0.83$) and early ripening ($h^2 = 0.73$) are inherited on the paternal side. Effectiveness of the target hybridization method developed earlier has been tested in this paper. Productive maternal plants with larger-sized fruits and early ripening dwarf paternal plants were involved in target crossings. F_1 hybrids and their F_2 progeny resulted from self-pollination of F_1 plants were produced. Analysis of dwarfism inheritance in three F_2 hybrid combinations using χ^2 criterion confirmed recessiveness of *d* gene. The tall plants and the dwarf plants of F_2 population segregated strictly by Mendel's low (3:1). By dispersion analysis of six parental forms and three hybrids, we selected the more productive plants with large fruit size among the dwarf plants, then obtained seed progeny of these plants and studied the heritability of two traits, the dwarfism and large fruit size, in the F_3 hybrids. It was found out that crossing between tall maternal plants with large fruit size and dwarf early ripening paternal plants resulted in lowering the plant height to that of dwarf father. This trait was maintained in F_3 that confirmed the correctness of conclusions have earlier been made by us. Heritability of average fruit weight on the maternal side also has been confirmed in F_3 progeny. In F_3 hybrids derived from crossing maternal plants Vspishka and Krainiy Sever with large fruit size the average fruit weight increased 2 times compared to the parental forms. This trait is maintained in progeny despite negative effects of *d* genes on some quantitative characteristics. Use of high productive maternal forms with small fruits size resulted in lowering average fruit weight in the hybrid progenies. So, dwarfism of *Solanum lycopersicum* L., desirable in multi circle hydroponic technology, is inherited on the parental side, and the fruit weigh is inherited on the maternal side. Thus, to obtain new tomato forms for multi circle hydroponics, the maternal plants with large fruit size and dwarf paternal forms should be crossed.

Keywords: tomato, breeding, heritability, dwarfism, fruit weight

Tomato (*Solanum lycopersicum* L.) is a crop which has been most genetically [1-5]. At the end of the last century, S.D. Tanksley and M.A. Mutschler compiled a classic map of 12 tomato chromosomes where they have indicated several linkage goups [6]. Wide-scale molecular and genetic studies have significantly advanced mapping tomato genome [5, 7-10] and provided for success in solving a number of selection problems, e.g. in mapping the dominant genes at simple trait inheritance [11, 12]. In other cases, traditional genetics is used in dealing with-inheritance of the main valuable traits [13-17].

Dwarfism in *Solanum lycopersicum* L. is controlled by the family of *d* genes located in the long arm of the chromosome 2, which are associated with biosynthesis of brassinosteroids and show 11 alleles [2, 6, 18]. Obtaining dwarf hybrids combining dwarfism and early ripening and high productivity is hindered

due to several characteristics of the *d* genes, i.e., negative effect on the fruit weight and recessiveness (this trait, according to Mendel's second law, is expressed only in a fourth part of F_2 progeny according to 3:1 segregation [2]. Positive characteristics of the *d* genes that can be used in selection are location in chromosome 2 close to the genes controlling early ripening (they can be inherited together due to linkage) [2] and early manifestation during plant development which allows for sporophyte selection, speeding up breeding three-fold [19].

Previously, in analysis of the inheritance of the main economically valuable traits in F_1 generation carried out in 2009 to 2011 based on the collection of tomato marker mutants (maternal forms), it has been shown that the main characteristics of productivity, average fruit weight ($h^2 = 0.99$) and average fruit number per plant ($h^2 = 0.96$) are inherited by maternal line [20],and dwarfism ($h^2 = 0.83$) and early ripening ($h^2 = 0.73$) are inherited by paternal line [21].

In this paper, we have for the first time demonstrated the efficiency of the target hybridization method developed earlier based on the pre-breeding data [15-19].

Our aim was to determine the character of manifestation of dwarfism and average fruit weight in F_2 and F_3 generations in the new forms of *Solanum lycopersicum* L. tomato meant for multi-circle narrow-shelf hydroponics.

Techniques. Basing on the pre-breeding data [19-21], we have carried out a target selection of maternal and paternal forms. The maternal forms were mostly selected by large fruits and productivity; the paternal ones were selected by dwarfism and early ripening. Part of these starting forms was used for target crossings, as a result of which the F_1 hybrids (2011) were obtained. The F_2 generation (2012) was obtained from the self-pollination of the hybrids.

The experimental material in 2013 was three F_2 hybrid combinations of varieties and samples: Mo 411 × Komnatnaya Grusha, Vspyshka × Tiny Team, Krainiy Sever × Komnatnyi, 6 parental forms and 3 F_3 hybrids. Morphological description of plants, biometry of the main parameters, assessment of productivity and average fruit weight were carried out using the weight method. After segregation in height in the F_2 hybrid progeny, the most productive and large-fruit plants from the dwarf ones were selected. In 2014, dwarfism and average fruit weight were determined in the F_3 progenies of three hybrid forms, the parents of which were 2 maternal forms with large fruits, 1 highly productive but small-fruit maternal form and 3 dwarf and early ripening paternal forms.

The studies were carried out in a polycarbonate greenhouse (Richel, France) in planting section (2013) and with the original installation of five-circle narrow-shelf hydroponics with a FITO, Russia medium unit (2014). The repetition of the experiments was 5- (2013) and 10-fold (2014).

The statistical processing of the data was carried out using dispersion analysis according to B.A. Dospekhov [22].

Results. The key characteristics of the starting parental forms used earlier in target hybridization are outlined in Table 1. After their crossings, the F_1 generation was obtained, from which the F_2 generation was obtained from self-pollination. The most large-fruited and productive samples were selected from the dwarf progeny segregated according to the plant height in the 3 (tall plants):1 (dwarf plants). As a result, in the F_2 7 samples were selected from the dwarf plants (Table 2) to obtain seeds.

The progeny of the plants No. 1 from F_2 Mo 411 × Komnatnaya Grusha, No. 8 from F_2 Vspyshka × Tiny Team, No. 1 from F_2 Krainiy Sever × Komnatnyi was used for analysis of the hybridization efficiency in F_3 in studying the degree of manifestation of key traits (dwarfism and average fruit weight). Cross-

ing of the large-fruited and tall maternal form with the dwarf paternal one has led to a decrease in the plant height in all hybrids to the parameters observed in the dwarf father. The trait was maintained in the F_3 generation (Table 3), which confirms the conclusions made in pre-breeding about the inheritance of dwarfism in the paternal line [21]. The inheritance of the fruit weight in the maternal line established in pre-breeding studies [20] was also confirmed in the F_3 generation. An increase in the average fruit weight (almost 2-fold compared to that in the small-fruited paternal form) was observed only in those F_3 hybrids that derived from the large-fruited maternal forms Vspyshka and Krainiy Sever (Table 3). The trait was maintained in the progeny despite the obvious negative effect of the *d* genes on some quantitative traits. The use of the highly productive but small-fruited maternal form Mo 411 has led to a decrease in the fruit weight in the hybrid (see Table 3).

1. Characterization of starting tomato (*Solanum lycopersicum* L.) parental forms resulted from pre-breeding for the multi-circle narrow-shelf hydroponics (Richel polycarbonate greenhouse, All-Russian Research Institute for Breeding and Seed Production of Vegetable Crops, Moscow Province, 2009-2011)

Sample	Plant productivity, g		Fruit weight, g		Number of fruits per plant, psc.		Plant height, cm		Sprouting—ripening, days	
	$\bar{X}$	Δ	$\bar{X}$	Δ	$\bar{X}$	Δ	$\bar{X}$	Δ	$\bar{X}$	Δ
St (Funtik)	221		53		4		46		110	
Maternal forms										
Mo 411	614	+393	16	–37	34	+30	91	+45	106	–4
Vspyshka (1C)	846	+625	79	+26	11	+7	75	+29	96	–14
Krainiy Sever	842	+621	95	+42	9	+5	88	+42	108	–2
	LSD_{05}	129	LSD_{05}	16	LSD_{05}	3	LSD_{05}	16	LSD_{05}	7
Paternal forms										
Komnatnaya Grusha (3C)	248,6	+27,6	11	–42	21	+17	42	–4	103	–7
Tiny Team (11C)	303,8	+82,8	12	–41	21	+17	36	–10	105	–5
Komnatnyi	292,2	+71,2	19	–34	20	+16	33	–13	108	–2
	LSD_{05}	76,0	LSD_{01}	12	LSD_{01}	10	LSD_{01}	16	LSD_{05}	7

Note. X — average trait value, Δ — standard deviation (Funtik variety).

2. Productivity and perspective dwarf tomato (*Solanum lycopersicum* L.) hybrids in F_2 generation (Richel polycarbonate greenhouse, planting section, All-Russian Research Institute for Breeding and Seed Production of Vegetable Crops, Moscow Province, 2013)

Plant No.	Productivity of one plant in repetitions, g					Σ_v	$\bar{X}$	Deviation from St	Group
	1st	2nd	3rd	4th	5th				
St	170	155	165	265	180	935	187		
Dwarf hybrids F_2 Mo 411 × Komnatnaya Grusha									
1[a]	234[a]	150[a]	275[a]	220[a]	285[a]	1164[a]	233[a]	+46[a]	I
2	80	105	75	98	85	43	87	–100	IV
6	15	15	35	15	22	102	20	–167	IV
14	72	86	88	90	60	396	79	–108	IV
21	133	175	185	115	205	813	163	–24	III
30	70	35	25	70	90	290	58	–129	IV
39	152	150	110	150	130	692	138	–49	IV
44	85	100	125	110	90	510	102	–85	IV
46[a]	225[a]	249[a]	200[a]	205[a]	265[a]	1144[a]	229[a]	+42[a]	I
47	50	60	65	110	70	355	71	–116	IV
48	170	128	135	207	125	765	153	–34	III
50	175	139	110	210	164	798	160	–27	III
LSD_{05}								38	
Dwarf hybrids F_2 Vspyshka × Tiny Team									
3	109	186	139	150	118	702	140	–47	III
5	140	134	232	135	190	831	166	–21	III
8[a]	108[a]	295[a]	253[a]	195[a]	190[a]	1041[a]	208[a]	+21[a]	II
10	64	60	65	78	80	341	69	–138	IV
22	150	135	194	140	120	739	148	–39	III
26	35	66	80	60	70	311	62	–125	IV

Continued Table 2

33[a]	255[a]	190[a]	145[a]	205[a]	152[a]	947[a]	189[a]	+2[a]	II
49	32	48	42	45	50	217	43	–144	IV
LSD_{05}								50	
Dwarf hybrids F2 Krainiy Sever × Komnatnyi									
1[a]	227[a]	220[a]	195[a]	250[a]	253[a]	1145[a]	229[a]	+42[a]	I
2	95	54	50	52	60	311	62	–125	IV
4	132	170	145	108	130	685	137	–50	IV
10[a]	175[a]	140[a]	248[a]	195[a]	180[a]	938[a]	188[a]	+1[a]	II
11	145	260	140	158	150	853	171	–16	III
28	125	120	145	130	100	620	124	–63	IV
38	140	130	141	90	120	621	124	–63	IV
46	55	40	35	90	75	295	59	–128	IV
47[a]	210[a]	230[a]	224[a]	190[a]	205[a]	1059[a]	212[a]	+25[a]	II
48	95	85	80	110	90	460	92	–95	IV
49	168	165	110	145	130	718	144	–43	IV
51	95	85	60	74	80	394	79	–108	IV
LSD_{05}								35	

Notes. St — standard (Funtik variety), Σ_v — sum of variants, X — average trait value; [a] — perspective samples selected for seed production. The groups mean deviations of the average square (dispersion) of the set mean value from the average square (dispersion) of the general mean value.

3. Plant height and fruit weight of tomato (*Solanum lycopersicum* L.) parental forms and hybrids in F_3 (Richel polycarbonate greenhouse, five-circle narrow-shelf hydroponics unit, All-Russian Research Institute for Breeding and Seed Production of Vegetable Crops, Moscow Province, 2014

Genotype	Repetition										$\bar{X}$
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	
Average plant height, cm											
Mo 411	95	90	93	90	88	92	90	90	93	95	91.6
Komnatnaya Grusha	38	33	35	35	35	33	35	38	35	37	35.4
F_3 Mo 411 × Komnatnaya Grusha	33	37	33	38	35	32	30	37	35	30	34.0
LSD_{05}											2,5
Vspyshka	53	57	57	55	55	60	50	55	53	53	54.8
Tiny Team	37	35	37	32	38	40	33	37	45	37	37.1
F_3 Vspyshka × Tiny Team	43	40	38	37	42	38	40	38	37	32	38.5
LSD_{05}											4,3
Krainiy Sever	58	50	55	55	58	57	53	57	55	57	55.5
Komnatnyi	32	28	30	27	28	27	28	28	28	28	28.4
F_3 Krainiy Sever × Komnatnyi	18	17	22	15	20	18	22	18	22	17	18.9
LSD_{05}											1.7
Average fruit weight, g											
Mo 411	14	18	12	15	12	15	16	17	13	17	14.9
Komnatnaya Grusha	10	12	8	10	12	9	11	11	8	10	10.1
F_3 Mo 411 × Komnatnaya Grusha	9	10	9	9	9	8	9	7	9	8	8.7
LSD_{05}											1.0
Vspyshka	74	84	77	84	74	71	84	79	88	84	79.9
Tiny Team	9	10	8	10	11	11	8	12	10	9	9.8
F_3 Vspyshka × Tiny Team	18	14	19	16	22	21	18	14	16	18	17.6
LSD_{05}											3.7
Krainiy Sever	27	48	37	46	50	46	38	26	56	50	42.3
Komnatnyi	7	8	7	9	10	7	8	6	10	9	8.1
F_3 Krainiy Sever × Komnatnyi	17	16	14	16	14	18	12	16	14	12	14.9
LSD_{05}											1.5

Note. X — average trait value.

Currently, most genetics papers are dedicated to mapping genes, analysis of their interaction and mapping quantitative trait loci (QTL), whereas in practical selection there is a lack of data about inheritance of selection valuable traits. Despite significant successes of biotechnological approach to realization of genetic information in the progeny [9-12], hybridization remains the key method for production of new forms and is inscribed in the modern organic agriculture concept [13-15]. Data about inheritance and heritability of traits are still required, although today these are few and obtained mostly in diallel crossing. Thus, Serbian scholars, studying the results of diallel crossings of six tomato genotypes, found that genotypes with high number of fruits per plant and high fruit weight can be effectively involved in crossings and selection for high yield

[23]. This corresponds to our data.

Thus, classic genetics is still successfully applicable in selection practice and do not contradict to necessity of genome mapping in the main agricultural crops. In *Solanum lycopersicum* L., dwarfism, a trait that is required for multi-circle narrow-shelf hydroponics technology of tomato growing, is inherited in paternal line, and fruit weight is inherited in maternal line. In order to obtain dwarf forms with 30 to 50 g fruits, the large-fruited tomato forms should be involved in crossings as maternal parents.

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AGRICULTURAL BIOLOGY,
2017, V. 52, № 5, pp. 1056-1064
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 634.722:581.1:632.112

doi: 10.15389/agrobiology.2017.5.1056rus
doi: 10.15389/agrobiology.2017.5.1056eng

PHYSIOLOGICAL FEATURES OF RED CURRANT VARIETIES AND SELECTED SEEDLING ADAPTATION TO DROUGHT AND HIGH TEMPERATURE

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The authors declare no conflict of interests
Received May 25, 2016

Abstract

Adaptation to weather conditions is an important feature of cultivars. To assess plant adaptability, we applied a physiological test of *Ribesia* (Berl.) Jancz. leaf resistance to abiotic factors during vegetation. The effect of high temperatures and drought on red currant plant photosynthetic apparatus and water regime was studied in 2011-2013 in the Central-Chernozem region (Orel region). Five varieties and three selected seedling genotypes of different eco-geographic and genetic origin were used including the derivatives of *Ribes petraeum* Wulf., *R. vulgare* Lam. and *R. multiflorum* Kit. (of these, six genotypes were originated in VNIISPK). Leaves of red currant plants are known to have a mesomorphic structure characteristic of *Ribesia* (Berl.) Jancz. subgenus. In this paper, it was shown that the morphological characteristics of leaves (i.e., shape, venation) are determined by the biological features of varieties while growing conditions alter the anatomical structure. We found a positive correlation between the leaf area and hydrothermal coefficient ($r = +0.99$) and negative correlation between the leaf area and environment temperature ($r = -0.97$). An increase in leaf thickness, the expansion of spongy parenchyma cells, a decrease in chlorophylls (both Chla and Chla + Chlb), as well as elevated carotenoid level are the response to stress factors during vegetation. The ratio coefficient of chlorophyll sum to carotenoids is considered as one of the adaptation indices. High coefficients were revealed in Hollandische Rote variety (5.14) and 1426-21-80 (5.51). Correlations between the chlorophyll sum and water loss ($r = -1.00$) as well as chlorophyll sum and fraction of available water ($r = +0.98$) were ascertained. The pigment content, fractional composition of water and water holding capacity of leaves are interconnected with the water shortage. The positive correlation of air temperature and water deficit ($r = +0.84$) has been noted. The total water content in red currant leaves depends on the shoot growth, leaf age, variety, meteorological conditions and is not the main indicator of resistance to high temperatures and drought. Ratios of bound and free water and water holding capacity of leaves vary depending on meteorological conditions and water availability. In 2012 the weather conditions were unfavorable, and in all red currant genotypes the increase of the coefficient of bound water to available water as well as drop in water loss were observed. According to the parameters of water regime, Hollandische Rote variety and 1426-21-80 displayed high adaptability. The laboratory diagnostic methods were confirmed by the field data of genotype resistance under high temperature and moisture deficiency. Thus, the physiological express tests are suitable for estimation of red currant plant adaptability to environmental factors in the course of breeding. A comprehensive assessment showed diverse ecological plasticity in the plants with different *Ribesia* (Berl.) Jancz. subgenus species in the pedigree. The derivatives of *Ribes petraeum* Wulf. (Hollandische Rote) and *R. multiflorum* Kit. (1426-21-80) showed the highest tolerance to drought conditions during the vegetation season in the Central Chernozem region. The varieties and seedlings derived from *R. vulgare* Lam. had low drought resistance.

Keywords: red currant, drought resistance, leaf, mesostrurture, pigments, fractional structure of water, water holding ability

Red currant is one of valuable berry crops having high yield, early maturity, and good food qualities of berries. A total of 50 to 80 % of its yield losses are accounted for the negative effect of natural climatic factors. The most important of these is an increase in the instability and stress of weather conditions [1, 2]. In the Central Black Earth Region, such stressors include drought caused

by high temperatures. An increase in temperature causes morpho-anatomical, physiological and biochemical alterations that affect the growth and development of plants and can lead to a reduction in commercial yield [3-5]. Studying the morphological features, the structure of the photosynthetic apparatus and water exchange of plants with regard to the growth area are necessary for solving fundamental and applied problems of the biology of red currant. Currently, physiological and biochemical express methods are appreciated in estimation of plant resistance to adverse weather and climatic factors that makes it possible to significantly optimize breeding, to minimize yield losses and to create genotypes resistant to the destructive effect of climatic anomalies [6].

Plants have various mechanisms of adaptation to stressors [8-10]. The xenomorphic leaf structure, alterations in the pigment complex and water balance are important diagnostic features of drought resistance [11-13]. Thus, at increasing drought, palisade mesophyll cells of black currant *Ribes americanum* Mill. increase in size, and the amout of retained water in cells also increases. In some red currant varieties derived from *R. vulgare* Lam. species (Gollandskaya rozovaya, Natali krasnaya), the content of the total a and b chlorophyll and photosynthesis decrese [14-16]. A similar regularity is characteristic of strawberry, grapes, chestnut, tea plants (*Camellia sinensis*) and olive trees (*Olea evropaea* L.) under increasing temperature [17-20]. In fruit, vegetable and leguminous crops, a decrease in water retaining during the light day, an increase in transpiration and alterations in the ratio of water forms in various plant parts were observed [21-23]. Adaptation of berry crops including red currant to stressors during vegetation period is poorly studied and requires a more detailed investigation.

This paper is the first reporting application of physiological methods to detect resistance of red currant leaf photosynthetic apparatus to adverse factors during vegetation.

The aim of our study was to investigate water regime, photosynthesis and productivity in various species of *Ribesia* (Berl.) Jancz. subgenus, and also to identify varieties and breeding forms resistant to drought and elevated temperature.

Techniques. The observations were carried out in 2011-2013 at the primary nursery for red currant (All-Russian Research Institute for Breeding Fruit Crops, ARRIBFC). Aridness during the vegetation periods was assessed by a hydrothermal coefficient (HTC). Eight red currant genotypes were studied, including 6 cultivars of ARRIBFC (Dana, Niva, Roza, 1518-37-14, 1426-21-80, 1432-29-98) and 2 foreign ones (Jonkheer Van Tets, Hollandische Rote). The samples had different genetic, ecological and geographical origins and were derivatives of the *Ribes petraeum* Wulf. (Hollandische Rote, 1518-37-14), *R. vulgare* Lam. (Jonkheer Van Tets, Niva, Roza), and *R. multiflorum* Kit. (Dana, 1426-21-80, 1432-29-98) species.

Morpho-anatomical structure of leaves was assessed by studying their mesostructure [24] using a microscope Eclipse 50i (Nikon, Japan, ×400). The pigment content was determined in 80 % acetone extract [25] using a Smart Spec^{TM}Plus (Bio-Rad, USA) spectrophotometer. The water regime parameters were determined according to the developed techniques [11, 26, 27].

Dispersion, correlation and regression analyses were carried out at 95 % significance level [28] using the Microsoft Excel 2010 software package.

Results. The weather conditions varied strongly over the years of studies. From May to June of 2011, there was a combination of high water supply and elevated temperatures (HTC = 0.93; 1.12; 2.10; +30.0...+31.5 °C). 2012 was the most arid year (HTC = 0.20; 0.82; 0.40; +31.2...+32.2 °C). The vegetation period of 2013 was intermediate according to hydrothermal conditions (HTC = 0.72; 0.89; 0.68; +28.6...+31.5 °C).

Morphological variability and ecological plasticity are characteristic of *Ribesia* (Berl.) Jancz. subspecies. Red currant belongs to mesophytes. All its varieties and forms are derived from wild species (*Ribes petraeum* Wulf., *R. rubrum* L., *R. vulgare* Lam., *R. multiflorum* Kit.), growing in the intermediate zone of the Northern hemisphere in the regions with high soil humidity and average air temperature during vegetation. Europe is believed to be the origin of the *R. vulgare* Lam., while *R. petraeum* Wulf. is distributed in mountaneous regions of Europe and North Africa. The origin of *R. multiflorum* Kit. is Southern Europe [29, 30]. Study of the assimilation apparatus in varieties and selection forms of various red current species in the Russian Central Region is of special interest because specific climatic conditions significantly affect the formation and functions of leaf apparatus.

Studies have shown that some morphological features (shape, veining) of red currant leaves depended on the biological features of the variety, and the anatomical structure was affected by growth conditions. In the periods with insignificant water supply, the leaf area reduced in all studied samples compared to the optimal conditions: it was 17.64 cm^2 on the average in 2011, 13.95 cm^2 in 2012, and 14.37 cm^2 in 2013. Thereby, maximum leaf area was noted in 2011 to 2013 in 1426-21-80 sample, the derivative of *R. multiflorum* Kit. A positive relationship was between the leaf area and HTC ($r = +0.99$) and a negative one was between the leaf area and the temperature ($r = -0.97$).

The leaves of red currant plants had a mesomorphous structure characteristic of *Ribesia* (Berl.) Jancz. subspecies. The spongy parenchyma was predominant over the palisade one and the epidermal cells appeared to be large enough, being larger on the upper side of the leaf than on the lower one. High temperature and drought differently affected the sizes of the adaxial epidermal cells and the mesostructure of a leaf. In 2012 and 2013 from May to July, the main cells of the adaxial epidermis in the Hollandische Rote variety (*R. petraeum* Wulf.) and the selection form 1426-21-80 (*R. multiflorum* Kit.) affected by high temperatures (+31.2...+28.6 °C in May, and +32.2...+31.5 °C in July) somewhat elongated, which was associated with reduced cell turgor. We have noted cell shrinkage in the tangential direction (parallel to the palisade surface) in the remaining samples (Fig. 1).

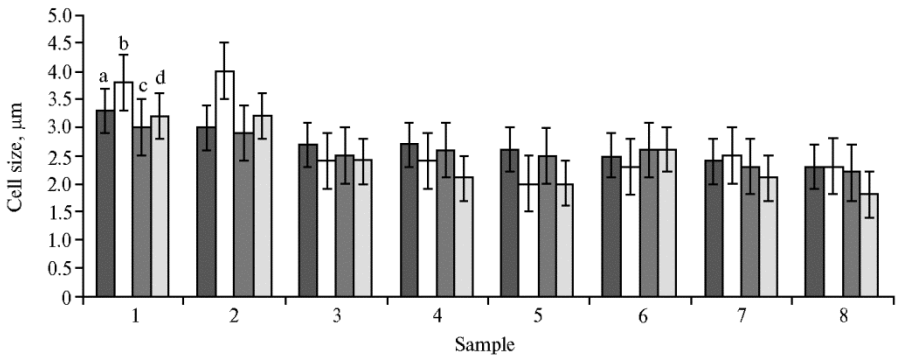

Fig. 1. Cell size in the leaf adaxial epidermis of red currant varieties and forms in May (a) **and in July** (b) **2012 and also in May** (c) **and July** (d) **2013:** 1 — Hollandische Rote, 2 — 1426-21-80, 3 — Dana, 4 — 1432-29-98, 5 — Niva, 6 — 1518-37-14, 7 — Jonkheer Van Tets, 8 — Roza (All-Russian Research Institute for Breeding Fruit Crops, Orel Province, Zhilina village).

The elevated temperature and drought in 2012 and 2013 has led to the growth of parenchymal cells and an increase in leaf thickness (Table 1). The growth of mesophyll cells occurred mainly due to the increase in the air-contained elements of spongy parenchyma which facilitated gas exchange be-

tween a leaf and environment. Cell growth in spongy parenchyma and an increase in leaf thickness compared to 2011 were found almost in all of the samples, except Niva variety, especially in Hollandische Rote plants and 1426-21-80. These changes should be viewed as a manifestation of a high adaptability of the anatomical leaf structure to stressors during the vegetation period.

1. Leaf mesostructure in the red currant varieties and forms as depended on year conditions (All-Russian Research Institute for Breeding Fruit Crops, Orel Province, Zhilina village)

Sample (B)	Year (A)								
	2011			2012			2013		
	PP	SP	TLT	PP	SP	TLT	PP	SP	TLT
Hollandische Rote	10.29	13.62	26.94	12.00	20.00	37.30	9.75	15.80	–
Niva	9.56	15.24	27.70	11.25	18.45	35.35	9.55	16.95	31.40
Dana	9.28	12.65	24.63	10.15	15.35	29.60	10.35	15.30	29.10
Jonkheer Van Tets	8.10	12.00	22.70	10.05	16.30	29.92	9.10	14.85	27.70
Roza	7.90	12.35	23.25	11.65	17.55	33.65	10.15	15.10	29.10
Hollandische Rote	7.70	14.68	25.38	9.15	18.15	30.80	8.55	16.90	29.15
Niva	7.30	10.10	20.33	10.50	15.90	30.05	9.00	12.30	24.80
Dana	6.80	11.23	20.53	7.95	13.85	25.60	8.10	13.80	25.10

N o t e. PP — palisade parenchyma, μm; SP — spongy parenchyma, μm; TLT — total leaf thickness, μm. LSD_{05} for palisade parenchyma: A = 1.57; B = 2.56; AB = 4.42. LSD_{05} for spongy parenchyma: A = 1.92; B = 3.14; AB = 5.43. LSD_{05} for total leaf thickness: A = 1.39; B = 2.27; AB = 3.93. Dash means no data.

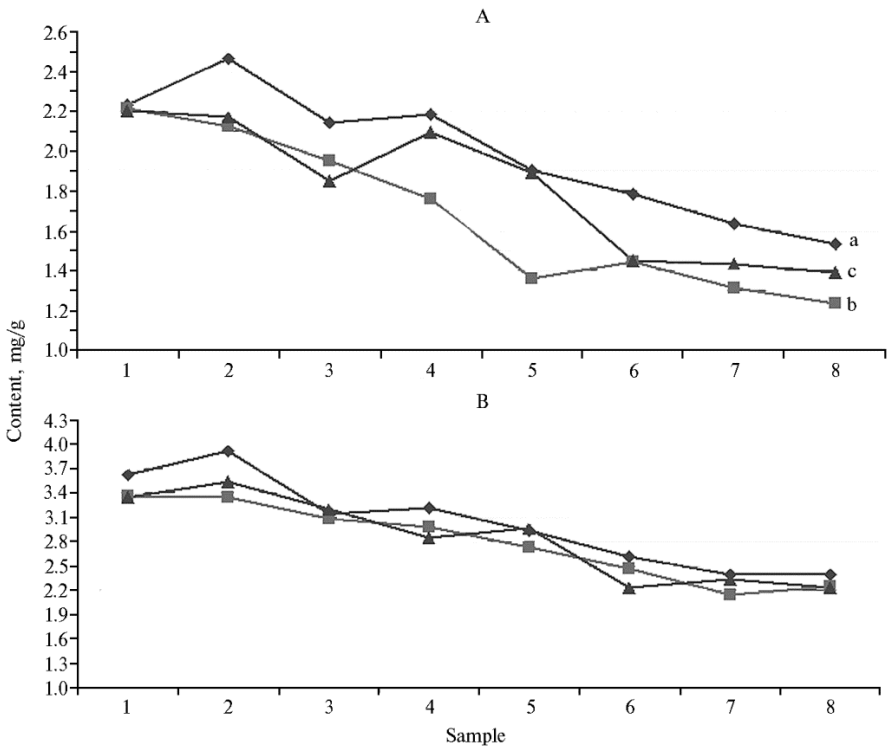

Fig. 2. Chlorophyll a (A) **and chlorophylls a+b** (B) **content during vegetation in 2011** (a), **2012** (b) **and 2013** (c) **in the leaves of red currant varieties and forms:** 1 — Hollandische Rote, 2 — 1426-21-80, 3 — Dana, 4 — 1432-29-98, 5 — Niva, 6 — 1518-37-14, 7 — Jonkheer Van Tets, 8 — Roza (All-Russian Research Institute for Breeding Fruit Crops, Orel Province, Zhilina village). A: a — LSD_{05} = 0.31; b — LSD_{05} = 0.26; c — LSD_{05} = 0.29. B: a — LSD_{05} = 0.61; b — LSD_{05} = 0.53; c — LSD_{05} = 0.58.

The weather conditions during vegetation have significantly affected assimilation apparatus. In the arid 2012, a reduction in chlorophyll a and a + b chlorophylls (1.23-2.21 and 2.15-3.36 mg/g, respectively) was noted in the leaves of all red currant cultivars compared to 2011 (1.53-2.46 and 2.40-3.92 mg/g)

(Fig. 2). It should be noted that a more drastic decrease in the pigment amount in 2012 occurred in all derivatives of the *R. vulgare* Lam. Species; the minimal one was in *R. petraeum* Wulf. and *R. multiflorum* Kit. plants. Insignificant drought relief in 2013 facilitated a small increase in the pigment content.

Drought together with high temperatures stimulated the formation of carotenoids in all of the samples of *Ribesia* (Berl.) Jancz. subgenus studied, which is consistent with findings about the protective function of this pigment group at stress [21, 31, 32]. We have found a positive correlation between the carotenoid content and temperature ($r = +0.77$). Some scholars in order to assess stressor resistance suggest using the coefficient of the total chlorophyll to carotenoid ratio [31, 33, 34]. Under stress conditions of 2012, the Hollandische Rote variety and 1426-21-80 plants with the coefficient of 5.14 and 5.51, respectively, were distinct in this parameter, which may indicate their resistance to drought and high summer temperatures. The hydrothermal regime affects productivity by affecting the functional state of the plants which was confirmed by high correlations between the chlorophyll a level and yield ($r = +0.85$), and total chlorophylls and yield ($r = +0.78$)

The pair correlation coefficients between total chlorophylls and water loss ($r = -1.00$), and total chlorophylls and free water amount ($r = +0.98$) confirmed the dependence of the pigment content on water regime parameters. These parameters (pigment content, water fractions, water-retaining leaf capacity) were associated with the development of water deficiency which depended on air temperature ($r = +0.84$). The relationship between the water regime parameters and the meteorological features of the vegetation period was described using multiple regression coefficients (Table 2).

2. Correlation between physiological parameters of the red currant samples and the meteorological factors (All-Russian Research Institute for Breeding Fruit Crops, Orel Province, Zhilina village)

Parameter	Regression coefficient
Water content	0.90 · F; r = 0.22
Free water fraction	0.84 · F; r = 0.33
Water loss	–0.88 · F; r = 0.30

N o t e. F — hydrothermal coefficient, r — standard experiment error.

It should be noted that the total water content in red currant leaves is not the main parameter of plant resistance to elevated temperatures and drought, because it depends on a number of factors (sprout growth, leaf age, berry formation, weather conditions and variety) [35] (Fig. 3).

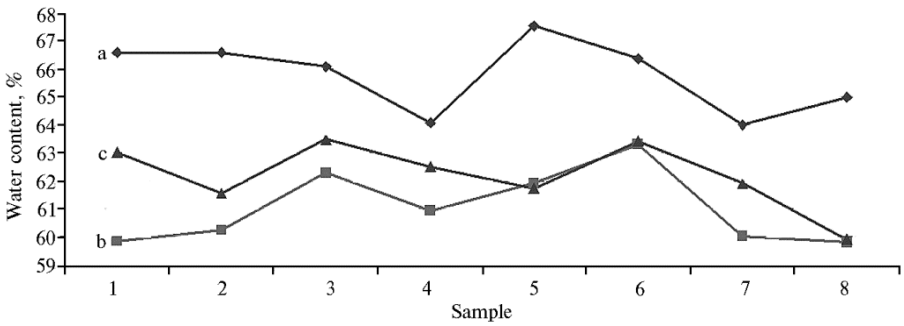

Fig. 3. Water content in leaves during vegetation in 2011 (a), **2012** (b) **and 2013** (c) **in the red currant varieties and forms**: 1 — Hollandische Rote, 2 — 1426-21-80, 3 — Dana, 4 — 1432-29-98, 5 — Niva, 6 — 1518-37-14, 7 — Jonkheer Van Tets, 8 — Roza (All-Russian Research Institute for Breeding Fruit Crops, Orel Province, Zhilina village). LSD_{05}: a — 4,38, b — 3,74, c — 3,98

The most labile parameters, depending on meteorological conditions and

water supply, are the ratios of the bound and free water and the water retaining capacity of leaves [11, 36, 37]. We assessed the water retaining capacity based on water loss. Among the studied traits in the stress period, significant differences were associated with variety and specific features. In 2012 with unfavorable HTC compared to 2011, an increase in the ratio of bound and free water and a decrease in leaf water loss were observed in all the samples. In 2013, this ratio was higher than that in 2011, but lower compared to the parameter of 2012 (Table 3). The highest values of the coefficient and minimal water loss were noted in the Hollandische Rote plants and the 1426-21-80 form; this gives grounds to believe that genotypes of these *R. petraeum* Wulf. and *R. multiflorum* Kit. samples manifest a higher adaptability to high temperatures under soil moisture deficiency.

3. Main parameters of water regime in the red currant varieties and forms aas depended on year conditions (All-Russian Research Institute for Breeding Fruit Crops, Orel Province, Zhilina village)

Sample (B)	Year (A)							
	2011		2012		2013		average	
	BW/FW	WL, %	BW/FW	WL, %	BW/FW	WL, %	BW/FW	WL, %
Hollandische Rote	1.33	35.82	2.09	20.36	2.03	21.11	1.82	25.76
1426-21-80	1.31	36.16	2.41	20.62	2.09	19.37	1.94	25.38
1432-29-98	1.12	30.17	1.42	25.01	1.20	26.98	1.25	27.39
Jonkheer Van Tets	1.01	38.10	1.31	21.85	1.04	27.40	1.12	29.12
1518-37-14	0.96	40.91	1.06	27.62	1.08	26.66	1.03	31.73
Niva	0.82	42.56	1.71	24.84	1.50	25.58	1.34	30.99
Dana	0.70	40.97	1.76	25.51	1.66	25.44	1.37	30.64
Roza	0.63	39.75	0.98	26.82	0.87	27.85	0.83	31.47
LSD_{05}	0.08	2.06	0.11	3.03	0.14	1.86		

Note. BW/FW — coefficient of the bound to free water ratio, WL — water loss over 24 h, %. LSD_{05} for BW/FW: A — 2.18; B — 4.59; AB — 7.95. LSD_{05} for WL: A — 0.13; B — 0.21; AB — 0.37.

Therefore, prospects of physiological methods in assessment of resistance to unfavorable weather factors have been shown in the members of *Ribesia* (Berl.) Jancz. subgenus. The obtained results are fully consistent with data about field resistance of red currant varieties and forms under elevated temperature and moisture deficiency. This allows using the said methods for express diagnostics that significantly intensify breeding for adaptability. The complex physiological assessment has shown that the derivatives of various species of *Ribesia* (Berl.) Jancz. subgenus have different ecological plasticity which is explained by differences in their genetic and ecological and geographic origins. The highest resistance to drought during vegetation in the Central Black Soil Region was manifested in the derivatives of *Ribes petraeum* Wulf. (Hollandische Rote) and *R. multiflorum* Kit. (1426-21-80) species. Low drought resistance was noted in the varieties and forms of *R. vulgare* Lam.

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