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. 57, Issue 5
September-October

2022 Moscow

A peer-reviewed academic journal for delivering current original research results and reviews on classic and modern biology of agricultural plants, animals and microorganisms
Covered in Scopus, Web of Science (BIOSIS Previews, Biological Abstracts, CAB Abstracts, Russian Science Citation Index), Agris

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

Publisher: Agricultural Biology Editorial Office NPO

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E-mail: felami@mail.ru, elein-k@yandex.ru **Internet:** http://www.agrobiology.ru

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], 2022, Vol. 57, № 5

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 821-831
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Reviews, challenges, advances

UDC 631.52:581.557:579.64:575

doi: 10.15389/agrobiology.2022.5.821eng
doi: 10.15389/agrobiology.2022.5.821rus

AGRICULTURAL MICROBIOLOGY AND SYMBIOGENETICS: SYNTHESIS OF CLASSICAL IDEAS AND CONSTRUCTION OF HIGHLY PRODUCTIVE AGROCENOSES

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (project № 19-16-00081P)
Received June 16, 2022

Abstract

Agricultural microbiology (AM) is presented as a discipline addressing the prokaryotic and eukaryotic microorganisms that influence operation of the major components of agrocenosis — plants, animals and soils. Development of AM is based on the synthesis of ideas and methods of microbiology, plant physiology, soil science and genetics. This synthesis is aimed to study the organization and evolution of biosystems in which symbiotic microorganisms perform adaptively important functions in cooperation with each other and with host organisms. Upon migration from environment into the endosymbiotic niches of plants and animals, microorganisms form with them multicomponent complexes — holobionts (E. Rosenberg, I. Zilber-Rosenberg, 2018). They possess own systems of heredity, symbiogenomes and hologenomes, which have become the subjects of a new discipline, symbiogenetics (I.A. Tikhonovich, N.A. Provorov, 2012). Microorganisms forming symbioses with plants perform the important adaptive functions — nutritional (N_2 fixation, absorption of soil nutrients, firstly phosphates), defensive (biocontrol of pathogens and phytophagans) and regulatory (synthesis of phytohormones that optimize plant development and improve their resistance to adverse environment) (I.A. Tikhonovich, N.A. Provorov, 2009). The broadly studied and practically important plant symbionts include: a) nodule bacteria or rhizobia (*Rhizobiales*) — N_2-fixing symbionts of legumes; b) arbuscular mycorrhizal fungi (*Glomeromycota*) — phosphate-mobilizing symbionts of a wide range (more than 80 % species) of plants (A. Berruti et al., 2016); c) rhizospheric and endophytic bacteria (e.g., *Azospirillum*, *Bacillus*, *Pseudomonas*) which stimulate the development of plants and determine their resistance to antagonists (pathogens, pests) and stresses (drought, salinity of soils, their contamination with xenobiotics or heavy metals) (M.A. Hassani et al., 2018). In animals, trophic symbionts determine the assimilation of plant biomass (intestinal or rumen microbiota), synthesis of essential amino acids and cofactors (intestinal and intracellular symbionts), and N_2 fixation (symbionts of some herbivorous animals) (E. Rinninella et al., 2019). The study of microbial effects on plants and animals makes it possible to create microbial preparations that improve the nutrition of hosts, their resistance to biotic and abiotic stresses, and increase the soil fertility. In crop production, preparations of N_2-fixing and growth-stimulating bacteria are widely used, which ensure a drastic reduction in application of environmentally hazardous nitrogen and phosphorus fertilizers. Preparations of microorganisms that are antagonists of phytopathogens — *Pseudomonas*, *Bacillus* (B.J. Lugtenberg et al., 2001; V.K. Chebotar et al., 2009), rodents — *Salmonella enteritidis*, *Serratia plymuthica* (A. Soenens, J. Imperial, 2019) or phytophagous insects — *Bacillus thuringiensis, Beauveria bassiana* (A.V. McGuire, T.D. Northfield, 2020) are used broadly for their biocontrol to significantly reduce the pesticide load on agrocenoses. By studying the integrative functions of agronomically valuable microorganisms, AM invests a significant contribution to the fundamental biological research, including the genetic and molecular interactions of prokaryotes and eukaryotes, evolution of cell and of its genome, and formation of supraorganismal genetic systems (I.A. Tikhonovich, N.A. Provorov, 2012). Based on these studies, methods of symbiotic engineering are being developed aimed at constructing the highly productive

biosystems, including the cereal and vegetable cultivars capable of symbiosis with rhizobia, as well as N_2-fixing plants.

Keywords: agricultural microbiology (AM), symbiogenetics, genetic engineering, symbiotic nitrogen fixation, biocontrol of pathogens and pests, microbial preparations, sustainable agriculture

Agricultural microbiology (ACM) emerged at the end of the 19th century as a research field applied to plant nutrition and the biocontrol of plant pests [as cited by 1, 2]. By the 1930s, ACM became a synthetic discipline using the methods of microbiology, plant physiology and soil science. In the 1940s, gene-for-gene systems were discovered that determined the relationship between phytopathogens and their hosts [3], as well as between rhizobia and legumes [4]. It was shown that the relationship between microorganisms and their hosts can be described in terms of gene interaction [5], i.e., the epistasis (parasitic systems) or complementarity (mutualistic symbioses) (Table 1). The deepest integration of partners, that is, the formation of combined signal-receptor complexes and biochemical pathways, is characteristic of mutualistic relationships [6]. The result of integrative evolution was the formation of holobionts, supraspecific complexes with their own systems of heredity (hologenomes), which provide plants and animals with various adaptive functions [7].

1. Types of gene interaction in free-living and symbiotic organisms

Type of interaction	Microorganisms	
	free-living (5)	symbiotic (3, 4)
Epistasis	The gene of one allelic pair suppresses the expression of the gene of another allelic pair	Gene-for-gene interactions in parasitic systems (host suppression of pathogens)
Complementarity	Involvement of multiple non-allelic genes in the development of a trait	Functional integration of genes in mutualistic systems (formation by partners of combined signaling and biochemical pathways)

The practical use of the ACM achievements began with the selection of effective strains of rhizobia for the inoculation of legumes and the coordinated selection of microorganisms and plants to create optimal combinations of their genotypes [8]. These works determined the development of a new field of biotechnology, symbiotic engineering, aimed at constructing eco-safe agrocenoses in which the adaptive functions of plants and animals are performed by their symbionts [9].

The ACM development in Russia was initiated by P.I. Kostychev and S.P. Kostychev, who found that bacteria and fungi are actively involved in plant nutrition and in the formation of fertile soils [10]. To date, microbial-plant symbioses divided into three types (trophic, protective and regulatory) is the most studied [6]. Their emergence has a long history associated with the joint (coevolutionary) emergence of plants and mycorrhizal fungi on land [11]. Fungi acted as intermediaries between the most ancient plants that lacked roots (rhyniophytes, psilophytes) and the soil, and possibly as donors of symbiotic bacteria that passed from fungi to plants [12].

In natural ecosystems and agrocenoses, microorganisms carry out all the main stages of the cycle of substances associated with plant nutrition, the assimilation of plant food by animals, as well as the transformation of organic residues into humic substances. The performance of agronomically significant functions by microorganisms is determined by their circulation in the soil—plants—animals ecosystems. Most plant symbionts originated from soil bacteria and fungi, and many inhabitants of the digestive cavities of animals evolved on the basis of the microflora of host plants [8]. Moving into the internal environment of the hosts, microbial communities fall under their control and become even more integrated and functionally active. This integration is associated with deep transformations of

the metagenomes of microbial communities that occur under the influence of plants and animals, which indicates the possibility of managing agrocenosis as a single genetic system.

The purpose of this paper is to generalize and analyze modern ideas about the integrative and adaptive functions performed by microorganisms during the formation of terrestrial ecosystems. Based on the knowledge of the genetic organization of functionally integral and self-sufficient microbial-plant and microbial-animal complexes (holobionts), methods for managing agrocenoses by modifying their microbial components that determine the vital activity of agricultural organisms, as well as the formation of fertile soils, will be proposed.

Bioengineering of agricultural microorganisms. In Russia, work on obtaining agronomically valuable microorganisms began in the laboratory of agricultural bacteriology, founded at the end of the 19th century in St. Petersburg with the aim of selecting strains for the control of murine rodents [2]. The creation of domestic preparations of rhizobia (nitragins and their modern modifications) is associated with the works of V.P. Israeli et al. [13] and E.N. Mishustin [14]. In 1930, the All-Union (now All-Russian) Research Institute for Agricultural Microbiology became the center of these studies, where in the 1970s the development of genetic breeding methods for constructing effective microbial-plant symbioses began. An important step along this path was formulated by L.M. Dorosinsky [15] the main principle of rhizobia selection, which should be based on the complementarity of partner genotypes, which plays a much greater role in determining symbiotic efficiency (SE, the ability to increase plant productivity) than the adaptability of bacteria to local soil conditions.

Developing this approach, we assessed the genotypic contributions of partners to the development of legume-rhizobium symbiosis and showed that an increase in SE can be achieved by narrowing the specificity of the interaction between bacteria and plants [16]. According to the results of the analysis of variance of data on the interaction of different genotypes of partners, the highest productivity of legumes inoculated with rhizobia is achieved with the maximum contribution of nonadditive cultivar—strain interaction to the variation of SE parameters, which serves as a measure of the specificity of symbiosis [17].

Based on these data, a methodology was proposed for coordinated breeding of plants and bacteria aimed at creating optimal combinations of their genotypes [17]. It should be also taken into account that native microbial populations interacting with leguminous plants in the field are heterogeneous and contain many strains that are useless for hosts and even parasitic strains that compete with industrial strains of rhizobia for the formation of nodules [18]. A change in the composition of the microbial population that has penetrated into the plant in favor of mutualist strains is possible due to the directed selection of these strains by the host from the soil or the rearrangement of the composition of the endosymbiotic population in favor of active N_2-fixers. Thanks to these mechanisms, competitive interactions of several types are carried out in the microbial-plant system.

The first type includes the competition of soil strains of rhizobia for the formation of nodules in leguminous plants. Its study made it possible to identify a number of *cmp* genes in bacteria that control nodulation competitiveness, which usually does not correlate with the activity of N_2 fixation [18]. Therefore, industrial strains of rhizobia introduced into the agrocenosis often cannot compete with inefficient, but virulent (aggressive) local strains. In a number of works, the preferential selection of certain genotypes of rhizobia from the soil by leguminous plants was noted [19], which usually does not provide targeted extraction from populations of strains with high nitrogenase activity [20].

The second type includes the competition of groups of bacteria located in

different nodules of a leguminous plant for the products of photosynthesis supplied to them. This competition is based on a positive feedback between N_2 and CO_2 fixation processes, which provides a preferential supply of carbon to microbial genotypes that have formed nodules actively fixing N_2 [21, 22]. Selection for mutualist strains is of a group nature and is effective under the condition of clonality of the endosymbiotic population of rhizobia, which is determined by infection of plants with individual bacterial cells through root hairs [6].

Finally, competition of the third type occurs between different holobiont plants for survival under conditions of soil nitrogen deficiency [23]. Its success depends on how efficiently individual legume genotypes select active N_2-fixers from the soil population of rhizobia and distribute photosynthesis products in favor of those microbial genotypes that formed actively N_2-fixing nodules.

The methodology of coordinated selection of symbiosis partners, based on the analysis of their natural variability, was used in the development of genetic methods for creating symbiotically active microbial strains. At the first stage of the work, it was shown that in alfalfa rhizobia (*Sinorhizobium meliloti*) hybridization (transformation, transduction, conjugation) is a more efficient source of active N_2-fixers than mutagenesis [24]. The selection of strains for an increase in nitrogenase activity turned out to be more effective than for an increase in SE, which indicates a different genetic control of these traits.

An important step in the creation of effective strains of rhizobia was the molecular labeling of symbiotically specialized (*sym*) genes, most of which are not active outside the host. Usually, this labeling is carried out by means of transposon (Tn5) mutagenesis [25], which makes it possible to identify two groups of symbiosis regulators, positive and negative (Table 2). Upon inactivation of the former, the symbiotic activity of bacteria decreases or is lost; upon inactivation of the latter, it increases (26).

2. Rhizobia genes for positive and negative regulators of symbiosis

Features	Symbiotic regulators	
	positive (27)	negative (28)
Functions	Synthesis of nitrogenase (*nif*) and ienergy supply (*fix, dct*)	Synthesis of reserve nutrients (*pha*C, *phb*A, *glg*A) and extracellular polysaccharides (*egl*C, *rkp*C), energy saving (red)
Localization in the genome	In extrachromosomal clusters	Dispersed
Expression outside symbiosis	Only shown for some genes (*dct*)	Common to most genes
Symbiotic efficiency:		
for gene inactivation	Lost or drastically reduced	Increased
for gene amplification	Increased	No data

To obtain agronomically valuable strains of rhizobia, amplification of positive symbiosis regulators is also used. For example, when additional copies of *dct*-genes, which determine the transport of dicarboxylic acids, are introduced into alfalfa rhizobia, the N_2-fixing (C_2H_2-reductase) activity of bacteria increases by 60-100%, but the mass of plants, the main SE indicator, increases only by 15-20% [27]. A balanced increase in nitrogenase activity and SE is ensured by the inactivation of negative regulators of N_2 fixation (for example, genes that determine the conversion of carbohydrates obtained from plants into reserve nutrients), which, in combination with amplification of its positive regulators, can be used to construct effective strains of rhizobia [28].

Construction of holobionts. The main result of the colonization of the internal environment of eukaryotic hosts by microorganisms is the formation of new biological units — holobionts. Using the legume-rhizobium symbiosis as an example, one can see that the integrity of the holobiont is determined by the partners' positive and negative feedbacks (Table 3). Negative feedback which acts at the early stages of interaction, increases the stability of the system, since, thanks

to it, the host strictly controls the size of the endosymbiotic population of rhizobia, limiting the number of nodules and the number of bacteria in each of them [29].

3. Feedback of partners in the system of legume-rhizobium symbiosis

Compared properties	Negative connections	Positive connections
Symbiosis stages	Early	Late
Interaction of partner genes	Epistasis (gene-to-gene)	Complementarity
Defined trait of symbiosis	Stability	Efficiency
Rhizobium genes	*nod*	*nif/fix*, *dct*
Plant genes	*NFR*	*GS/GOGAT/AAT*, *PEPC*
Controlled symbiotic processes	Host recognition and infection by bacteria, development of nodules	Formation of combined pathways of nitrogen-carbon metabolism

N o t e. Rhizobia genes control synthesis of lipo-chito-oligosaccharide Nod-factors (*nod*), the synthesis and regulation of nitrogenase activity (*nif/fix*), and the transport of plant-derived dicarboxylic acids (*dct*) [27]. Plant genes control the reception of Nod factors (*NFR*) [6], the assimilation of N_2 fixation products (*GS/GOGAT/AAT*), and dark CO_2 fixation (*PEPC*) [21, 22].

The positive feedback realized at the late stages of symbiosis plays an important role in the determination of SE, since the receipt of fixed nitrogen by the plant stimulates the supply of photosynthesis products to the nodules, which are used to ensure the nitrogenase reaction and bacterial reproduction [30]. Taken together, these connections determine the stability and integrity of the symbiosis, which, as shown by the results of mathematical and experimental modeling, are closely related to its adaptive functions. Indeed, the highest SE is achieved when plants and bacteria respond in a coordinated manner to external factors that affect the vital activity of partners [31].

The use of models of microbial-plant symbiosis showed that when moving from the external environment to the internal niches of plants or animals, soil microbiomes significantly change in composition and become more integrated, falling under the regulatory influence of the hosts [32]. Due to this regulation, symbionts coexist stably with the host, despite their diversity and rapid reproduction.

While in plants endosymbiotic microbiomes are formed on the basis of organisms that come mainly from the soil, in animals they are formed from organisms obtained from food. Holobionts formed by animals are characterized by deep integration necessary to perform symbiotic functions. For example, acquired immunity systems in vertebrates regulate the structure of microbiomes inhabiting the digestive organs (rumen, intestines), where symbionts degrade biopolymers obtained with food (primarily cellulose and pectin) and synthesize protein. The composition of these microbiomes is determined by the age of animals, their physiological state, and feeding regimen [33, 34] and is largely preserved during host reproduction [35]. The functional structure of the animal endosymbiotic microbiome associated with adaptive functions can be characterized using mathematical models based on fractal analysis. Their use for the analysis of the avian gut microbiome has shown [36] that gut microbiome integrity correlates with host productivity and may be a criterion for selecting bacterial strains used as feed probiotics.

The structural and functional integrity of holobionts is determined by the formation of superorganismal systems of heredity, the study of which is the subject of a new discipline, symbiogenetics [37, 38]. These systems were designated as symbiogenomes (only partner genes specialized for interaction participate) or hologenomes (all partner genes participate). Symbiogenomes arise as a result of the functional integration of partners, which is characteristic of facultative symbioses. As the mutual dependence of microsymbionts and hosts increases, the partners can move to structural integration, which is characteristic of obligate symbioses and leads to the formation of hologenomes [39].

A qualitatively new stage in increasing the integrity of holobionts is associated with the emergence of mechanisms for the regular (vertical) transfer of

micro-partners during host reproduction. It usually causes the genetic reduction of microorganisms, which are transformed into hereditary eukaryotic determinants that implement the strategy of pangenesis, i.e. the inheritance of acquired traits [40]. The deepest integration with the host cell is characteristic of its permanent organelles—mitochondria and plastids, many organelle genes were transferred to nuclear chromosomes [41], resulting in the formation of eukaryotic multicomponent genomes of mosaic origin.

The study of supraorganismal systems of heredity opens up the possibility of creating algorithms for their design, symbiotic engineering [9] as one of the most important areas of biotechnology. Its goal is to obtain new agronomically valuable biosystems, such as N_2-fixing plants. However, the direct way to solve this problem (introduction of *nif* genes into the plant genome) is not productive, since *nif* genes do not function in the eukaryotic cell [42]. The incorporation of *nif* genes into mitochondria or plastids which are evolutionarily associated with N_2-fixing bacteria is more realistic [43]. Deeply reduced cell organelles (hydrogenosomes, mitosomes, nonphotosynthetic plastids) which lack their own genomes and maintain anaerobic conditions necessary for nitrogenase activity can be considered as promising recipients of these genes [44].

Ecologically friedndly agricultural technologies. Microorganisms that carry out the main stages of metabolism between soil, plants and animals play a key role in the formation of natural ecosystems. One of the main tasks of agricultural microbiology is the study of the circulation of microorganisms in agrocenoses as a factor in their stability and productivity [8]. By interacting with microorganisms, plants and animals are able to develop with minimal agrochemical impact due to the symbionts fulfilling the functions of feeding the hosts, protecting them from pathogens and stresses, and increasing soil fertility [45-48].

An important condition for the development of environmentally friendly agricultural technologies is to increase the efficiency of symbiotrophic plant nutrition, including the assimilation of sparingly soluble phosphates by mycorrhizal fungi and rhizospheric bacteria [49]. It is of interest to develop combined preparations containing mineral fertilizers and microorganisms that allow plants to make the most of the nitrogen and phosphorus compounds introduced into the soil, reducing the accumulation of harmful products of their transformation [50].

Symbiotic microorganisms play an equally important role in the nutrition of animals, especially herbivores. Being in the digestive organs, microbes destroy entering biopolymers and synthesize protein and metabolites which are deficient in host plants [51]. To optimize the microflora in animal's body, probiotic preparations are actively used, primarily lactobacilli which increase the digestibility of feed and carry out biocontrol of pathogenic organisms [33, 34].

Structural-functional and genetic integration of prokaryotes and eukaryotes is one of the main areas of symbiotic engineering aimed at the formation of supraorganismal complexes for agricultural and environmental purposes [9]. Analysis of the mechanisms of interaction of microorganisms with plants and animals should be considered as a necessary condition for the development of algorithms for constructing effective agrocenoses. Conderning plants, it includes the improvement of natural (nodule, endophytic, epiphytic) symbioses, aimed at creating microbial preparations that replace environmentally hazardous agrochemicals. The construction of new biosystems, including transgenic plants capable of synthesizing metabolites used as edible vaccines, is also being discussed [52].

As a promising area of symbiotic engineering, one can consider the creation of fundamentally new photosynthetic systems for use in green energy. The development of approaches to solve this problem is associated with the study of

symbioses formed by pro- and eukaryotic phototrophic microorganisms with animals, protozoa, and fungi [53]. An example of the emergence of new systems of photosynthesis in nature are kleptoplasts, plastids extracted by animals from the plants they eat and maintained in cells by new hosts for a long time as effective sources of carbon (54).

Heterotrophic protozoan *Alveolata* groups, which apparently originated from organisms that previously had plastids, and contain numerous genes derived from cyanobacteria, are promising for the construction of new photosynthesis systems [55]. Obviously, such organisms are pre-adapted to the maintenance of photobionts and can be used to create new CO_2 fixation systems with a high biotechnological potential.

The creation of agricultural technologies of the future should be based on the study of the processes of evolution of natural biosystems, aimed at increasing their integrity, environmental sustainability and productivity. The appearance of these systems was the result of the co-evolution of symbionts and their hosts, during which partners formed unified genetic systems. As a result of human-directed evolution [56], the problems of plant transition to symbiotrophic nutrition can be solved in the near future, which will lead to partial, and under certain conditions, to complete replacement of mineral fertilizers with environmentally friendly microbial preparations. The introduction of endophytic microorganisms into plants, which make the hosts immune to infection by phytopathogens, also seems promising. The solution of these problems requires the integrated use of microbiology, symbiogenetics, and genetic engineering approaches, the combination of which is a priority task for agricultural biology.

Thus, the use of methods of agricultural microbiology and symbiogenetics is associated with the development of environmentally friendly agricultural technologies based on the use of symbiotic microorganisms that provide symbiotrophic nutrition for plants and animals and protection from parasites and pests. The solution of these problems will allow to partially, and in some cases completely abandon environmentally hazardous fertilizers and protective equipment. The design of ecologically safe agrosystems should be based on the formation of stable supraorganismal complexes (holobionts) which have their own systems of heredity, resulting from the genetic integration of plants and animals with beneficial microorganisms.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 832-851
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.012:575.174.015.3

doi: 10.15389/agrobiology.2022.5.832eng
doi: 10.15389/agrobiology.2022.5.832rus

THE SOURCES OF GENOME VARIABILITY AS DOMESTICATION DRIVERS

(review)

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The authors declare no conflict of interests
Acknowledgements;
The authors express their sincere gratitude to L.M. Fedorova, Ph.D, for the interest shown in the work, fruitful discussion and useful advice in preparing the article for publication.
Received June 22, 2022

Abstract

Plant and animal domestication is the key event in the history of mankind, its mechanisms have attracted the attention of many researchers, especially in recent decades due to the well-known decline in biodiversity, including in agricultural species. According to the definition proposed by Melinda Zeder (M.A. Zeder, 2015), domestication is the stable mutualistic relationship in a number of generations in which the domesticator influences the reproduction of the domesticates, optimizing their lifestyle for the supply of the needing resource to human, and thanks to which the domesticates gain advantages over other individuals of the species. Such relationships are accompanied by interspecific coevolution, they are present not only in humans and domestic species of plants and animals, but also in representatives of wild species, for example, insects, fungi. As a universal feature of domestic species in comparison with closely related wild ones, a high phenotypic diversity is considered, which was noticed by Charles Darwin (Ch. Darwin, 1951). Pairwise genomic comparisons of such species as domestic dog and wolf, wild and domestic cat, domesticated and wild rabbit reveal a relatively increased density of a number of mobile genetic elements in domesticated animals compared to wild ones. In recent years, mobile genetic elements, or transposons (TEs), have been considered as the main factors of genomic transformations, gene, genomic duplications, genomic and gene reconstructions, as well as horizontal exchanges of genetic information (K.R. Oliver, W.K. Greene, 2009). The number of comparative genomic studies of TEs in domesticated species is small, and the role of such elements in domestication, as a rule, is not discussed. However, it can be expected that universal mechanisms of genome variability underlie all evolutionary events, including in response to the new niche-construction during domestication. The presented review systematizes such mechanisms. TEs providing deep genomic transformations, active and passive forms of their interactions with the host genome are considered (K.R. Oliver et al., 2009). Examples of the emergence of new genes based on TEs, such as the synticin gene, are described (C. Herrera-Úbeda et al., 2021), the synaptic plasticity regulator gene *arc* (Activity Regulated Cytoskeleton Associated Protein) (C. Herrera-Úbeda et al., 2021), the *bex* gene family encoding, in particular, the neuron growth factor receptor (E. Navas-Pérez et al., 2020; R.P. Cabeen et al., 2022). Conflict and cooperative interactions with the host genome during retrotransposon movements and different mechanisms of their effects on gene expression profiles are discussed. The participation of TEs in the formation and variability of networks of genomic regulatory elements, in particular microRNAs, is considered. Examples of the involvement of microRNAs in the control and formation of economically valuable traits in domesticated plants and animals are presented. The accumulated data suggest that the leading source of large phenotypic variability of domesticated species is the relatively high saturation of their genomes with mobile genetic elements and, as a consequence, an increase in the variability of genomic regulatory networks in the formation of a new niche during domestication by humans.

Keywords: domestication, genomics, variability, transposons, regulatory networks, microRNAs

Finding the genetic foundations of domestication as the entry of plants and animals into new habitat and reproduction conditions, purposefully formed by man, has theoretical and practical aspects. Theoretical aspect is related to the fact that only the domestication is a direct experimental model of micro- and macroevolution available for human study [1], as pointed out by Charles Darwin [2]. The practical aspect of the problem is due to the fact that a lack of understanding of the mechanisms of domestication does not allow the development of methods for managing genetic flows in domesticated species, which is becoming increasingly important due to the growth of the Earth population, the reduction of fertile lands and biodiversity [3].

To date, the least controversial definition of domesticators formulated by M.A. Zeder [4] describes it as a stable, multi-generational, mutualistic relationship in which humans (domesticators) ensure, to a significant extent, the reproduction control and care of plants/animals (domesticants) for a more predictable supplying with the resource of plant/animal interest, whereby the plant/animal is able to increase its reproductive success compared to individuals not participating in such a relationship, thereby increasing the fitness of both humans and target domesticated species. The concept of the "domestication syndrome" that crosses taxonomic differences has been formed. For example, in annual plants, this concerns traits associated with seed germination and spreading, e.g., the changes in germination rate, seed size, seed shedding, wall thickness, as well as in the timing and morphology of spreading mechanisms [5, 6]. In animals, these traits include characteristics of the neural crest, which are associated with the behavior of animals (in particular, social activity), adaptive potential, including indicators of fertility, and the variability of those traits that are required by the domesticator and are associated with animal productivity [7].

It should be noted that quite often, both in plants and animals, the characteristics that are classified as domestication syndromes are associated with polymorphism of different genes involved in similar metabolic pathways, which is expected, since most of the signs of domestication syndrome are polygenic [8].

The accumulated data indicate that the only common feature for all domesticated species is a relatively increased phenotypic variability, which serves as a source of response to the factors of natural and artificial selection that appear when representatives of a particular species are involved in the sphere of human interests. In this case, the main issue in studying the mechanisms of domestication becomes the elucidation of the genetic basis for such a large variability [9].

Mobile genetic elements (transposons, transposable elements, TEs). In recent years, mobile genetic elements, or transposons (TEs), have been considered as the main factor in genomic transformations, in gene and genomic duplications, genomic and gene reconstructions, as well as in horizontal exchanges of genetic information [10-12]. In some studies, taking into account the phylogenetic and biological similarity of TEs to viruses, it is proposed to designate the totality of such sequences in genomes as endovir [13]. A certain correlation was found between the density of the genomic distribution, the activity of TEs, and the intensity of speciation (abundance of species) in different taxa [14]. However, the possibility of involvement of TEs in increased phenotypic variability in domesticated species is generally not considered.

Mobile genetic elements are divided into two main classes. Class I consists of retrotransposons whose distribution through the genome involves RNA, while autonomous retrotransposons have a gene encoding reverse transcriptase. Class II includes DNA transposons that do not use RNA as an intermediate for transposition. Each class has autonomous and non-autonomous members. As a result, the following main classification has been adopted for mobile genetic elements

(transposons) (MGEs, TEs) [15]. Class I which includes retrotransposons is represented by autonomous endogenous retroviruses (ERV) with long terminal repeats (LTR), long interspersed nuclear elements (LINE), as well as non-autonomous elements, the short dispersed nuclear elements. elements (short interspersed nuclear elements, SINE) and composite retrotransposon (recombination products between retrotransposons and microsatellites, SVA). Class II represented by DNA transposons includes autonomous elements that carry a DDE-amino acid motif typical for transposase/integrase of most families of autonomous DNA transposons, except for Helitrones (the DNA transposons that replicate via the ring model and use an enzyme with endonuclease and helicase domain), as well as a non-autonomous element, a miniature inverted repeat transposable element (MITE).

Mobile genetic elements are called drivers of evolution, and their significant contribution to evolution, in particular, in primates, is known [16]. TEs have been found in the genomes of various taxonomic groups, from bacteria to mammals. Their ubiquitous presence is due to a pronounced tendency to spread along the genome, as well as to colonize other genomes. Some TEs (e.g., SINE) may arise spontaneously from non-mobile DNA sequences in the genome, while others may be horizontally transferred between species. TEs have an ancient origin dating back to prokaryotes. DNA-TEs (class II) are associated with sequences of bacterial inserts, retro-TEs are associated with introns of the second group [16, 17].

Some TEs appear to have been present in eukaryotes since the very beginning of their existence, perhaps even more than a billion years ago. TEs often make up the largest, if not the largest, portion of the eukaryotic genome. For example, sequenced mammalian genomes are composed of at least a third of TEs in non-primates and about half in primates. TEs appear to be an important determinant of genome size, with organisms with very large genomes (e.g. plants) often having a much higher abundance of TEs (> 60%) compared to species with relatively small genomes (yeasts, nematodes, insects and birds), in which the proportion of TEs is significantly lower.

Thanks to whole genome sequencing powered by software for the analysis of nucleotide sequences, data on the presence of TEs in different taxa have been accumulated (Fig. 1) [18]. These results indicate a wide distribution of TEs and that their number varies significantly between species, making an obvious contribution to the differences in the size of their haploid genome. Significant differences can be found even between closely related organisms. For example, in the genus *Entamoeba*, the *E. histolytica* genome is ~ 20% TEs, while the *E. dispar* genome is ~ 10%. Similarly, in the genus *Oryza*, the genome of the wild rice species *O. australiensis* more than doubled in size for 3 million years due to amplification of TEs, resulting in approx. 3 times the genomes of some of its closest relatives. On the contrary, a number of taxonomic groups show only minor differences in the content of TEs, which may reflect certain restrictions on the size of the genome or, conversely, a relatively high tolerance for genome expansion. For example, birds have a relatively conserved genome size (possibly due to the metabolic costs associated with active flight), despite the fact that TEs are active in most studied species of this taxonomic group. In salamanders, on the contrary, extreme and independent amplification of TEs occurred, which led to the formation of giant genomes (see Fig. 1).

It is interesting to note that the largest genome size and, accordingly, the contribution of TEs to it was found in lungfish and amphibian, that is, species in which aromorphosis (survival in two environments) is realized (see Fig. 1). Also noteworthy are the data on the unequal representation of different TEs variants in plants and animals: in plants, endogenous retroviruses are more common than in animals, and in the latter, the frequency of occurrence of SINEs is higher [18]

(see Fig. 1).

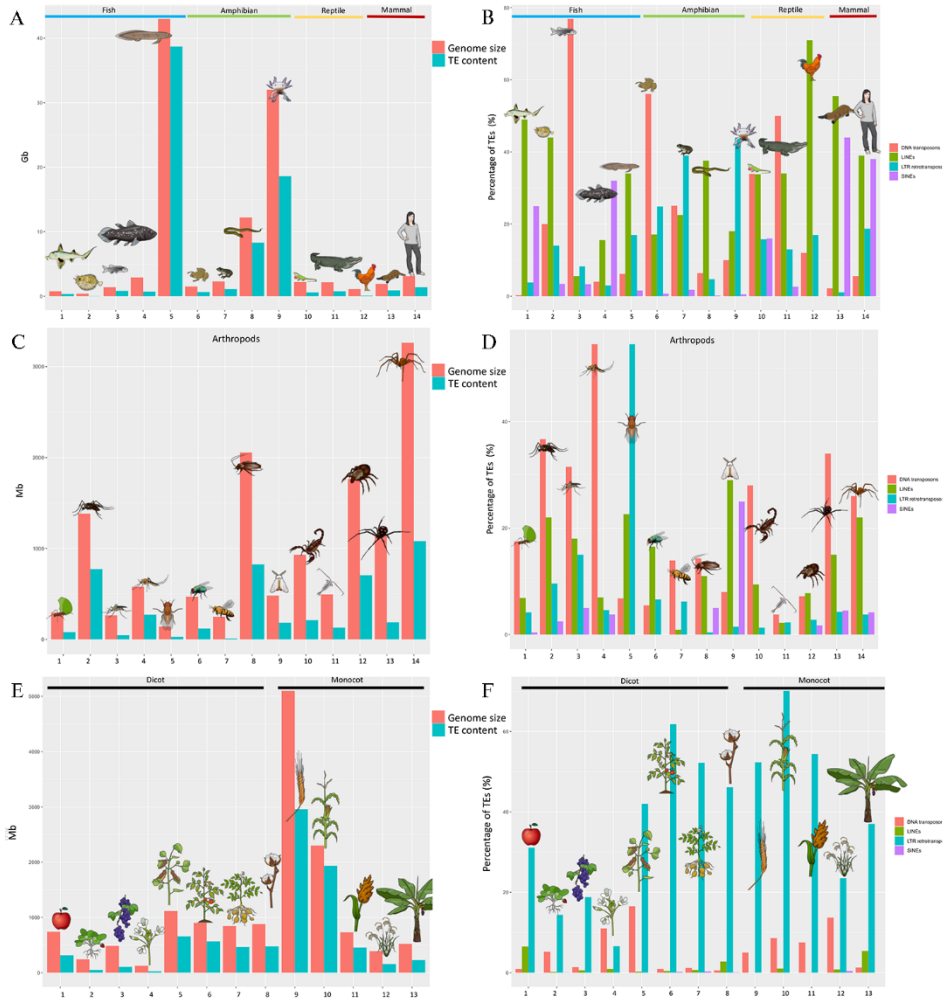

Fig. 1. The prevalence of TEs transposons in the genomes of various species [18].

A. Size of the genome (red) and the TEs it contains (blue) in vertebrates from left to right: 1 — *Callorhinchus milii*, 2 — *Tetraodon nigroviridis,* 3 — *Danio rerio*, 4 — *Latimeria chalumnae*, 5 — *Neoceratodus forsteri*, 6 — *Xenopus tropicalis*, 7 — *Nanorana parkeri*, 8 — *Chthyophis bannanicus*, 9 — *Ambystoma mexicanum*, 10 — *Anolis carolinensis*, 11 — *Alligator mississipiensis*, 12 — *Gallus gallus*, 13 — *Ornithorhynchus anatinus*, 14 — *Homo sapiens.*

B. Proportion (%) of different types of TEs: red — DNA transposons, green — LINE, lilac — SINE, blue — endogenous retroviruses (ERV) of vertebrates (see species A).

C. Genome size (red) and TEs it contains (blue) in arthropods:1 — *Acromyrmex echinatior*, 2 — *Aedes aegypti*, 3 — *Anopheles gambiae*, 4 — *Culex quinquefasciatus* (москиты), 5 — *Drosophila melanogaster* (плодовая мушка), 6 — *Lucilia cuprina*, 7 — *Apis mellifera*, 8 — *Blattella germanica*, 9 — *Bombyx mori*, 10 — *Centruroides exilicauda*, 11— *Eurytemora affinis*, 12 — *Ixodes scapularis*, 13 — *Latrodectus hesperus*, 14 — *Loxosceles reclusa*.

D. Proportion (%) of different types of TEs in arthropods: red — DNA transposons, green — LINE, lilac — SINE, blue — endogenous retroviruses (ERV) of vertebrates (species see C).

E. Genome size (red) and TEs it contains (blue) in plants: 1 — *Malus domestica*, 2 — *Fragaria vesca*, 3 — *Vitus vinifera*, 4 — *Arabidopsis thaliana*, 5 — *Glycine max*, 6 — *Solanum lycopersicum*, 7 — *Solanum tuberosum*, 8 — *Gossypium raimondii*, 9 — *Hordeum vulgare*, 10 — *Zea mays*, 11 — *Sorghum bicolor*, 12 — *Oryza sativa*, 13 — *Musa acuminate.*

F. Proportion (%) of different types of TEs in plants: red - DNA transposons, green - LINE, lilac - SINE, blue - endogenous retroviruses (ERV) of vertebrates (see species E).

Active and passive mechanisms of interaction of transposons with the host genome, the emergence of new genes. TEs,

as already noted, are powerful factors in the evolution of the genome (and hence phenotypic diversity) because they are able to induce genetic changes on a large scale. TEs can change the way genomes work both actively and passively. Species with active TEs or abundant uniform inactive TEs that can passively influence genome function by inducing ectopic recombination are potentially fertile and adaptable. Conversely, taxa deficient in TEs or having heterogeneous populations of inactive TEs are often well adapted in their niche but tend to be stagnant for a long time and may be at risk of extinction due to a lack of adaptability to changing conditions or diversification [17].

The mechanisms of influence of TEs on the genome functioning include a number of genomic effects of TEs, divided into active and passive influences [17].

Approx. 50 cases of neogenes have been described the nucleotide sequnsces of which are largely the TEs derivatives [17]. These genes include *TERT*, *CENPB*, *RAG1/2*. These neogenes made possible some extraordinarily complex evolutionary events that otherwise might not have occurred, such as the formation of recombination signaling sequences involved in rearrangements of the V(D)J Ag receptor. These sequences, like the RAG1/2 recombinase genes themselves, seem to be descended from the ancient DNA-TE.

*Exons (partial exons), protein-coding region*s. TEs often form independent exons within genes [17].

Extragenic sequences. TEs contribute to the formation of various extragenic sequences, such as centromeres, telomeres in Drosophila and some protozoa, sites of DNA replication initiation in yeast, regions associated with the framework of the interphase nucleus, and chromosome matrix in humans [17].

*Direct contribution to gene regulatio*n. *Full and partial promoters, enhancers, silencers.* Many TEs control gene expression, often tissue-specific [17]. In addition to influencing individual genes, TEs, apparently, turned out to be mobile carriers of ready-made promoters (enhancers) for the wide distribution of discrete regulatory elements throughout the genome. This provides a regulatory network by which an entire set of genes can be co-regulated to create new pathways for cellular development or improve existing ones.

Regulatory (micro)RNAs. Many exonized TEs encode miRNAs. Fifty-five human miRNA genes have been identified that derived from TEs and are capable of regulating thousands of genes [17].

*Indirect regulation: retrotransposition/transduction of gene sequences, gene duplication, exon shuffling, distribution of regulatory elem*ents. Some families of retro-TEs (e.g., LINE and LTR elements) tend to transduce host DNA due to their weak transcriptional termination sites. Gene duplication can also occur through the assignment of the TEs (*pol* reverse transcriptase gene) retrotransposition apparatus by host mRNA transcripts. In human, there are more than 1000 retrogens that have arisen in this way, some of which have developed very useful functions, for example, the *GLUD2* (*Glutamate Dehydrogenase 2*) gene is important for the utilization of the main excitatory neurotransmitter glutamate during neurotransmission [17].

Passive mechanisms. Stimulation of duplications (or losses) of DNA sections, gene duplication, exon duplication, segmental duplication. The mere presence in the genome of a large number of inactive TEs-like elements creates many highly homologous sites, which typically causes ectopic (non-allelic) DNA recombination due to such homology. This probably explains most of the ongoing effects of TEs in organisms with low TEs transcription activity, high TEs abundance and low diversity [17].

DNA duplication events are especially important in evolution because they

create functional redundancy and the potential for enhancing gene function and/or expression.

Stimulation of karyotypic changes by ectopic recombination, intra- and interchromosomal DNA rearrangements. TEs can passively support major chromosomal rearrangements by creating highly homologous regions scattered throughout the genome that are prone to ectopic recombination. For example, Alu-mediated translocation t(11;22)(q23;q11) is the most common constitutional translocation in humans [17].

TEs and new host genes. Active influences of TEs on evolutionary events include, in particular, examples of the incorporation of proviral genes in the host genome. For example, approximately 8% of the human genome consists of endogenous retroviral sequences. In the course of evolution, most of the genes from these sequences lost their function, but some of them were captured and "domesticated" via so-called exaptation. Among the domesticated viral genes, there is a group of syncytin genes that most clearly influenced the evolution of mammals [19]. Syncytins are captured viral proteins, products of the envelope gene of hereditarily endogenized retroviruses. The envelope glycoprotein (*Env* gene) is crucial in the process of virus entry into the cell and induces the fusion of the virion envelope with the plasma membrane of the cell. Several *Env* genes are found in the human genome, but only two of them, which induce the formation of syncytium, have placental-specific expression. Since it is the presence of the placenta that underlies the allocation of placental mammals to a separate taxonomic group, one would expect that the syncytins responsible for the development of this unique organ, which is formed only during pregnancy, would be orthologues in different species, but this is not the case. Primate and mouse syncytins are not orthologues, and there is evidence pointing to independent uptake events of the respective provius genes in the ancestors of each clade, as well as in *Scincidae* of the genus *Mabuya*. In fact, in mammals, gene capture events of various retroviruses can be associated with four main types of placental structures. It can be seen that the differences between the lizard placenta of the genus *Mabuya* and the mammalian placenta are due to different *Env* genes uptake events [19]. It is important to emphasize that the formation of syncytium with the participation of syncytins can occur due to different syncytins, which provide intercellular fusions of the envelope proteins of many retroviruses (including HIV, bovine leukemia virus), which is necessary for the reproduction of the virus [19].

Another example is the *Arc* gene encoding the Activity Regulated Cytoskeleton Associated Protein which is of particular interest because it seems to be the main regulator of synaptic plasticity [19]. The Activity Regulated Cytoskeleton Associated Protein is released from neurons in extracellular vesicles that mediate the transfer of *Arc* mRNA to new target cells where *Arc* mRNA can be translated. It was found that this protein is necessary for forms of long-term memory dependent on its synthesis and is involved in the development of depression in humans. The protein accumulates in weak synapses (probably to prevent their undesirable strengthening), participates in postsynaptic transport and processing of beta-amyloid A4 (APP). In addition to its role in synapses, it is involved in the regulation of the immune system: it is specifically expressed in migrating dendritic cells, thereby participating in the activation of T cells [19].

The authors of these studies [19] note that *arc* gene products mediate intercellular communication and synaptic plasticity through extracellular vesicles and are largely homologous to group-specific retroviral antigen (Gag) polyproteins. In retroviruses, capsids are essential for cellular infection and their assembly is mainly mediated by Gag. The similarity between Arc and Gag is not limited to amino acid sequence, as Arc is able to spontaneously assemble into a capsid-like

structure. In fact, Arc not only forms these capsid-like structures, but also encapsulates any mRNA present. Such processes ensure the movement of RNA molecules between the cells of the nervous system. Regarding the uptake and evolution of this viral protein, phylogenetic analyzes have identified at least two independent uptake events that occurred in tetrapod ancestors and in schizophorans. In both cases, arc co-optation resulted in similar functions of the RNA transporter protein in the nervous system. In both lines, the Ty3/gypsy retrotransposon was the closest to the putative initial ancestral variant, but according to the homology of the Ty3/gypsy sequences, tetrapods clustered with fish, while flies clustered with insects, which, apparently, indicates the independent origin of arc from retrotransposons. Ty3/gypsy in each line, despite the significant homology of its product with the Gag protein [19].

The results of transposon domestication also include the appearwnce of a new *Bex/Tceal* cluster (consisting of 14 genes, located in the X chromosome of the placental ancestor) after the divergence of the marsupial and placental clades. The *bex* gene family, encoding, in particular, the neuronal growth factor receptor, emerged through the introduction of placental retrotransposons HAL1b, L1ME-like, and the Hnmph1 endogenous retrovirus in placental progenitors [20].

Analysis of the functional activity of the *Bex/Tceal* genes was performed in vitro and on mouse lines mutant for the *bex3* gene. The lines homozygous for the mutation of this gene showed deviations in the morphology of the skull, the size of the cerebellum and brain decreased, which may be associated with the behavioral defects observed in mutant mice. They showed impairments in social interactions, nest building, working memory, and object recognition memory. This mutant phenotype may mean that the *bex3* gene subproduct interferes with the interaction of the TSC1/2 complex (TSC Complex Subunit 1, a tumor-suppressing gene encoding the growth-inhibiting protein hamartin) with the target of rapamycin in small-feeding mTORC1/mTORC2, inhibiting this pathway. mTORC2 belongs to the phosphatidylinositol kinase family. They mediate cellular responses to stress (in particular, in response to DNA damage). This kinase is a component of two different complexes, the mTORC1 which controls overall protein synthesis, cell growth and proliferation, and mTORC2 which serves as a regulator of the actin cytoskeleton and promotes cell survival [21].

mTOR inhibitors are used in organ transplantation as immunosuppressants and are being evaluated for therapeutic potential in SARS-CoV-2 infections [20]. Mutations in the *mTOR* protein gene are associated with Smith-Kingsmore syndrome (characterized by macrocephaly, mental retardation, seizures) and somatic focal cortical dysplasia type II [21]. The *ANGPTL7* gene (Angiopoietin Like 7, involved in the negative regulation of carpillar network development) is located in the *mTOR* intron. It has been suggested that *ANGPTL7* dysregulation under the influence of *Bex3* is associated with autism-type disorder in humans.

The following scheme was proposed to describe the stages of gene cooptation [19]. At the first stage, the proto-BGW motif (Bex/GASP/Wex element common to the genes *bex*, *gasp*, and *wex*) [21] was present in the X chromosome of the placental and marsupial ancestor in a position upstream of the alpha-galactosidase gla gene promoter (pα). At the second stage, in the placental lineage, the retrotranscribed endogenous retrovirus Hnrnph1 was inserted next to the *bgw* motif in positions above *gla*, which led to the appearance of the retrogen *hnrnph2*. At the third stage, the region containing the co-opted motifs *bgw* and *hnrnph2* underwent duplication, and retrotransposons similar to HAL1b and L1ME were inserted nearby. At the fourth stage, *bgw* and the open reading frame (ORF) appeared upon the introduction of retrotransposons formed the nucleotide sequences that corresponded to proto-*Bex/Tceal* with the preserved YY1 binding site from

HAL1b. At the next stage, the BGW motif and the YY1 binding site of the *Bex/Tceal* gene were duplicated in a position upstream of the retrocopy of the *armc10* gene. This led to the appearance of the inherited *armcX* gene belonging to the family of signaling genes located on the X chromosome, which encode the Armadillo repeat in proteins and possess tumor- overwhelming activity. At the final sixth stage (even before the diversification of the placental lineage of mammals), the *bex/tceal* and armcx gene families expanded, forming the BGW cluster.

The X chromosome contains a disproportionate number of genes associated with mental functions, as evidenced by the prevalence of mental retardation in men. However, all nine X-linked genes that, when mutated, lead to mental impairment have orthologues in fish or even earlier eukaryotes. It also turned out that all bex, wex, and gasp genes are expressed in the brain [21]. Thus, these placental species-specific genes can be considered as possible candidates for the adaptive evolution of the neocortex, a region of the forebrain that is unique in mammals [22].

The presence of the conserved BGW element within the 5'UTR of the bex, wex, and gasp genes suggests its involvement in the regulation of translation [21]. The rationale is that, as previously shown, the rate of translation is influenced by regulatory regions, including the consensus sequence, secondary structures preceding the AUG (site of translation initiation), internal ribosome entry sites, and the site of recognition of sequences specific for regulatory factors, such as protein or RNA. The presence of translational control for the bex, wex, and gasp genes may indicate that the proteins encoded by them are used under certain physiological conditions, at certain developmental stages, or in subcellular compartments. Another possible role for the BGW element could be the regulation of alternative splicing [21].

The same authors identified genes with a coding sequence overlapping more than 50% with annotated TEs and present in more than one species (28 genes in humans and 9 genes in mice) [21].

In plants, various genes have been described whose mutations are involved in artificial selection and are caused by the incorporation of TEs either into coding sequences or into regulatory motifs [23-25]. The same events are found in the genes of different animal species, in particular in chickens [26, 27].

Retrotransposons LINE and SINE in genomic changes. The scale of involvement of retrotransposons in genomic rearrangements was recently revealed by comparing the results of whole genome sequencing of the human genome [28]. This paper considers the transduction of human genomic elements associated with such retrotransposons as L1 and SVA. During their transcription, the signal of its termination at the 3'-end can be ignored by RNA polymerase. As a result, transcription is completed at the host genomic element, creating a chimeric transcript. The work analyzed 3202 sequenced genomes from 26 population groups from different countries and identified 7103 polymorphic L1 and 3040 polymorphic SVA. As a result, 268 and 162 transduction variants of 3'-regions from 7 to 997 nucleotides in length were found involving sequences homologous to L1 and SVA, respectively. In the chromosomes X, 6 and 7, specific loci with the most widely represented L1 and SVA were identified, which, among other things, determined the largest number of transductions.

Of particular importance are the processes associated with TEs which lead to a change in the copy number of genes (loss or increase in their number) [29].

Interactions between TEs and the host genome are complex and include a large number of different mechanisms in each specific case [30]. Multiple variants of TEs insertion into the host genome and interactions with its various genomic elements can lead to different consequences, including both conflicting and

favorable mutually beneficial relationships [31]. Examples of conflicting relationships include insertion of TEs into exons, which leads to frameshift mutations and disturbances in protein structure and function. TEs are also able to increase genomic instability by forming structures that create regions of homology along chromosomes, which leads to chromosomal rearrangements, i.e., duplications, deletions, inversions and translocations. When TEs are inserted into regulatory regions, such as 5'-, 3'-regions or gene introns, epigenetic modifications occur, which cause inappropriate activation or suppression of gene expression [31].

An example of cooperative relationships [31] is the use of TEs by the host genome to generate new regulatory signals or coding sequences. This process is called molecular domestication [31]. TEs are able to introduce new enhancer sequences for transcription factors that change the spatiotemporal regulation of gene expression. In Drosophila, after the loss of telomerase, autonomous (LTR-deprived) retrotransposons HeT-A, TAHRE, and TART are actively involved in telomere maintenance. TEs can help maintain the architecture of the genome by providing binding sites for the CTCF protein, which is responsible for the creation of topologically associated domains (TADs). Topologically associated domains are directly involved in the assembly of gene expression programs [32)], which significantly depends on the distribution and movement of various TEs throughout the genome.

It should be noted that LINEs are widely represented in almost all eukaryotes [33]. LINE1 long dispersed nuclear elements are the most successful family of TEs in terrestrial mammals. The length of LINE1 varies within 6 kb. LINE1s carry the genes for two main proteins, ORF1 and ORF2 (similar to the *gag* and *pol* genes of exogenous retroviruses), which are involved in transposition mechanisms. The product of ORF1 is an RNA-binding protein, ORF2 has endonuclease (EN) and reverse transcriptase (RT) enzymatic activities. Despite their abundance, most LINE1 elements are not transposed due to the accumulation of mutations. Of the approximately 8,868,000 and 599,000 LINE1 elements in the human and mouse genomes, respectively, only 80-100 and approximately 2300 LINE1s are transpositionally competent [34].

Insertion of LINE1 occurs mainly through endonuclease-dependent reverse transcription. LINE1 RNA forms a complex in the cytoplasm with several ORF1p homotrimers and at least one ORF2p dimer to form a ribonucleoprotein (LINE1 RNP). The LINE1 RNP enters the cell nucleus, where the ORF2p endonuclease releases the 3'-hydroxyl group. The free 3'-hydroxyl group is then used by the ORF2p reverse transcriptase as a primer for the synthesis of the LINE1 cDNA, starting from the LINE1 polyA-mRNA tail. The non-autonomous short dispersed genomic element (SINE) polyA-mRNA tail can compete with LINE1 polyA-mRNA for the LINE1 ORF2p reverse transcriptase, exploiting the LINE1 mechanism for transposition. In addition, LINE1 ORF2p can also retrotranspose unique protein-coding mRNAs and small nuclear RNAs.

LINE1 retrotranspositions depend on other cellular proteins. A number of positive transposition regulators have been identified, including, in particular, nucleolin and heterogeneous nuclear ribonucleoproteins (hnRNPs), mitogen-activated protein kinases, and cyclin-dependent kinases. To complete the LINE1 insertion, a DNA repair mechanism is required. There are several mechanisms for limiting LINE1 transposition in mammalian genomes at the transcriptional, post-transcriptional, and posttranslational levels. Transcription of LINE1 is downregulated by methylation of CpG DNA regions and repressive histone modifications at the LINE1 promoter. Various KRAB-ZFPs (zinc finger DNA interaction proteins) selectively recognize ERV and LINE1 and recruit KAP1 (heterochromatin protein). Post-transcriptional suppression of LINE1 mRNA is mediated through

RNA interference by small RNAs. LINE1 retrotransposition may also be post-translationally limited by a number of interferon-stimulated genes [34].

The estimated frequency of new LINE1 inserts is approximately 1 per 100 births in humans and 8 in mice. The possibilities of LINE1 influence on the work of the genome are numerous and include, in particular, the induction of genomic insertions, the control of retrotransposition of SINE/Al elements (which themselves act as the main modulators of genomic variability). The endonuclease activity of LINE1 ORF2 is a potential inducer of mutagenic effects, regardless of retrotransposition. The LINE1 and SINE elements can contribute to changes in the number of repeats in microsatellite loci, especially those rich in AT, which are abundant in genomes.

Thus, due to the abundance in genomes, LINE1 and SINE elements can induce large-scale genomic changes, such as duplications and inversions. It is generally accepted that, on average, any two human haploid genomes differ by approx. 1000 TEs, mainly from the LINE1 or Alu families [34].

Epigenetic effects of LINE and SINE transpositions. The inherent self-assembly property of L1 (LINE) and B1/Alu (SINE) repeats provides multiple trigger points for the formation of nuclear subregions in the interphase nucleus. Repetitive DNA sequences also serve as anchor sites necessary for the functioning of transcription mechanisms, for the binding of regulatory proteins and RNA. TEs can influence gene expression profiles due to the fact that their DNA or RNA transcripts can interact with DNA and/or RNA binding proteins. The accumulation of molecules can lead to aggregation of repeats containing the same type, forming separate compartments in the nucleus and, thus, changing the genome packaging. Nuclear segregation of compartments rich in L1 or B1/Alu can be further enhanced by binding their DNA sequences to subnuclear structures (nuclear speckles or nucleolus, respectively) that serve as scaffolds to stabilize the nuclear architecture [32].

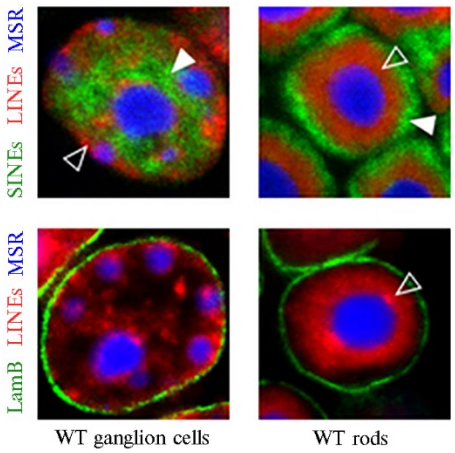

Fig. 2. Distribution of heterochromatin markers LINE (red)**, euchromatin SINE** (green)**, microsatellite sequences MSR** (blue)**, LamB** (lamina protein - a protein network underlying the nuclear envelope, green) **in the nuclei of nerve ganglion cells** (on the left, WT ganglion cells) **and in cylindrical cells of retinal photoreceptors** (on the right, WT rods) (35).

Apparently, the most illustrative example of the involvement of tandem and dispersed repeats in the regulation of changes in gene expression programs through dynamic changes in the architectonics of the interphase nucleus was described in a study performed on the interphase nuclei of cylindrical photoreceptor cells in the retina of nocturnal mammals.

They had an inverted pattern of localization of heterochromatin and euchromatin compared to other nuclei: heterochromatin was located inside, euchromatin was located on the periphery of the nucleus under the lamina (Fig. 2) [35]. The authors of this study came to the conclusion that a significant contribution to such differences can be made, in particular, by contacts between homologous dispersed repeats localized in different regions of chromatin.

In a group of closely related species of the bristled rat, pronounced interspecies differences were found in the chromosomal positioning of the LINE and SINE retrotansposons, which made it possible to suggest their involvement in

speciation [36].

It is interesting to note that the involvement of the mutual positioning of chromosomes and their regions (domains) in interphase nuclei in interspecific differentiation was discussed quite a long time ago by V.N. Stegniy [37] on the example of polytene chromosomes of the malarial mosquito. He also revealed the participation of mobile genetic elements, in particular LINE, in the contacts of chromosomes with the lamina of the interphase nucleus [38].

The wide distribution of LINE1 elements in mammalian genomes makes them good candidates for centers of local chromatin organization and higher-order chromatin architecture. Interestingly, the LINE1 and SINE elements, whose number in the genome depends on the LINE1 ORF2 activity, have opposite genomic distributions. LINE1s are more common in silent, almost gene-free, heterochromatic, AT-rich regions, while SINE elements predominate in gene-rich, expressed, euchromatic, GC-rich regions. A similar general subdivision of genomic regions is observed between compartments B and A (non-expressed and expressed regions of the genome, respectively) in the interphase nucleus. It is assumed that the presence of LINE1 and SINE elements correlates with the presence of compartments B and A, respectively, and LINE1 and SINE can directly participate in the formation of chromosomal regions of these two compartments. The high association of RNA repeat sequences, including LINE1 RNA, with chromatin, and evidence that chromatin-associated RNA promotes global chromatin organization, support this hypothesis [35].

Iinterestinly, it is shown that the regulatory effects of LINE insertions into the host genome may differ depending on the function of the genes [39]. A comparative analysis of the localization of LINE1 and LINE2 in regulatory sequences (promoters, enhancers) in genes whose expression is tissue-specific and common genes (housekeeping genes) revealed a relatively increased rate of evolution of tissue-specific genes, in which the inserted LINE1 retrotransposon is actively involved. The more ancient retrotransposon LINE2 is more often present in the regulatory sequences of housekeeping genes [39].

TEs, in particular LINE1 and various ERVs, are globally demethylated and expressed during major waves of epigenetic erasure that occur during preimplantation and germline development [34, 39]. Similarly, weakening of TEs repression in somatic cells upon demethylation of H3K9me2 occurs in artificially induced pluripotent stem cells [39]. Many families of TEs act as important cis-elements of gene regulation [39]. However, it becomes clear that TEs gene products also play a significant role in the early development of an organism. RNA derived from the transcription of elements of the endogenous retrovirus HERVH is important for maintaining the undifferentiated state of human embryonic stem cells (perhaps by acting as a long non-coding RNA, the lncRNAs involved in regulatory networks of gene expression). Rec protein encoded by the endogenous retrovirus HERVK is highly expressed in human embryos where it increases antiviral resistance by upregulating the IFITM1 (interferon-induced transmembrane protein 1) proteins [39]. Rec also forms complexes with a subset of endogenous RNAs, thus regulating their binding to ribosomes and, as a result, protein expression levels, which may be important for early embryonic development.

It is assumed that the mechanisms of limiting the mutagenic activity of TEs can be considered from the point of view of the balance between the beneficial and negative functions of TEs, which ensures the reproducible development of the organism up to reproductive age, while maintaining the ability to quickly generate genetic novelty in a changing environment. However, such metastability can in some cases lead to disease as a result of adverse retrotransposition events. In other words, the potential reactivation of TEs in adult somatic cells and their association

with diseases such as cancer may be the price to pay for the importance of TEs for the development and evolution of organisms [40].

Endogenous retroviruses. Endogenous retroviruses exhibit many functions that affect the normal biology of host cells [41]. Some of these functions are directly related to interactions with exogenous retroviruses. These may include receptor interference, immune self-tolerance, recombination, and the simultaneous action of restricting and stimulating exogenous retroviral infection using various mechanisms.

Endogenous retroviruses (ERVs) are divided into different classes and are direct descendants of exogenous retroviruses. Retroviruses (exogenous and endogenous) are divided into three classes (I, II and III) each of which corresponds to the expanded groups ERV1, ERV2 and ERV3. Class I retroviruses are endogenous (ERV1) and exogenous gamma and epsilon viruses, class II includes ERV2 and exogenous alpha, beta, delta retroviruses and lentiviruses, class III are spumaviruses and ERV3 (ERV3/ERVL mammals). The ERV4 group now includes the previously identified and described CrocERV in crocodilians, as this ERV differs from any other known groups of retroviruses and, at the same time, shares homologous regions with all [42].

Invertebrate eukaryotic genomes also contain retrovirus-like LTR-containing elements, the so-called LTR retrotransposons. They are divided into three groups, the *Pseudoviridae* (Copia/Ty1) group, found in plants and fungi, the *Metaviridae* (Gypsy/Ty3) group, which is also found in plants and fungi, and the *Semotivirus* (Bel/Pao) group, found in metazoans. The most diverse group is the *Metaviridae*, which includes about 10 subgroups. One of them (chromoviruses) has a wider range of hosts (plants, fungi and vertebrates). Chromoviruses got their name because their pol gene encodes an integrase with a chromodomain (chromatin organization modifier domain), the nucleosome-binding portion of which can mediate a provirus insertion specific to the host genome sequence [43].

There are regiond of homology between various taxonomically distant retroviruses, including pathogenic ones, in particular with human immunodeficiency viruses, bovine leukemia viruses, chromoviruses (in centromeres more often in plants), polychaete retroviruses, which indicates a wide scale of recombinations between these viruses [43].

A unique example of the integration of an exogenous retrovirus into the koala genome, which occurred under the supervision of researchers, has been described [44]. It is clearly shown how endogenous retroviruses arise as a result of infection of cells that form gametes. During retroviral infection, the RNA of the viral genome undergoes reverse transcription into proviral DNA, which is subsequently integrated into the host genome. Sometimes integration can also occur in primary embryonic cells, which can lead to the formation of an embryo and then progeny with an integrated provirus in all cells. Over many generations of the host, proviral DNA undergoes significant mutational changes (single nucleotide polymorphisms, insertions and deletions), which usually result in an inability to produce an infectious virus [45].

It should be noted that TEs are widely involved in epigenomic variability, including changes in methylation pattern, histone modification, miRNA formation, and transgenerational inheritance [46, 47]. It can be expected that the construction of new niches in which humans and domesticated species participate contributes to the activation of TEs and the formation of new regulatory networks based on them [48-50].

Non-coding RNA (ncRNA), miRNA. *Major sources of non-coding RNA.* In addition to the functions of messenger, ribosomal, and transfer RNAs, many other RNAs (non-coding RNAs, ncRNAs, ncRNAs) play a regulatory role

in eukaryotes [51]. They act as regulators of the functional activity of nucleic acids, recognizing their specific target sequences by homology, and are involved in the regulation of growth, development, and stress reactions in animals and plants. Regulatory ncRNAs ranging from short to long (lncRNAs) control a wide range of biological processes. Depending on the mode of biogenesis and function, ncRNAs have evolved into various forms, including microRNAs (microRNAs), small interfering RNAs (siRNAs), microRNA variants (isomiRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), and derivatives of non-coding RNAs [51]. One of the elements of regulatory networks is microRNA, which has a hairpin structure and is a derivative of TE and other genomic elements [51].

In eukaryotic cells, gene expression is regulated at several levels. At the post-transcriptional level, regulation is modulated by various trans-acting factors that bind to specific sequences in mRNA. This affects various processes such as the rate of degradation and the efficiency of mRNA translation, splicing, and localization [52]. MicroRNAs in combination with the Argonaute enzyme form an RNA-induced silencing complex (miRISC), which uses a complementary nucleotide sequence to suppress the target transcript. RNA-binding proteins (RBPs) promote post-transcriptional control by affecting mRNA stability and translation when bound to cis elements in the mRNA transcript. RBPs influence gene expression through miRISC or its interaction with the target mRNA [52].

LINE1 and endogenous retroviruses (ERVs) induce the formation of dsRNAs formed from convergent transcripts or hairpin structures as a result of end-to-end transcription of head-to-head/tail-to-tail elements. Alu elements (SINEs) are much shorter and form hairpin structures as well as open dsRNA hybrids [53].

MicroRNAs have become the object of in-depth research in recent years. A large amount of data has been accumulated on the involvement of various microRNAs in the regulation of developmental stages and responses to stress in plants [51], as well as in the control of feed payment in some main species of farm animals [54, 55].

Epigenetic mechanisms such as DNA methylation, histone modification, and expression of non-coding RNAs appear to be particularly important in the response of multicellular organisms to environmental stressors [54]. In addition, abiotic stress, such as heat shock, can induce suppression of TEs, causing destruction of RISC by the inducible Hsp70 chaperone, which directs the RISC complex to lysosomes.

In connection with the involvement of miRNA molecules in epigenetic variability, studies of their genomic distribution in a number of domesticated mammalian species (cow, dog, horse, pig, and rabbit) were performed. Data have been obtained on the predominant localization of miRNAs in introns and intergenic spaces [55]. It has been found that in the process of evolution, new miRNAs appear and the existing ones are lost [55].

The results of a comparative analysis of the so-called young and old orthogroups (common in origin) of miRNAs in different tissues in the studied species indicated that the expression of young groups has more pronounced tissue-specific features compared to old groups [55]. Approximately 20% of the new orthogroups are localized to the brain, and their target targets appear to be enriched in genomic elements to ensure neuronal activity and processes of their differentiation.

Changes in the microRNA regulatory network were also found during domestication. Thus, a comparison of the extinct predecessors of cattle *Bos primigenius* with representatives of the modern species *B. taurus* showed that noncoding miRNAs became key regulators of the spatiotemporal expression of target genes that control the growth and development of mammals [56]. During the domestication process, the selection of mutational changes in microRNAs and/or microRNA

binding sites could provide a mechanism for generating some of the traits that distinguish domesticated cattle from their wild predecessors. An open reading frame DNA sequence analysis was performed for 19,994 pairs of orthologous protein-coding genes from existing *Bos taurus* genomes and one extinct *B. primigenius* genome. Polymorphisms of miRNA-binding sites in the 3'-UTR were identified in 1620 of these orthologous genes. The identified 1620 genes with miRNA binding sites that differ between *B. taurus* and wild progenitors are candidate genes associated with domestication. These 1620 candidate genes have been found to be involved in the control of pigmentation, fertility, neurobiological processes, metabolism, immunity, and animal performance characteristics (including milk quality and feed conversion efficiency) [56]. These results suggest that the directed selection of miRNA regulatory variants was important in the domestication and subsequent artificial selection that gave rise to modern European cattle [56].

It has been shown that domesticated species differ from closely related wild ones in terms of an increased frequency of deletions [57], i.e. mutations, the nature of which is also closely related to retrotransposons [58].

There are also direct results of experimental studies indicating significant differences in the distribution of TEs in species such as the domestic dog (*Canis lupus familiaris*), gray wolf (*Canis lupus*) and red wolf (*Cuon alpinus*) which are associated with domestication processes [59]. Differences between the genomes of these species were revealed. Thus, TEs in the dog account for 41.75% of the nucleotide sequences in the genome which is higher than that of the gray wolf (39.26%) and red wolf (38.51%). The most divergent components of TEs in these genomes are long dispersed nuclear elements LINE1 (L1) and microsatellites, which distinguish the dog from the gray wolf by 86.1%, from the red wolf by 83.2%.

A comparison of the distribution of transposable genetic elements between the domestic rabbit (*Oryctolagus cuniculus domesticus*) and the pika (*Ochotona princeps*) showed a markedly higher frequency of occurrence in the domestic species compared to the wild one [60]. Similar differences were found when comparing a domestic cat (*Felis silvestris catus*) and a closely related wild species (Felis silvestris silvestris) [61].

Therefore, key questions of domestication are where does the large range of phenotypic variability come from and why some species are amenable to domestication unless others. In our opinion, the answer may be that during formation of a new multicomponent niche human (domesticator)—plants/animals (domesticants), an increased density of mobile genetic elements occurres in the genome of the domesticant due to a reduced resistance of genomes to transposon integration. This assumption is supported by the involvement of transposons in epigenetic variability [31, 51] and in the organization of the interphase nucleus architectonics [35], by the high rate of TEs evolution [34], the frequency of recombinations between TEs [43, 58], and the differences between domestic and wild species in terms of prevalence of some transposons in genomes [56, 59-61]. In our previous studies, we attracted attention to the fact that the main unresolved issue in the search for leading genomic features in domesticated species when compared to closely related wild species is the source of variability that distinguishes these closely related forms [62, 63]. The accumulated data on the leading role of transposons in evolutionary transformations, in the formation and modulation of regulatory networks that control gene expression profiles, suggest that mobile genetic elements are also essential for domestication processes.

Thus, in our opinion, the high ability of mobile genetic elements (TEs) to generate variability, their relatively increased frequency of occurrence in the genomes of domesticated species compared to closely related wild ones, and the

involvement of TEs in the formation of regulatory networks of gene expression profiles suggests that the formation of new habitat conditions and human-controlled reproduction leads to increased contacts of domesticated forms with a wide range of exogenous viruses new to them. The events ultimately cause an increased variability (including regulatory networks) which contributes to phenotypic diversity and the effectiveness of artificial selection. This or a close mechanism, apparently, should be involved in all evolutionary processes associated with the emergence of new forms.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 852-881
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 632.4.01/.08:579.64:577

doi: 10.15389/agrobiology.2022.5.852eng
doi: 10.15389/agrobiology.2022.5.852rus

ERGOT *Claviceps purpurea* (Fries) Tulasne ALKALOID DIVERSITY AND VIRULENCE: EVOLUTION, GENETIC DIVERSIFICATION, AND METABOLIC ENGINEERING

(review)

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The authors declare no conflict of interests
Acknowledgements:
The work was done within the framework of topic 10.6. Plant protection and biotechnology (FGUU-2022-0014)
Received June 7, 2022

Abstract

Claviceps purpurea (Fries) Tulasne is a valuable source of many bioactive metabolites (alkaloids) for pharmaceutical industry and a unique plant—parasite model but also a serious adversary for plant growing, feed and livestock industries causing significant economic damage in different countries. Ergot appeared in South America in the Paleocene, the age of the genus *Claviceps* is 20.4 million years (K. Píchová et al., 2018). Intraspecific diversity and divergence of indole alkaloid production gene cluster occurred in accordance with the evolutionary "hourglass model" (M. Liu et al., 2021). Ergometrine, ergosine, ergotamine, α-ergocryptine, ergocornine, ergocristine and 8-S(-inine-) epimers are the major identified ergoalkaloids which account for approximately 50 % of the ergot alkaloid metabolome. *Claviceps* alkaloid gene clusters consist of varying numbers of genes, posses two or three copies of *dmaW*, *easE*, *easF* genes, and there are many facts of frequent gene loss and acquisition (M. Liu et al., 2021). Differences in metabolomic profiles of *C. purpurea* indole alkaloids correlate with the *lpsA* gene variability. Diversity of the ergot alkaloids is a result of sequence diversity in the *easH/lpsA* tandem-duplicated region (C. Hicks et al., 2021). The *lpsA1* and *lpsA2* genes derived from recombination events (S. Wyka et al., 2022), i.e., the *lpsA* genes are supposed to be due to reshuffling (C. Hicks et al., 2021). *C. purpurea* has a relatively large accessory genome (~ 38 %), high recombination rates ($\rho = 0.044$), and transposon-mediated gene duplication (S. Wyka et al., 2022). A transgenic yeast line is capable of producing enantiopure D-lysergic acid up to a level of 1.7 mg/l (G. Wong et al., 2022). Genetically engineered cultures of *Metarhizium brunneum* can produce 86.9 % lysergic acid and 72.8% dihydrolysirgic acid (K. Davis et al., 2020). Expression of the *trpE* and *dmaW* genes is quantitatively related to intensity of alkaloid synthesis in saprophytic *Claviceps* cultures (M. Králová et al., 2021). Pectin is the main target of CAZymes proteins responsible for cell wall degradation during *C. purpurea* and *C. paspali* infection (B. Oeser et al., 2017; H. Oberti et al., 2021). Polygalacturonase, MAP kinase, transcription factor CPTF1 (*Cptf1* gene), GTPase (*Cdc42* gene) make the main contribution to *Claviceps* virulence (B. Oeser et al., 2017; E. Tente et al., 2021). Ergot affects the auxin, ethylene, and cytokinin pathways in plants, with varying effects depending on tissue type and time after inoculation (E. Tente, 2020; Tente et al., 2021). Wheat resistance is due to mutations in DELLA proteins (E. Tente, 2020; A. Gordon et al., 2020) while rye resistance is due to pectinesterase activity, cell wall modification, and modulation of pollen tube growth (COBRA-like protein and pectinesterase inhibitor) (K. Mahmood et al., 2020).

Keywords: *Claviceps purpurea*, ergot, alkaloids, biosynthesis pathways, toxicity, virulence, genotype, gene clusters, *Claviceps*, *C. purpurea*

Recent identification of genes encoding proteins that catalyze the successive steps in alkaloid biosynthesis in fungi are due to advances in genomics and molecular biology [1]. The diversity of fungal secondary metabolites can be expanded by activating silent gene clusters in artificially cultivated strains or by replenishing existing collections of fungal cultures in order to obtain new natural biological products [1, 2].

Ergot, a parasitic fungus *Claviceps purpure*a (Fries) Tulasne, is an important source of biologically active substances for the production of drugs [3-5]. Natural ergoalkaloids, as well as their semi-synthetic derivatives, are used as pharmaceuticals in modern medicine, for example in obstetrics and gynecology [6] as inhibitors of prolactin release [7, 8], as well as for the treatment of a number of neurological diseases [9], in particular parkinsonism [[9, 10], dementia [9, 11] and hypertension [9, 12]. Recent studies point to the high promise of ergot alkaloids (metergoline derivatives) for the creation of new antimicrobial agents [13]. In Russia, with the participation of VILAR, a number of drugs based on ergot alkaloids have been developed: abergine (α-, β-ergocriptines), novocristine (dihydroergocristine), bellataminal (ergotamine), ergometrine maleate (ergometrine) [14].

The VILAR collection includes five strains of the ergot parasitic culture *Claviceps purpurea* (Fries) Tulasne (producers of α-, β-ergocriptines, ergotamine, ergotoxin, ergocristine, and ergocornam). All parasitic strains are maintained in an active state, including the stage of seasonal cultivation in the field on winter rye crops (variety Moskovskaya 12) and an intermediate in vitro stage in the form of an axenic culture. In addition, two saprophytic ergot strains (producing ergocriptine and ergotamine) have been registered, which are also maintained in a live collection [15-17].

In agriculture, ergot causes significant economic losses. Ergot is a health hazard not only for animals, but also for humans [18, 19]. In world practice, contamination of products, animal feed, and pasture crops with ergot toxins is a fairly common occurrence, and the toxic effect of ergoalkaloids on animal metabolism has been fairly well studied [20-22], including in vitro [23, 24]. In addition, not only alkaloids, but also other secondary metabolites of ergot, such as ergo pigments [25), lecanoric acid and its derivatives [24], indole-diterpenes [25-28], epipolytiodiketopiperazines [29], can have a toxic effect.

The quantitative characteristics and composition of the metabolome of ergoalkaloids can vary greatly depending on the specific conditions for the development of the fungus and its species, which makes it difficult to monitor the content of ergoalkaloids and, therefore, assess the degree of contamination of agricultural products with ergotoxins [30, 31]. In wheat and barley plants, alkaloids also penetrate the healthy grain, which is formed above and below the infected areas of the inflorescence [32].

Issues of toxicology and secondary metabolism of ergot have become more and more relevant in the last decade. Scientific reviews consider the technological and physiological features of ergot cultivation [15, 16], functional characteristics of key genes and gene clusters involved in the biosynthesis of alkaloids in different genera of the Ergot family (*Clavicepitaceae*) [30, 33-35], and discuss the identification and classification of intermediate products. synthesis [36, 37], construction of pathways for the formation of ergoalkaloids in the fungus and various heterologous organisms, strategies for optimizing the creation and use of recombinant producer strains. Ergot virulence factors have been described [32, 38, 39]. Published analytical materials (39) are refined and supplemented [40, 41].

In the present review, we have detailed and systematized the results of studying the mechanisms of alkaloid biosynthesis and ergot virulence in different members of the *Claviceps* genus, taking into account new data on the role of alkaloid biosynthesis and virulence cluster genes in the evolution and spread of ergot, paying attention to methodological aspects, including metabolic engineering. Such an integrated approach to understanding the genetic diversification of ergot, the associated diversity of alkaloids, toxins, and knowledge of the molecular mechanisms of their biosynthesis expand our understanding of the biology of the *Claviceps* genus and form the basis for solving practical problems both in agriculture

and in the creation of drugs based on ergot metabolites. .

Evolution, distribution and role of ergot toxic metabolites. Molecular genetic technologies make it possible to study the biology of ergot at the level of gene clusters, genomes, transcriptomes, metabolomes, using a wide arsenal of tools. For the analysis of the ergot genome, pyrosequencing is used by the method of single/paired-end pyrosequencing) (42), paired-end sequencing (PEs) [43, 44], Sanger sequencing [45], shotgun pyrosequencing and mate-pair sequencing [46]. Thus, PEs were used to analyze the transcriptome during signaling interactions of *C. purpurea* with the host *Secale cereale* [47] and sequencing the genome of *Claviceps paspali* [48], single-end multiplexed sequencing was used to analyze the wheat transcriptome upon infection with *C. purpurea* (49).

The study of the evolutionary history of the genus *Claviceps* has recently begun [43, 50]. It has been established that the center of origin for ergot in the Paleocene was South America [50]. Four sections are classified according to ecological, morphological, and metabolic features: *Citrinae*, *Paspalorum*, *Pusillae*, and *Claviceps* [43, 50], which split in the *Paleocene* and *Eocene* [50]. The speciation of *Pusillae* occurred during the Eocene, Oligocene, and Miocene and was associated with warm-season herbs from the subfamilies *Panicoideae*, *Aristidoideae*, *Chloridoideae*, *Micrairoideae*, *Arundinoideae*, and *Danthonioideae* [43, 50] with subsequent spread from South America to Africa [43, 50]. The age of the genus *Claviceps* is estimated at 20.4 Myr [50], and its division and further expansion into North America is associated with the ecological features of new host plants (transition from parasitizing Cyperaceae sedges to cold-resistant plants from the subfamilies *Pooideae*, *Bambusoideae*, and *Oryzoideae* [syn: *Ehrhartoideae*] [50-52]. This was followed by the worldwide spread of *Claviceps* [43, 50].

Citrinae, *Paspalorum*, and *Pusillae* are characterized by a narrow range of distribution and low toxicity, while the section *Claviceps* is considered to be evolutionarily more successful and adaptable, since it has the widest range of host plants and range [43, 50, 53]. Currently, the classifications and systematic position of natural parasitic strains of ergot continue to be refined [54-56].

The ergot population in the US differs from European isolates [57]. It is claimed that in the USA there are no subpopulations of this parasitic fungus, formed depending on the host plant [54, 57, 58]. In the US, there are moderate levels of genotypic diversity (H = 3.43-4.23) and gene diversity (H_{exp} = 0.45-0.57) [59]. There is genetic differentiation between North American populations from different host plants (22%), but it is geographically mixed. The standardized association index ranged from 0.007 to 0.122 for four groups (two regions and two hosts, Kentucky bluegrass and perennial ryegrass) [59]. Three genetic lines of *C. purpurea* have previously been identified [60], reflecting ecological differentiation and adaptation [53, 61, 62]. Lines designated as G1, G2, and G3 ecotypes have been differentiated based on conidial morphology, alkaloid profiles, RAPD (random amplified polymorphic DNA) markers, and the ability or inability of sclerotia to maintain buoyancy [53, 61, 63]. At present, the ecotypes of *C. purpurea* G1, G2, and G3 are described as three independent species, the *C. purpurea sensu stricto*, *C. humidiphila*, and *C. spartinae*, respectively [53, 60].

The reasons for the evolutionary success and high adaptability of the section *Claviceps* are not completely clear [43]. To some extent, this can be explained by the production of a large number of toxic metabolites, which limits the consumption of plants affected by the fungus by vertebrates and invertebrates and is important for the mutualistic relationship between the parasite and the host, which provides plant protection from phytophages [50]. However, this issue remains the subject of debate [40].

For the divergence of of the indole alkaloid cluster (EAS) genes, phylogenetic analysis and analysis of DNA polymorphism revealed a correspondence to the evolutionary hourglass model (HGM) in the intraspecific diversity of ergot [44]. The description is borrowed from the ontogenesis when morphological divergences on the middle stages of embryonic development are more conservative than those at earlier and later stages [44, 64, 65]. Thus, the rate of evolution of the genes for the early stages of synthesis of the *dmaW* and *easF* alkaloids is much higher than that for the genes for the intermediate stages *easA*, *easC*, *easD*, and *easE* [44]. This pattern, supported by genomic studies, is consistent with the hourglass model [44, 66]. The HGM model suggests developmental constraints [44, 67]. Metabolic pathways for the synthesis of alkaloids have been viewed as unusually inefficient due to the fact that many intermediates accumulate in excess of what is needed to form the final products [44, 68]. However, this redundancy of intermediates is likely to serve as a factor in the stabilization of the biosynthetic pathway and hinder the selection of variants with changes in the corresponding genes [44].

The variety of *Claviceps* alkaloids has been deribed from three main processes. i.e., the acquisition of genes, the loss of genes, and a change in the sequence of genes for the biosynthesis of alkaloids [35, 46].

Changes in the architecture and plasticity of the genome can shape the direction of the evolutionary process of fungi and their adaptability [43]. Presumably, it is the secondary metabolites of ergot that serve as the factors of the primary influence on the diversification and promotion of the species into new ecological niches and help maintain its global distribution and a wide range of hosts [42]. The composition of the alkaloid synthesis gene cluster and unique polymorphisms indicate that *C. purpurea* is currently undergoing a process of adaptation resulting in a wide variety of peptide alkaloids [45]. Thus, the evolution of ergot is directed from specialized genomes (*Citrinae* and *Paspalorum*) to adaptive ones (*Pusillae* and *Claviceps*). This is facilitated by the joint localization of the transposed elements around the effectors. It is also suggested that in section *Claviceps* there was a loss of the repeat-induced point mutation (RIP), which led to unrestricted tandem gene duplication that corresponds to an increase in the potential for expansion of the range of hosts and speciation [43]. The results show the absence of gene duplication in sections *Citrinae* and *Paspalorum*, probably due to the presence of RIP-like mechanisms, which corresponds to the high host specificity and low species diversity of *Paspalorum* [43]. It is not clear whether such rearrangements gave *Claviceps* an advantage in moving to new hosts and new climatic conditions when separating sections and leaving South America, or were a consequence of this event [43].

C. purpurea showed significantly high recombination rates ($\rho = 0.044$), a relatively large accessory genome (38%), and transposon-mediated gene duplication [42]. It is important to note that the total content of transposable elements in the ergot genome is relatively low (8.8%), the genome size does not vary [42], and recombination is not the main duplication factor in *C. purpurea* [42]. Pseudogenization and neofunctionalization can also be significant processes. Due to the absence of RIP mutations, the increase in the number of transposable elements is probably controlled by high recombination rates [42].

We believe that the “hourglass” model in the evolutionary trajectory of the ergot alkaloid productivity gene cluster most likely indicates that parasitic strains that are promising in terms of alkaloid bioproduction can be weakened by natural selection and lost.

Identification of ergot strains. SSR (simple sequence repeats, short simple repeats, microsatellites) markers have been developed for rapid

identification of *C. purpurea* strains. In addition to distinguishing *C. purpurea* isolates, these SSRs can differentiate isolates of three other *Claviceps* species, the *C. pusilla*, *C. paspali*, and *C. fusiformis* [69]. For genotyping, multilocus sequence typing (MLST) can be used in combination with quantitative real-time polymerase chain reaction (qPCR) [70].

Some of the alkaloids are specific to certain strains of *C. purpurea* and can be used as chemotaxonomic markers for identification [71].

Ergot alkaloids and their producers. At present, the production of ergot alkaloids with various pharmacological activities is based mainly on fermentation using saprophytic cultures [9, 17, 36]. Approximately 60% of commercially produced ergoalkaloids are obtained by submersible cultivation of specially created mutant or recombinant strains of *C. purpurea* or heterologous organisms on liquid nutrient media, the remaining 40% are obtained by cultivation of ergot in the field [9, 72, 73].

The main problems in the production of ergoalkaloids are a wide variety of synthesized alkaloids, which complicates the subsequent extraction and purification from by-products and increases the cost of production [9, 36], as well as the instability and tendency to degradation of saprophytic strains during cultivation and storage [9, 36, 72, 73]. Saprophytic strains lose the sclerotio-like cellular morphology of the mycelium with an irreversible loss of the ability to produce alkaloids [72, 73]. Modern methods for the chemical synthesis of D-lysergic acid include long multistage reactions under harsh conditions and are not always enantioselective [3, 9, 74, 75].

The parasitic stage of the ergot life cycle is characterized by the formation of sclerotia, the only resting vegetative organ of the fungus, in which alkaloids are synthesized [76]. Ergot sclerotia collected in different geographical areas can serve as a valuable starting material for the selection of highly productive strains [77].

For the cultivation of ergot, the alternation of the parasitic stage of the life cycle and the axenic culture (re-sowing of sclerotium on a nutrient medium in vitro with the possibility of obtaining saprophytic mycelium with sclerotia-like morphology and purple pigmentation) is extremely important [40]. Morphological selection in axenic culture makes it possible to obtain a plectenchymatic form of mycelium, which resembles the early sclerocial stage of rye infection, producing lysergic acid and peptide alkaloids under submerged cultivation conditions [40, 78, 79]. Lysergic acid is also produced in vitro by isolates of parasitic strains selected in atypical habitats [40, 80]. However, this is a rare phenomenon: selection based on morphological features requires the cultivation of a large number of sclerotia in axenic culture, the detected mycelium is poorly separated, and the resulting isolates are extremely sensitive to cultivation conditions, which makes the process very time consuming and depends on a large number of different factors [17, 40].

UV mutagenesis [7, 16, 81-83] or chemical mutagens [7, 16], in particular nitrous acid [7, 84] or N-methyl-N'-nitro-N-nitrosoguanidine [7, 85] are applicable to obtain auxotrophic mutant strains of ergot or mutants with altered production of ergoalkaloids [7].

Among the methods for obtaining recombinant ergot-producing strains, the CRISPR/Cas9 genomic editing technology [7, 86, 87], polyethylene glycol (PEG)-mediated transformation [7, 88], and agrobacterial transformation using *Agrobacterium tumefaciens* (ATMT) [27] should be mention. Improvement of genetic engineering methods based on homologous recombination (HR) [89-91] makes it possible to obtain designer lines of ergot [8, 47] and heterologous organisms [73], including those with increased synthesis of target alkaloids [8, 73].

Methods for sampling, isolation, purification, detection, and quantification of ergot alkaloids in food, feed, plant materials, and animal tissues have been

extensively described, standardized, and validated [92-94]. Liquid chromatography with fluorescence (LC-FLD) or mass spectrometric (LC-MS/MS) detection is used for quantification [92, 94]. Less commonly used liquid chromatography with UV detection (LC-UV) [92) and enzyme-linked immunosorbent assay (ELISA) [92, 94, 95]. Express methods are also used, e.g., quantitative analysis with Van Urk reagent (colorimetric non-selective quantification of indole alkaloids) [40, 94] and thin layer chromatography [40].

The diversity and synthesis biology of ergot alkaloids is important to consider when they are found in agricultural products [96]. Quantitative comparison of ergoalkaloid production in 13 *Claviceps* species from natural communities and grass and cereal agrocenoses in Europe, North America, New Zealand and South Africa [25] drew the authors to the conclusion that doses and mechanisms of toxicity of ergot secondary metabolites must be reconsidered. According to various sources, in *C. purpurea s. l.* the average accumulation of alkaloids in sclerotia varies from 0.01-1.3 mg/g [61, 96-98] to 2.88-7.26 mg/g [99], but sometimes reaches 5-10 mg/g [61, 96, 97, 100].

Mass spectra of 67 peptide alkaloids of ergot were obtained [71]. The main identified ergoalkaloids were ergometrine, ergosine, ergotamine, α-ergocryptine, ergocornine, ergocristine and their 8-S(-inine-) epimers, which accounted for at least 50% of the total isolated alkaloid metabolome [25, 71, 95]. Matrix-assisted laser desorption ionization mass spectrometric imaging (MALDI-MSI) assessed the distribution of two representative alkaloids (ergocristine and ergometrine) produced by different strains of *C. purpurea* upon rye infection. Ergometrine shows a relatively even distribution throughout the sclerotium, while ergocristine is concentrated in the proximal region [101].

Cluster of alkaloid bioproduction genes. The cluster organization of alkaloid biosynthesis genes in ergot was first reported in 1999, and in particular, the importance of the *dmaW* gene for biosynthesis was shown [8, 102]. Clusters of genes for the biosynthesis of ergoalkaloids have been found in various fungi [30], for example, in *Clavicipitaceae* [30, 103, 104], in particular, in *Claviceps* [30, 102, 103], *Epichloe* [20, 30], *Periglandula* [3], *Metarhizium brunneum* [86, 105], *Neotyphodium lolli* [106], *Balansia cyperi*, *Balansia obtecta* [30]; in *Aspergillus* [107, 108], in particular, in *Aspergillus fumigatus* [107, 110], *A. leporis*, *A. homomorphus*, *A. hancockii* [111] and *A. japonicus* [112, 113]; in *Clavulinopsis fusiformis* [106]; in *Arthroderma benhamiae* [114, 115]; in *Penicillium camemberti* and P*enicillium biforme* [116].

Ergoalkaloids are represented by three main classes [34, 36, 37]. In clavine-type alkaloids (ergoclavines), the structural framework is formed by the aldehyde hanoklavin-I. Hanoklavin-I, as well as some other ergoclavins, such as agroclavin and elimoclavin, also serve as intermediates in the synthesis of ergoamides and ergopeptides. The second class is the enantiomers of lysergic acid and ergoamides: D-lysergic acid and its amides, including ergometrine. Lactam alkaloids and ergopeptides are the most complex compounds, representing the most numerous and diverse class of ergoalkaloids [34, 36, 37]. The pharmacological effect of ergot alkaloids is attributed to the molecular similarity between their ergoline backbone and the monoamine neurotransmitters adrenaline, dopamine and serotonin [9, 117, 118]. All ergoalkaloids have the common structure of a tetracyclic system (ergoline) containing an indole nucleus, in which rings A and B are formed from tryptophan, and rings C and D are formed as a result of cyclization of dimethylallyl pyrophosphate and tryptophan [25, 36, 72].

The alkaloid synthesis gene cluster in ergot is represented by 12-14 genes [8, 44], the *cloA*, *dmaW*, *easA*, *easC*, *easD*, *easE*, *easF*, *easG*, *easH*, *lpsA*, *lpsB*, *lpsC*; the *lpsA* gene has two homologues, *lpsA1* and *lpsA2*, the *easH* gene

is presented as *easH1*, and the reduced pseudogene *easH2* [8, 44-46]. The alkaloid biosynthetic cluster genes are responsible for the functioning of all the enzymes necessary for the formation of biosynthetic end products, ergotamine and ergocriptine, from tryptophan in some strains of *C. purpurea* [44, 46]. Two additional genes *easP* and *easO* were found in *C. paspali* [44].

The *dmaW*, *easF*, and *easC* genes are expressed at the four initial stages of ergot alkaloid biosynthesis, in addition, the *easE* gene is responsible for ergoline C-ring closure, followed by the formation of tetracyclic clavines, which requires the activity of the *easD*, *easA*, *easG*, and *cloA* genes. Later stages are synthesis of lysergic acid amides, dihydroalkaloids and complex peptides with the participation of the *lpsA-C* and *easH* genes (Fig. 1) [34, 35, 44].

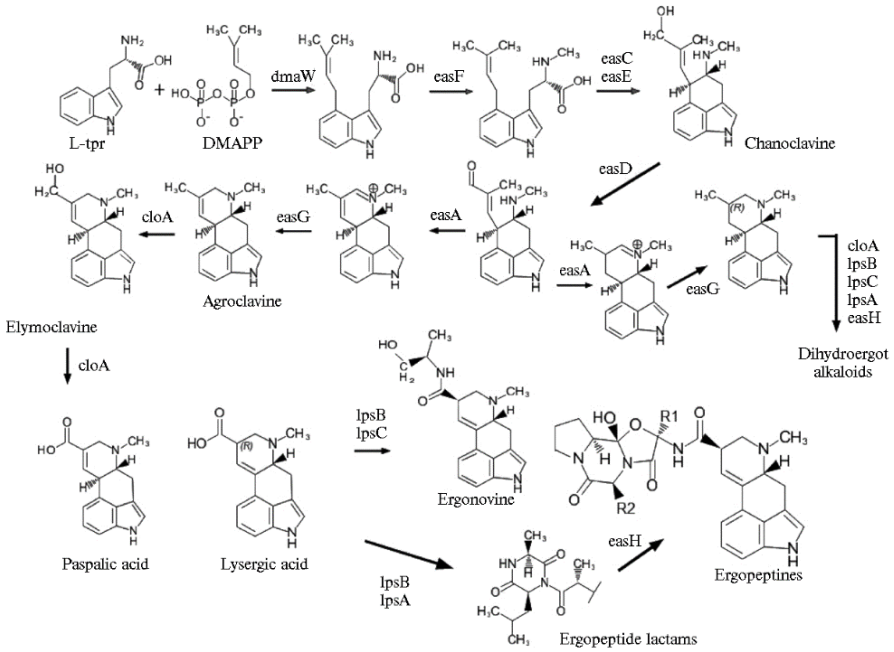

Fig. 1. Metabolic pathway for the biosynthesis of ergoalkaloids in *Claviceps* spp. (with addendum by S. Robinson and C. Young, edited by M. Liu) [34, 35, 44].

The results of studies have shown the presence of a different number of indole cluster genes in representatives of *Claviceps* (44,119). The presence of two or three copies of the *dmaW*, *easE*, and *easF* genes has been established, as well as a generally high frequency of gene acquisition and loss [44]. Homologues of nine cluster genes were found in *C. fusiformis* (44). In some *C. paspali* isolates, the *easG* gene may be absent [55] and *easE* may be non-functional [44]. In *C. paspali*, the alkaloid biosynthesis gene cluster is unstable and subject to partial elimination, and isolates capable of producing the predicted but not yet identified alkaloids have been identified [55]. Some strains of *C. africana* have eight genes for alkaloid biosynthesis (*cloA*, *easH2*, *lpsB*, and *lpsC* are missing), *C. lovelessii* has ten genes, in particular, *lpsC* and *easH2* are missing, and *easH1* and *lpsB* carried mutations leading to the appearance of stop codons [44]. In some strains of *C. maximensis* and *C. citrina* (*Citrinae*), in the absence of production of certain alkaloids, the same genes were not detected [44]. Only representatives of *Claviceps* had *lpsC* and *easH2*, although representatives of *C. perihumidiphila*, *C. ripicol*a, and *C. arundinis* lacked *lpsC*, and *C. capensis*, *C. cyperi*, *C. humidiphila*, and *C. monticola* had a partially identical *lpsC* gene sequence. Three strains of *C. purpurea* and three strains of *C. quebecensis* did not contain *easH2* [44].

Ways of biosynthesis of alkaloids and their genetic control. The metabolomic profiles of ergot alkaloids consist of complex mixtures of minor stereoisomers, constitutional isomers and transition products [45]. Changes in the operation of the alkaloid biosynthesis gene cluster can lead to the accumulation of various intermediates and by-products of the metabolic pathway and affect the rate of accumulation of alkaloids, especially those whose synthesis is regulated by feedback intermediates [8, 34, 35, 120]. The metabolic pathway for the biosynthesis of ergoalkaloids has several branch points. An optimized biosynthetic pathway excluding such points is shown in Figure 2 [9] as an example of the formation of D-lysergic acid from tryptophan. The accumulation of intermediate and final products in different species and strains of ergot is not the same: clavine derivatives, dihydro derivatives are formed, lysergic acid isomerization and lysergine amides, peptide alkaloids and their epimers are synthesized [34, 121, 122].

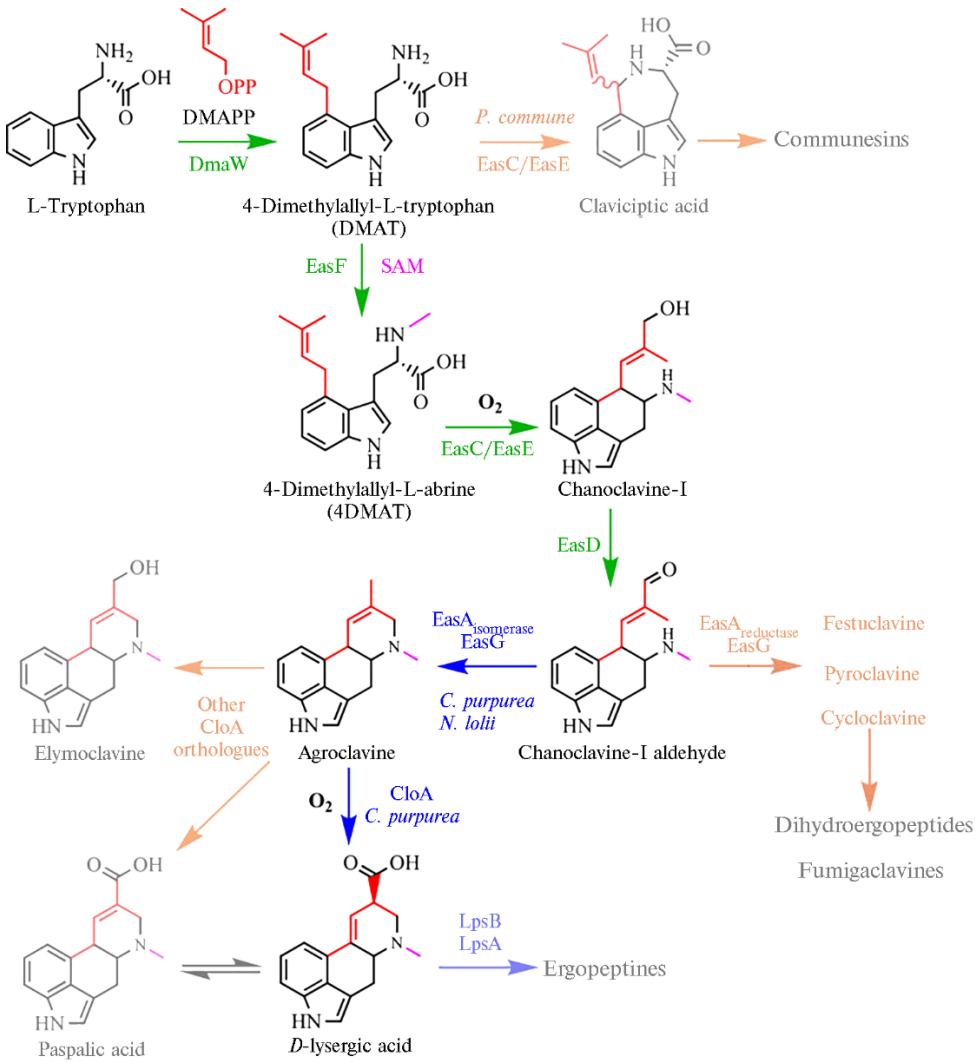

Fig. 2. A reconstructed optimal pathway for the biosynthesis of D-lysergic acid from tryptophan, excluding branch points [9].

It is believed [37] that the early stages of the biosynthesis of ergoalkaloids are conservative. Later stages are controlled by unique genes, which cause modifications and provide a large variety and species specificity of alkaloids in different

taxonomic groups of ergot [37, 114, 123].

Tryptophan synthesis in *C. purpurea* (as well as in other fungi) involves five steps carried out by three enzymatic complexes controlled by four different genes [8, 124, 125]. The most important is the anthranilate synthase complex, which consists of two subunits, AAS-I (α-subunit encoded by the *TrpE* gene) and AAS-II (a trifunctional peptide containing the β-subunit of anthranilate synthase, phosphoribosyl anthranilate isomerase and indole-3-glycerol phosphate synthase, *TrpC* gene) [8, 124]. While the α-subunit, which contains different sites for substrate binding (chorismate) and feedback inhibition by tryptophan, provides for the synthesis of anthranilic acid directly from chorismate in the presence of a large amount of ammonia, the β-subunit, together with AAS-I, catalyzes the conversion of chorismate to anthranilate in the presence of glutamine [8, 126].

The biosynthesis of alkaloids in *Claviceps* species begins with the prenylation of L-tryptophan by dimethylallyl diphosphate (DMAPP), which leads to the formation of 4-(γ,γ)-dimethylallyltryptophan (4-DMAT) [8, 127]. The 4-(γ,γ)-dimethylallyltryptophan synthase (DMATS) gene *dmaW* encodes the enzyme of the first stage of the biosynthetic pathway [109], DMAT synthase limits the rate of ergoline formation and is positively regulated by tryptophan, negatively by the intermediates agroclavine and elimoclavine [8, 36, 127]. In the promoter region of the active *dmaW* gene, binding sites were identified for CreA [8, 128], a regulatory protein involved in the repression of carbon catabolite, nitrogen metabolism regulator AREA [8, 129], transcription regulation factor PacC which is modulated depending on pH and is associated with virulence in *Aspergillus* [8, 130] as well as phosphorus deficiency-induced transcription factor NUC-1 [8, 102, 131]. The next step in the biosynthesis of ergoalkaloids is catalyzed by DMAT-N-methyltransferase (*easF* gene) [8, 132]. The subsequent reactions leading to the formation of the simplest clavin, chanoclavin-I, are catalyzed by chanoclavin-I synthase (*easE* gene) [8] and bifunctional catalase/decarbosylase (*easC* gene) [8, 106, 133]. The *easE* gene contains two exons and one intron and is 1503 bp long; the protein it encodes consists of 483 amino acid residues [36]. The protein region formed by amino acid residues 14-161 has been identified as a flavin adenine dinucleotide (FAD) binding domain, suggesting that EasE may function in a FAD-dependent manner [36, 110]. Ergot alkaloids of the lysergic and peptide classes are formed from the aldehyde hanoklavin-I as a common precursor [122]. The oxidation of hanoklavin-I to the aldehyde hanoklavin-I in the presence of NAD^+ is catalyzed by chanoklavin-I dehydrogenase encoded by the *easD* gene [134].

After five conserved steps in the formation of the chanoclavin-I aldehyde, the ergot alkaloid biosynthetic pathway branches. From the chanoclavin-I aldehyde, either festuclavin (the dihydroalkaloid pathway) or agroclavine (the D-lysergine pathway) is synthesized [34]. This process is regulated by the *easA* gene, which encodes flavin-dependent oxidoreductase [8, 34, 108], and by the *easG* gene which encodes reductase [8]. Festuclavin is formed in *C. africana*, agroclavin in *C. paspali*, *C. purpurea*, and *C. fusiformis*. Then agroclavine in *C. purpurea* is converted via elimoclavine into D-lysergic acid, and in *C. paspali* it is converted into paspalic acid [8, 34].

The enzyme CLOA (clavine oxidase, *cloA* allele, cytochrome P450-dependent monooxygenase) plays a key role in the oxidation of its substrate agroclavine to D-lysergic acid [135]. This is a process requiring cumulative six-electron oxidation and double bond isomerization [34, 136]. Deletion of *cloA* blocks the conversion of elimoclavin to D-lysergic acid (the mutant accumulates agroclavin, elimoclavin, and chanoclavin in significant amounts, but not ergopeptides). CLOA acts as a critical enzyme and links biosynthetic pathways for two different groups of ergot alkaloids [137]: agroclavine is oxidized to paspalic acid via elimoclavine by

cytochrome P450-dependent monooxygenase in the presence of NADPH and oxygen, and paspalic acid can spontaneously isomerize to D-lysergic acid [36, 137].

D-lysergic acid is converted to lysergic acid amides and ergopeptides with the participation of four lysergyl peptide synthetases (LPS), the trimodular LPS1 (LpsA1) with three domains with catalytic properties (*lpsA1* gene), monomodular LPS2 (LpsB, *lpsB* gene), monomodular LPS3 (LpsC, *lpsC* gene) and trimodular LPS4 (LpsA2, *lpsA2* gene). The LPSB/LPSC complex catalyzes the formation of ergometrine, the LPSB complex with LPS1 or with LPS4 mediates the assembly of ergopeptides [8, 138-141]. The biosynthesis of ergoamides and ergopeptides is initiated by the LpsB enzyme. D-lysergic acid, after being recognized as a substrate, is activated to form AMP ester and binds to the carrier protein LpsB. LpsA or LpsC compete for binding to LpsB [141]. LpsC can add one amino acid with the production of ergoamides, LpsA can add three amino acid residues with progressive elongation of the molecule and the formation of ergopeptams which are converted into ergopeptides by the monooxygenase EasH (*easH1* gene) [8, 142] into ergopeptides through oxidation followed by spontaneous cyclization [34]. Differences in the metabolomic profiles of *C. purpurea* alkaloids correlated with changes in the lpsA gene [45]. The study of the *lpsA1* deletion mutant led to the assumption that LpsA1 is responsible for the formation of phenylalanine-containing ergotamine and ergocristine ergopeptides [45, 140].

Biochemical analysis of extracts of *C. purpurea* sclerotia after infecting four cereal crops divided the isolates into two classes based on the content of aliphatic hydrophobic residues (I) and phenylalanine-containing alkaloids (II) in metabolomic profiles [45]. Ergotamine and ergocristine were the predominant sclerotia ergopeptides in class II, ergocryptine and ergocornine in class I. Both ergocornine and ergocryptine were found in both class I and II in samples from all four hosts, however class II sclerotia accumulated ergocornine and ergocryptine in much greater numbers [45]. Ergot alkaloid profiles specific to each of these classes are the result of nucleotide sequence variability in the tandemly duplicated *easH/lpsA* region [45, 103, 137].

In the ergoline biosynthetic cluster, the *lpsA1* and *lpsA2* genes were the result of a recombination event [42]. The presence of mobile transposable elements (TEs) similar to DNA transposons of the MULE and TcMar families and found in the intergenic space of the lpsA genes strongly suggests that TE-mediated transposition or mutations associated with TE inserts potentially contribute to the gene *lpsA* variability [45]. Highly polymorphic *lpsA1/lpsA2* intergenic spaces, rich in repetitive elements, associated with different strains of C. purpurea are closely related to divergent *lpsA2*. Domain-specific and highly variable *lpsA1/lpsA2* regions found in strain comparisons suggest that *lpsA* genes are likely to undergo recombinational shuffling [45, 143, 144].

Metabolic engineering of alkaloid biosynthesis. In a *C. purpurea* strain, overexpression of two genes of interest involved in alkaloid biosynthesis was described. These are the *trpE* gene for anthranilate synthase with *S76L* mutation generated to overcome inhibition by excessed tryptophan [8, 145] and the *dmaW* gene encoding dimethylallyltryptophan synthase, an enzyme involved in biosynthesis of the key intermediate of alkaloid production in ergot. Both manipulations led in a significant (up to 7-fold) increase in ergot alkaloids in submerged cultures [8].

Metabolic engineering of ergot may use the reconstruction of target biosynthetic pathways in convenient heterologous hosts, for example, in *Aspergillus nidulans* [104, 146]. Reconstruction of the ergot metabolic pathway with enzymes directing metabolic flux to desired branch points in a heterologous system ensures that there is no variation in the profile of ergot alkaloids produced [9,

136].

Significant progress has been made in the construction of pathways for the synthesis of ergoalkaloids in various heterologous hosts, such as *Aspergillus nidulans*, *Aspergillus fumigatus*: important intermediates and end products or new derivatives of ergot alkaloids have been obtained [112, 123, 136, 146].

A transgenic yeast line carrying eight genes responsible for the synthesis of D-lysergic acid (*dmaW*, *easF*, *easC*, *easE*, *easD*, *easA*, *easG*, and *cloA*) was developed (see Fig. 2) and enantiopure D-lysergic acid synthesized in a bioreactor riched in concentrations up to 1.7 mg/l [9].

Using genomic editing (CRISPR/Cas9), recombinant cultures of *Metarhizium brunneum* were obtained [86]. Their relative yield of D-lysergic acid (86.9%) and dihydrolysergic acid (72.8%) was much higher than that of engineered strains of *Neosartorya fumigata* (2.6 and 2.0%, respectively) [86]. For *C. purpurea*, a CRISPR/Cas9 genomic editing system has been developed with an editing efficiency of 50 to 100%. It successfully knocked out three target genes that are closely related to uridine biosynthesis (*ura5*), hyphal morphology (*rac*) and ergoalkaloids production (*easA*), which made it possible to obtain a uridine auxotrophic mutant (Δ*ura5*), a mutant with an altered phenotype in axenic culture (Δrac) and a mutant that did not produce alkaloids (Δ*easA*) [87].

We draw attention to the importance of several critical points in the biosynthetic pathway that can affect the amount of ergoalkaloids produced in vitro. These points are the synthesis of tryptophan from precursors and its involvement in the biosynthetic chain at the first stages of the metabolic pathway; the synthesis of clavines; the stage of agroclavine-D-lysergic acid; and three branch-points to target components, including synthesis of clavin derivatives, dihydro derivatives of alkaloids, isomerization of D-lysergic acid and stage D-lysergic acid—ergopeptides.

Ergot virulence and interaction with the host plant. When developing methods to reduce damage from damage by herb and ensure toxicological safety, it is important to take into account not only the ability of the fungus to synthesize ergoalkaloids, but also its virulence and mechanisms of interaction with the host plant. As already noted, ergoalkaloids played a significant role in the spread of ergot and the expansion of the range of this ascomycete. *C. purpurea* infects a number of economically important crops, including rye, wheat and barley [19, 49, 147]. Ergot infects cereals during flowering, infecting the tissues of unfertilized inflorescences of female plants and replacing seeds with sclerotia [53, 147]. Thus, cross-pollinating cereals that exhibit open flowering, such as rye, are particularly at risk of infection [49, 148]. Ergot is also infectious for hybrid forms of barley or wheat that have received sensitivity as a by-product of selection [49, 149, 150]. *C. purpurea* infections affect hexaploid soft wheat (*Triticum aestivum* L.) and tetraploid durum wheat *T. turgidum* subsp. *durum* (Desf.) Husn. [151, 152]. Sensitivity to *C. africana* has been described in sorghum, sensitivity to *C. africana* in millet [39, 153, 154]. The interaction of the fungus with the host can vary from antagonistic to mutualistic [155, 156]. Ergot is considered a biotrophic organism [47], but necrotrophic properties have been suggested [49].

The main quantitative indicators of ergot virulence are the amount of honeydew, which characterizes the efficiency of infection at the conidiospore stage, the size of the sclerotia and the total number of sclerotia per spike (the effectiveness of parasitism and the level of production of ascospores) [152, 157, 158]. A positive linear relationship has been noted between the size of sclerotia and the number of ascospore-containing stromas produced [159].

In some cases, *C. purpurea* exhibits weak host specificity [61, 160]. Wild isolates parasitizing on *Dactylis* representatives, when infected rye, did not cause

the usual sphacelial conidia formation but supported the growth of thin sclerotia, sometimes two per inflorescence, with a low alkaloid content, however, the sclerotia acquired their usual appearance after two cycles of axenic (in vitro) and parasitic (on rye) cultivation with successive selection of plectenchymatous axenic mycelium [40]. In the axenic culture, the unpigmented mycelium was nonpathogenic, but the pigmented (purple) plectenchymatous form showed the ability to parasitize rye inflorescences [40]. The host specificity of ergot is not the same in different phases of fungal development: at the sphacelial stage and during the formation of honeydew, the specificity is wider, and the formation of mature sclerotia is more limited by the range of hosts [161, 162].

It has been suggested that sclerotia and ascospores may be the primary inoculum [59, 163, 164]. Differences have been found in the relative contribution of ascospores and conidia to the spread of ergot [59]. Analysis of *C. purpurea* populations from different areas showed that isolates collected from one seed head accounted for 66% of genetic variability. This is considered as an indication of the infection of a significant part of the seed heads bearing multiple sclerotia with ascospores, but not with conidia. At the same time, most of the clonal isolates (they had identical multilocus genotypes) were also collected from the same seed head, indicating a role for conidia (paddy) in secondary infections within the seed heads [59].

Ergot is a homozygous organism [40]. Ascospores (stage of sexual reproduction) are dispersed mainly by wind [59, 164]. Conidia (the vegetative stage of the cycle) are introduced to healthy inflorescences by rain or irrigation, mechanically (by plant-to-plant contact) or insects, which can lead to multiple cycles of infection during flowering [19, 59, 165, 166]. For North American isolates, under a wide host range [167], earlier-flowering plants (e.g., rye) that become infected with ergot in early spring have been shown to be able to serve as a reservoir of conidia and a source of infection for late-flowering plants [59, 164]. .

Ergot infestation is reduced by immediate pollination of female inflorescences, closed flowering (cleistogamy) and physiological resistance [39]. Pollen sterility is one of the main factors facilitating ergot infection [102, 168]. Gametocidal treatment of rye increases the productivity of ergot when cultivated in planta in the field [169]. It is widely believed that *C. purpurea* causes infection by mimicking the growth of pollen tubes [39, 102]. At this stage, growth of *C. purpurea* occurs mainly intercellularly, but invasive hyphae are also found that are completely covered by the host's plasma membrane [47, 102].

Several loci of partial ergot resistance have been identified in wheat [152, 157, 170, 171]. Thus, resistance genes were found in chromosome 6B in the Kenya Farmer variety and in chromosomes 1B, 3B, 4B, and 5B in the Carleton variety [172]. Two ergot resistance QTLs were identified in hexaploid wheat cv. Robigus (located on chromosomes 2A and 4B, QCp.niab.2A and QCp.niab.4B) and two in cv. Solstice (chromosomes 4D and 6A, QCp.niab.6A and QCp .niab.4D) [171]. Four ergot resistance loci (QCp.aafc.DH-1B, QCp.aafc.DH-2A, QCp.aafc.DH-5A, QCp.aafc.DH-5B) were detected [n chromosomes 1B, 2A, 5A and 5B in the variety in Greenshank durum wheat [152], QCp.aafc.DH-2A (significantly reduces honeydew production) and QCp.aafc.DH-5B (reduces the total number of sclerotia per ear) contribute the most to resistance [152]. Male-sterile sorghum lines show little but consistent resistance to *C. africana* [173]. In sorghum, nine loci have been identified that affect the percentage of infection with this fungus, of which QTLs located on the chromosomes SBI-01, SBI-02, SBI-06, SBI-07, and SBI-08 make the greatest contribution to resistance [174]. In barley, genetic variability in ergot resistance has been described. Laurier, Maskot and Sabina have been reported to be the most resistant to infection (less than 0.1% sclerotia), while

Albany, Leger, Symko and Morrison are the most susceptible [149]. Partial resistance of wheat to ergot is associated with dwarfism gene alleles located at the *Rht* loci encoding DELLA proteins (*Rht-B1* and *Rht-D1*, chromosomes 4B and 4D, respectively) [32, 171]. *Rht-B1b* and *Rht-D1b* are mutations that determine the loss of sensitivity of DELLA to gibberellic acid [32]. A decrease in the amount of honeydew, as well as in the size and weight of sclerotia, was found in lines carrying mutant alleles of dwarfism and semi-dwarfism *Rht-D1b*, *Rht-D1c*, *Rht-B1c* [32]. The association between *Rht-B1b* and *Rht-D1b* and ergot resistance points to a role for gibberellic acid in *C. purpurea* infection [32].

Reprogramming of the hormonal pathways of the host plant and differential expression of auxin, ethylene, and cytokinin depending on tissue type and time after *C. purpurea* inoculation have been established [32, 49, 57]. An increase in the content of hyberrellic acid, auxin, and dihydrozeatin-type cytokinins (DHZ) has been found [32]. Gibberellic acid-mediated degradation of DELLA proteins and suppression of jasmonic acid signaling pathways have been shown to increase the incidence of ergot infection in wheat [175]. When infected with ergot, wheat genes associated with auxin become most differentially expressed in the early stages of infection. The *AUX/IAA* (auxin/indole-3-acetic acid) family genes encode known transcriptional repressors of auxin response genes, while the *GH3* (glycoside hydrolase 3) gene family encodes auxin conjugating enzymes that regulate the auxin pool through negative feedback. Both *AUX/IAA* and *GH3* are responsible for the early response to auxin. Suppression of auxin signaling by upregulation of the *AUX/IAA* genes and binding of excess auxin by GH3 family proteins serves as a direct host plant response to *C. purpurea* infection [49, 175]. Among the genes associated with ethylene, the highest activation was found in the 1-aminocyclopropane-1-carboxylate oxidase (ACO) and 1-aminocyclopropane-1-carboxylate synthase (ACS) genes. A number of genes for the biosynthesis and signaling pathways of jasmonic acid (JA) were expressed differently in response to infection. Thus, for 12-oxophytodienoate reductase (OPR) and allene oxide synthase (AOS), which catalyze the first stage of JA formation, it was noted that in the case of OPR, expression increased, AOS increased only in one gene, and the rest were suppressed [49]. Cytokinins are also involved in *C. purpurea* wheat infection with activation of cytokinin oxidase/dehydrogenase and cytokinin glycosyltransferase in plant tissues [49]. Mechanisms regulating the metabolism of gibberellic acid (GA) are induced upon infection: the gibberellin-2-beta-oxidase gene is activated at an early stage of infection, the GA receptor gene GID1 is also activated within 24 h after infection, and then it is suppressed within 48 and 72 h [49]. Salicylic acid, cytokinin, and auxin are involved in the interaction between C. purpurea and *Brachypodium distachyon* [176, 177]. Other protective mechanisms were also active upon wheat infection with *C. purpurea* (in particular, there was a steady increase in the expression of chitinase genes) [49].

Rye showed significant differences in ergot resistance [178-180]. It is believed that the main contribution to the resistance of rye is due to pectinesterase activity and metabolic processes of cell wall modification and pollen tube growth [180]. A comparative analysis of the transcriptome in rye hybrids sensitive (DH372) and moderately resistant (Helltop) to ergot showed that 12 and 8 genes, respectively, are activated differently in hybrids in response to infection [180]. Among them, six genes (*XLOC_059237*, *XLOC_1003867*, *XLOC_118963*, *XLOC_1220465*, *XLOC_1387037* and *XLOC_386424*) cell wall modification and pectinesterase metabolic pathways (180). COBRA-like protein (*XLOC_1432429*) and a putative pectinesterase inhibitor (*XLOC_118963)* show the highest differential expression [180]. In cell wall modification pathways, three genes *XLOC_1343481*, *XLOC_1343482*, and *XLOC_145869* are associated with polygalacturonase [180]. Polygalacturonase is

known as a pathogenicity factor in the interaction between *C. purpurea* and rye [180, 181], and its inhibition is one of the main mechanisms of plant resistance to fungal pathogens [182]. It has been shown that the activity of both *C. purpurea* polygalacturonases (*cppg1* and *cppg2* genes) decreases upon infection of transgenic plants with a high degree of pectin methylesterification [183, 184]. The defense responses associated with the cell wall are of decisive importance for the basal resistance of plants to fungal pathogens [180, 185]. It is also interesting to note that a COBRA-like protein, which is involved in the response of rye plants to *C. purpurea* infection, mediates the directed growth of pollen tubes in *Arabidopsis thaliana* [186]. Knockout of the COBRA-like protein gene caused gametophytic male sterility [186]. Mutant ergot strains with attenuated virulence induce the expression of other rye defense-related genes [47], in particular the chitinase gene (*Sc2Loc00083431.2*), a gene with high homology to the fungal xylanase inhibitor gene (*Sc4Loc00580338.2*), and a putative resistance protein (*Sc4Loc01458017.2*) [47].

In ergot, there are significant differences in virulence due to the rate of mycelium growth [157, 187, 188]. Hyphae elongation is determined by polysaccharide metabolism [189]. Deletion of the *Mid1* gene leads to a decrease in growth rates and a complete loss of virulence. At a certain stage of the infectious process, these signs correlate [190]. During cultivation, in some species of *Claviceps*, the mycelium grows faster than in others: for example, in the Japanese isolate of *C. sorgicola*, the rate is on average 1 mm/day [191], in the Indian isolates of *C. africana* and *C. sorghi*, 0.1 mm/day [192].

Ergot secretes plant cell wall degrading enzymes, signaling molecules and effector compounds [177, 193]. Approximately 90% of the most highly expressed ergot genes are genes that code for proteins involved in growth and development [47]. The *C. purpurea* genome contains more than 400 genes encoding putative infection development effectors; many of these genes are clustered and highly redundant. It has been suggested [47] that the high redundancy of genes in the clusters of effector compound synthesis serves as a mechanism preventing gene loss [47]. Functional analysis of some ergot effector metabolites showed that at least one of them accumulates in the plant apoplast [47]. Ergot has an extensive effector network influencing the course of infection, but a significant contribution to the virulence of any particular effector compound has not yet been established [47]. Ergot has enzymes (CAZymes) that break down the plant cell wall [47, 194]. Ten of the most expressed *C. purpurea* genes are the CAZyme protein genes [47]. *C. paspali* isolates secrete CAZyme-like proteins [195] main target of which is pectins [47, 195]. Signaling components include MAP kinases [47, 181, 196, 197], NADPH oxidases generating ROS (reactive oxygen species) (*Cpnox2* gene; controls the infection process and reduces damage in the host) [198], as well as cytokinins necessary for the successful development of infection [199, 200]. Cytokinins are required to establish and maintain plant-fungal interactions [199, 200]. Enhancement of absorptive activity and influx of nutrients to the foci of infection is the most important role of ergot cytokinins [177). Virulence-reducing cytokinin deficiency can be achieved by deleting the isopentenyltransferase gene, which is also involved in the regulation of translation [177, 200]. However, it is important to bear in mind that alternative pathways for cytokinin activation have been established [177]. The ergot polygalacturonase gene (virulence factor in the interaction of *C. purpurea* and rye) [47] *cppg1* (*cp6977*), the MAP kinase *cpmk1* gene (*cp1700*), and the superoxide dismutase *cpsod1* gene (*cp7438*) belong to the group of genes with a high level of expression, but the role superoxide dismutase is not significant in the development of infection [201].

Two virulence factors were of particular interest in terms of the interaction between *C. purpurea* and rye. These are a transcription regulation factor encoded

by the ergot gene *Cptf1* (homolog of the yeast *Ap1* gene) and a small GTPase encoded by the C*pcdc42* gene. Deletions of these genes reduce ergot virulence [47, 202, 203]. The small GTPase (*Cdc42* gene) is involved in the organization of the cytoskeleton in fungi. In the wild strain of *C. purpurea*, the expression of the heterologous GTP-ase gene of *Colletotrichum trifolii* (Ct*cdc42*) had a significant effect on the vegetative differentiation of ergot. Expression of the dominantly active Ct*cdc42* (DA) allele led to the loss of conidia and aberrant cell shape, while the dominant-negative (DN) allele of *Ctcdc42* (with such mutations, the altered product disrupts the ratio of wild-type proteins during co-expression of genes) stimulated branching and conidiogenesis. The deletion of the Cp*cdc42* gene was not lethal and resulted in a phenotype comparable to that of negative transformants; the DeltaCp*cdc42* mutants were non-pathogenic and did not cause symptoms of the disease (invasive growth stopped at an early stage) [203].

Ergot mutant lines, auxotrophic for tryptophan, are non-infectious for rye, probably due to reduced production of plant auxins, which are synthesized from indole-3-glycerol phosphate via tryptophan-dependent and tryptophan-independent biosynthetic pathways and help the fungus to colonize the host plant [7].

It is worth emphasizing once again that the definition of parasitic fungi as plant mutualists or pathogens remains very vague, with new data published periodically supporting both the first and second statements [204]. It has been experimentally shown that infection with ergot in red fescue (*Festuca rubra*), which forms a symbiosis with the endophytic fungus *Epichloe festucae*, reduces the damage of plants by aphids (*Sitobion* sp.) by 4.5 times [204, 205]. At the same time, plants containing a symbiotic endophyte were more susceptible to *Claviceps* infection (45% of E^+ plants vs. 31% of E^- plants) [204]. The presence of *Epichloe occultans* in Lolium multiflorum and *Epichloe gansuensis* in *Achnatherum inebrians* correlated with a decrease in the incidence of *C. purpurea* infection, suggesting that, in some plant species, members of the genus *Epichloe* are likely to mediate protection against *C. purpurea* [204, 206-208]. In these cases, the growth of resistance to pathogens may be the result of increased host immunocompetence or direct competition between ergot and an endophytic strain of *Epichloe* [204, 209, 210]. The absence of differences in the number and taxonomic composition of the mycobiota between plants infected and not infected with ergot partially supports the hypothesis of the benefit of this pathogen for plants [211].

Studies of 25 years of ergot susceptibility in major crops across Canada have shown that rye is most affected by ergot, followed by bread and durum wheat, followed by barley and oats [212]. An interesting fact was that the incidence and severity of outbreaks changed annually, and over time the infection spread more and more, but its harmfulness and degree of damage did not change [212].

An important subject of study remains the relationship between ergot virulence and ergo alkaloid content [213, 214]. In rye genotypes less susceptible to ergot, the content of ergoalkaloids is reduced [213], but this was not affected by gametocidal treatment of rye [169]. A negative relationship has been found between the mass of one sclerotium and the accumulation of ergoalkaloids in winter rye and spring wheat [214]. In Russia, two varieties of wheat (Novosibirskaya 18 and T-66) immune to ergot, and 13 relatively resistant (with a lesion of no more than 5.2% and an admixture of sclerotia in the grain of no more than 0.3%), have been identified. In rye, 10 varieties demonstrate relative tolerance with damage varying from 5.8 to 33.0% and grain contamination by sclerotia of 0.3 to 1.4% [214]. Genotypes have been revealed in which resistance to ergot damage is potentially combined with the absence of accumulation of ergoalkaloids in sclerotia: these are the varieties of winter rye Rumba, Symphoniya, Harmoniya and spring wheat Epos [214].

Hybrid breeding in rye has been shown to increase grain yield while enhancing ergot susceptibility associated with cytoplasmic male sterility (CMS), which is maternally inherited [215, 216]. The close relationship between the presence of fertile pollen and susceptibility to ergot poses a breeding problem [213, 215, 217].

Thus, *C. purpurea* is, on the one hand, the most important producer of a large number of biologically active compounds (alkaloids) and a unique model of the parasite-host system [218-221]. On the other hand, it is a pathogen that causes significant economic damage to grain producers, feed and livestock industries around the world [222, 223]. While medical and biotechnological research has focused on the beneficial effects of ergot alkaloids [224] and other secondary metabolites and how they are produced [224-228], the hazards of ergoalkaloids remain an acute and worrying global problem in the crop, animal and food industries [229-232]. Its severity is exacerbated by the fact that *Claviceps* alters the profiles and intensity of alkaloid production under changing environmental conditions [231].

In recent years, the management of symbioses of plants and endophytes to optimize the profile and concentration of produced secondary metabolites (including those toxic to humans and farm animals) can be considered as a promising approach [231, 233]. Phylogenetic studies and studies of the evolutionary variability of ergot remain significant [234]. The revealed patterns can be useful in obtaining new highly productive recombinant saprophytic strains-producers of *C. purpurea*, in breeding ergot-resistant varieties, and in optimizing plant protection techniques.

An analysis of publications indicates that different plants show a similar response to *C. purpurea* infection, but resistance mechanisms (for example, in rye and wheat) appear to be different. With respect to historically and evolutionarily determined hosts with open flowering (for example, rye), *C. purpurea* is a biotroph, and partner interactions are mutualistic. In the case of atypical hosts (eg wheat) and new breeding forms sensitive to ergot, necrotrophy and classical parasitism of *C. purpurea* are possible.

Thus, here, we reviewed data on gene clusters that regulate the mechanisms of virulence and biosynthesis of alkaloids in the ergot pathogen *Claviceps purpurea* in the context of evolutionary variability, speciation, and identification of *Claviceps* strains, as well as in view of the achievements and prospects of genetic engineering. The ergot pathogen *C. purpurea* is a very variable and adaptable. This provides for the wide variety of synthesized alkaloids and the wide range of host plants. As a result, there is an abundance of strains of the fungus. The pathways for the biosynthesis of ergot alkaloids have several branch-points with accumulation of intermediate metabolites. In commercial farming, such a redundancy requires additional purification, and in wild strains, it increases toxicity to farm animals and humans. Knowledge of the regularities and genetic control of secondary metabolism in *C. purpurea* is important for the effective technologies for production of ergoalkaloids. The ultimate practical goal of ongoing genomic, transcriptomic, and metabolomic studies of *C. purpurea* and the *C. purpurea*—host plant system is to reduce the toxicity and virulence of the fungus, and limit the spread of ergot to new areas and new plants, including by creating resistant breeding forms.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 882-896
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 633.72:581.19:575

doi: 10.15389/agrobiology.2022.5.882eng
doi: 10.15389/agrobiology.2022.5.882rus

GENETIC MECHANISMS OF THE BIOSYNTHESIS OF CATECHINS, CAFFEINE AND L-THEANINE IN THE TEA PLANT *Camellia sinensis* (L.) Kuntze

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, project No. 22-16-00058
Received August 15, 2022

Abstract

Catechins, caffeine and L-theanine are the main secondary metabolites of the tea plant *Camellia sinensis* (L.) Kuntze. They play a key role in shaping the taste, nutritional and medicinal value of tea (W.J.M. Lorenzo et al., 2016; Z. Yan et al., 2020). In addition, they are involved in the regulation of plant life, in particular, in the processes of adaptation to extreme environmental conditions (Y.S. Wang et al., 2012; L.G. Xiong et al., 2013; G.J. Hong et al., 2014). The above determines the interest in the physiological, biochemical and molecular mechanisms of the production of catechins, caffeine and L-theanine, to increase their accumulation in the plant (R. Fang et al., 2017; W. Kong et al., 2022), as well as to studying their participation in plant response to stress (P.O. Owuor et al., 2010). In the recent 5 years, a lot of new knowledge has been gained on the genes for the biosynthesis of catechins, L-theanine and caffeine, but there are no new reviews that generalize these new data and connect them with new data on the regulation of stress responses in tea. The purpose of this review is to analyze and summarize current data on the genetic mechanisms of the biosynthesis of catechins, L-theanine and caffeine in tea plant tissues, as well as their relationship with genes that regulate abiotic stress responses. The biosynthesis of catechins is carried out along the phenylpropanoid and flavonoid pathways (A. Laura et al., 2019; S. Alseekh et al., 2020) with the participation of the chalcone synthase (*CHS*,), anthocyanidin synthetase (*ANS*), anthocyanidin reductase (*ANR*,) and leucoanthocyanidin reductase genes (*LAR*) (J. Bogs et al., 2005). The accumulation of catechins in the tea plant involves transcription regulation factors of the MYB family, which regulate the expression of the *PAL*, *F3'H*, and *FLS* genes (C.-F. Li et al., 2015). Caffeine formation occurs mainly in tea leaves during purine modification (H. Ashihara, 2015) involving the *IMPDH* (Inosine monophosphate dehydrogenase), *SAMS* (Synthetase gene family), *MXMT* (7-methylxanthine methyltransferase), and *TCS* (tea caffeine synthase) genes. There are already 132 known transcription factors belonging to 30 families (including those encoded by genes of the *bZIP*, *bHLH* and *MYB* families), which are associated with the expression of caffeine biosynthesis genes (C.-F. Li et al., 2015). In C. sinensis, the biosynthesis of L-theanine from glutamate with the participation of pyruvate is controlled by a cascade of genes, the main of which are GS (glutamine synthetase), *GOGAT* (glutamate synthase), *GDH* (glutamate dehydrogenase), ALT (alanine transaminase), *ADC* (arginine decarboxylase), and *TS* (theanine synthetase) (C.Y. Shi et al., 2011; Y. Li et al., 2019). The regulation of these genes is conducted by more than 90 transcription factors — members of the *AP2-EREBP*, *bHLH*, *C2H2* and *WRKY*, *bZIP*, *C3H*, and *REM* families (C.-F. Li et al., 2015). The influence of stress conditions (drought, cold, salinity, nutrient deficiency) on accumulation of these biologically active substances is discussed. Nevertheless, the relationships between the expression of the metabolism genes of the studied compounds and transcription factors remain insufficiently studied; as well as changes in regulatory networks for the biosynthesis of valuable metabolites of tea plants under various environmental stresses.

Keywords: *Camellia sinensis* (L.) Kuntze, secondary metabolites, alkaloids, amino acids, catechins, L-theanine, caffeine, metabolite genes, gene expression, transcription factors, drought, low temperatures, salinity, nutrients

A drink from the young shoots of tea plants (*Camellia sinensis* L.) is widespread throughout the world and is highly valued due to a wide range of beneficial properties of a complex of substances (phenolic compounds, alkaloids, essential oils, essential amino acids, carbohydrates, mineral salts, vitamins, pectins, pigments, enzymes) [1]. Many of these components (approx. 700) are biologically active [1]. The substances contained in tea affect the heart activity and the function of the human nervous system [2], increase the efficiency of muscle tissues [3], induce vigor and stimulate mental activity [4, 5], strengthen the walls of blood vessels and capillaries [6], exhibit anti-radiation, bacteriostatic and bactericidal properties [7-10], activate the immune system and contribute to the prevention of certain types of cancer [9].

Catechins, which are phenolic compounds, and the alkaloid caffeine (these substances are secondary metabolites), as well as the unique amino acid L-theanine (found only in tea plants and not synthesized in the human body) play a key role in the formation of taste, food and nutrition. medicinal value of tea [2, 8, 10]. These plant metabolites are used in the manufacture of pharmaceuticals, food supplements, flavorings and other products [11, 12]. The content of these substances in the tissues of tea plants (and, accordingly, in the resulting products) is determined by the genotype [13, 14], growing area [15-18], harvesting season [19-22], elemental composition of leaves [23-26], the age of the tea leaf [27], the terms and methods of its processing and storage [28-30]. In addition, the accumulation of biologically active substances is significantly affected by the amount and composition of nutrients entering the soil with fertilizers [31-35], which creates opportunities for managing this process.

Secondary metabolites exhibit adaptogen properties, mitigating the effects of stress that plants experience when exposed to high and low temperatures [36-39], ultraviolet radiation [40, 41], in vitro osmotic shock [42-44], pathogen infection [45, 46], mineral deficiencies [47, 32], lighting levels and genetic factors, other factors [48-51].

The main pathways for the biosynthesis of catechins, L-theanine, and caffeine from the tea plant have been detailed in recent decades, but the mechanisms of regulation of ongoing biochemical processes have not yet been sufficiently studied [41, 52-55]. Thus, the gene networks responsible for this in C. sinensis have been identified relatively recently [56, 57]. Transcriptome studies have identified metabolic pathways and key genes involved in the biosynthesis, transport, and metabolism of catechins, caffeine, and L-theanine [58-61], which are discussed in more detail below.

Over the past 5 years, a lot of new knowledge has been obtained about the genes for the biosynthesis of catechins, L-theanine, and caffeine, but there are no new reviews in the world literature that summarize this information and link it with new data on the regulation of stress responses in tea.

The purpose of this review is to analyze and summarize current data on the genetic mechanisms of the biosynthesis of catechins, L-theanine, and caffeine in tea plant tissues, as well as their relationship with genes that regulate abiotic stress responses.

Biosynthesis of major secondary metabolites in tea plants. Catechins. These phenolic compounds belong to one of the most common classes of plant secondary metabolites. Catechins make up 12-24% of the dry weight of a tea leaf [58] and determine the strength and astringency of the resulting drink by 70-75% [62, 63]. Tea plant catechins are represented by four simple forms, the

(+)-catechin (C), (–)-epicatechin (EC), (+)-gallocatechin (GC), (–)-epigallocatechin (EGC), and their galloyl derivatives (–)-catechin-3-gallate (CG), (–)-epicatechin-3-gallate (ECG), (–)-gallocatechin-3-gallate (GCG), (–)-epicalocatechin-3-gallate (EGCG). The properties of catechins are mainly determined by the number and position of the hydroxyl group, which provides binding and neutralization of free radicals [64, 65]. In vitro, tea catechins have been shown to serve as electron donors and effective quenchers of reactive oxygen species, including superoxide anion, peroxyl radicals, and singlet oxygen [66].

The biosynthesis of catechins, which is currently well understood, occurs via the phenylpropanoid and flavonoid pathways [41, 67, 68]. The flavonoid biosynthetic pathway begins with the formation of chalcone with the participation of chalcone synthase encoded by the *CHS* gene, the expression of which correlated with the content of flavonoids in plants [69-71]. In several plant species, more than one copy of *CHS* has been identified in the genome [72, 73]. In *C. sinensis*, three copies of the *CHS* gene (*CHS1*, *CHS2*, and *CHS3*) were found, the expression of which also correlated with the accumulation of catechins in leaves and shoots [74], and the expression profiles, according to G.E. Mamati et al. [75], depended on leaf age. However, it is still unknown which of the three *CHS* genes plays a key role in catechin biosynthesis in *C. sinensis*.

In addition to *CHS*, the genes of flavonoid 3'-monooxygenase (*F3'H*), flavanol synthase (*FLS*), anthocyanidine synthetase (*ANS*), anthocyanidin reductase (*ANR*), and leucoanthocyanidin reductase (*LAR*), phenylalanine ammonium lyase (*PAL*) genes are involved in the biosynthesis of catechins. The *PAL* gene product catalyzes the first step of metabolism in the phenylpropanoid pathway. *F3'H* and *FLS* encode enzymes for the synthesis of flavonols in the flavonoid pathway. ANS catalyzes the conversion of leukocyanidins to anthocyanidins [76]. The *ANR* gene is involved in the biosynthesis of flavan-3-ol monomers, namely, in the conversion of anthocyanidin to epicatechin [77]. The *LAR* gene product catalyzes the conversion of leukocyanidin, leukodelphin, or leukopelargonidin to the corresponding 2,3-trans-flavan-3-ols [78]. All these genes play an important role in determining the composition of catechins in tea leaves [41, 79-81]. L. Zhang et al. [82] found a positive correlation between the expression intensity of the *CHS1*, *CHS3*, *ANR1*, *ANR2*, *LAR* genes and the total content of catechins. The expression level of the *ANS* gene had a positive relationship with the accumulation of simple catechins, while the *ANR1*, *ANR2*, and *LAR* genes had a positive relationship with the content of (–)-epigallocatechin-gallate and (–)-epicatechin-gallate [82]. It has been suggested that the most important genes for catechin biosynthesis in tea plants are the *F3'H* and *ANS* genes, the expression of which significantly increased in the autumn period simultaneously with the accumulation of catechins [83]. However, there is still very little data on the relationship between the expression of these genes and the composition and quantitative ratio of catechins in the tea plant. It is assumed that the expression of genes involved in the biosynthesis of phenolic compounds is regulated by transcription factors *MYB*, *bHLH*, *WRKY*, and other transcription factors associated with ABA-mediated plant response to stresses [84-87]. Thus, the *MYB* family genes are involved in the regulation of the expression of flavonoid biosynthesis genes (*PAL*, *F3'H*, and *FLS*) in the tea plant, which confirms the importance of MYB transcription factors in the control of flavonoid accumulation. In particular, in tea plants, the genes *CsMYB8*, *CsMYB99*, *MYB23* (*MYB* family), *bHLH96* (*bHLH* family), and *NAC008* (*NAC* family) are involved in the regulation of flavonoid biosynthesis, including catechins, anthocyanins, and flavonols [88, 89]. Increased expression of these transcription factors has been

positively correlated with catechin accumulation [88, 89]. A total of 206 transcription factors from 33 families have been reported to be associated with changes in the functional activity of 36 flavonoid biosynthetic genes [57]. It is recognized that at present the mechanisms of regulation of the biosynthesis and transport of flavonoids and anthocyanins are still insufficiently studied [89].

Caffeine (1,3,7-trimethylxanthine). This bioactive compound, synthesized by *C. sinensis*, is a purine alkaloid widely used as a stimulant and drug ingredient [90]. Both in terms of accumulation in the plant and in terms of pharmacological action, it is the dominant among all tea alkaloids [1]. Caffeine adds astringency to the tea infusion and also significantly affects its strength [29]. In addition, the caffeine content characterizes the activity of physiological processes in plants, in particular, redox and enzymatic reactions, and protein metabolism [90]. The caffeine content averages 3% of the dry weight of the tea leaf and, depending on a number of factors (environmental conditions, genetic and geographical factors), ranges from 1.5 to 4.5% [30, 90].

Caffeine is predominantly synthesized in young plant leaves from purine nucleotides in the reactions of adenine metabolism [91]. The main pathway of caffeine biosynthesis includes a series of sequential transformations xanthosine (XR) → 7-methylxanthosine (7-mXR) → 7-methylxanthine (7-mX) → theobromine (Tb) → caffeine (Cf) with the participation of the N-methyltransferase enzyme encoded by the *NMT* gene , which is also called the *TCS* (caffeine synthetase) gene [92-94]. N-methyltransferase exhibits transmethylation activity in two steps, catalyzing the conversion of 7-mX to Tb and Tb to Cf [95]. The genes *IMPDH* (inosine-5-monophosphate dehydrogenase), *SAMS* (S-adenosyl-L-methionine synthase), *MXMT* (7-methylxanthine methyltransferase) are also involved in the biosynthesis of caffeine. The study of the activity of allelic variants of the *TCS1* gene in tea plant populations confirmed that the caffeine synthetase enzyme determines the caffeine content in plant tissues. According to P. Li et al. [88], the *MYB* family includes the main transcription activators of the *TCS1* gene, while *CsMYB184*, *CsMYB85*, and *CsMYB86* play a key role in the regulation of caffeine biosynthesis [96]. Transcription factors of the *AP2/ERF*, *WRKY*, *bHLH*, *MYB*, *bZIP*, *TFIIIA*, and AT-hook families regulate the expression of structural genes of related synthetases involved in alkaloid biosynthesis [97]. For example, gene products of the *GATA* and *bHLH* families bind to the transcription initiation sites of 12 major caffeine biosynthesis genes of the AMPD family (encoding adenosine 5′-monophosphate deaminase enzymes), affecting their expression [98]. Recognition sites have recently been identified for the *MYB184* gene product, which exhibited high promoter activity, increasing *TCS1* gene expression by 4.7 times [96]. Transcriptomic studies of tea plant tissues at different stages of development have revealed regulatory networks that include 132 transcription factors from 30 families associated with the expression of 24 genes for caffeine biosynthesis [57]. Most of these transcription factors belong to the *bZIP*, *bHLH* and *MYB* families.

An analysis of tea varieties with different caffeine content showed that transcription factors of the *NAC* family are associated with the biosynthesis of purine alkaloids (99). One of the genes of this family is *CsNAC7*, according to W. Ma et al. [100], positively regulates the activity of the gene of the main enzyme of caffeine biosynthesis, tobacco N-methyltransferase *yhNMT1*. An analysis of the functional activity of CsNAC7 showed that its transient overexpression could significantly enhance the expression of yhNMT1 in tobacco leaves [96]. However, the relationship between the functional activity of caffeine metabolism genes and transcription factors requires further study. In particular, there is insufficient data

on changes in the regulatory networks for the biosynthesis of this alkaloid under nitrogen deficiency, which affects the productivity of tea plants.

L-Theanine (5-N-Ethylglutamine). This amino acid accounts for up to 50% of the total amino acids in black tea and 1-2% of the dry weight of green tea [101-103]. It gives a sweet and savory flavor to the tea drink [9, 104]. L-theanine is formed in roots, from where it is transported through the phloem to growing shoots and accumulated in young leaves [90, 105]. In *C. sinensis*, the formation of L-theanine from glutamate with the participation of pyruvate is controlled by a cascade of genes, the main ones being *GS* (glutamine synthetase), *GOGAT* (glutamate synthetase), *GDH* (glutamate dehydrogenase), *ALT* (alanine transaminase), *ADC* (arginine decarboxylase), and *TS* (threanine synthetase) (56). L-theanine can be hydrolyzed to ethylamine and then reused as a precursor in catechin biosynthesis, which has been noted with prolonged exposure to sunlight [54]. The conversion of glutamine and ethylamine to L-theanine in *C. sinensis* is carried out by the enzyme theanine synthetase (TS), which has a very high degree of homology with glutamate synthetase (GS) [54]. Glutamine, a precursor of L-theanine, is synthesized with the participation of glutamine-2-oxoglutarate aminotransferase and glutamate dehydrogenase [105]. Another precursor of L-theanine, ethylamine, is formed by the decarboxylation of alanine (Ala), which is catalyzed by the enzyme alanine decarboxylase AlaDC [106]. Alanine and acetaldehyde can be precursors of ethylamine in plant tissues [107, 108], while alanine precedes acetaldehyde in biosynthetic pathways [109]. Although the key genes for L-theanine biosynthesis are known, their transcriptional regulation remains poorly understood [110]. More than 90 transcription factors from the *AP2-EREBP, bHLH*, *C2H2* and *WRKY*, *bZIP*, *C3H*, *MADS*, and *REM* families have recently been found to be involved in the regulation of L-theanine biosynthesis [57]. According to P. Li et al. [88], the transcription factor genes *CsMYB9* and *CsMYB49* are involved in the control of L-theanine biosynthesis, and the expression of the transcription factor gene *CsMYB73* negatively correlated with the accumulation of L-theanine during leaf maturation. In tobacco leaves, the *CsMYB73* gene product binds to the promoter regions of the *CsGS1* and *CsGS2* genes and suppresses their transcription [110]. In addition, the transcription factor *CsWRKY40* activated the key gene for L-theanine hydrolysis, *CsPDX2.*1 (pyridoxal-5'-phosphate synthase). Upon wilting and loss of moisture, abscisic acid accumulated in the leaves, and the content of L-theanine decreased against the background of activation of *CsWRKY40* and *CsPDX2.*1 expression [111].

Thus, a total of 339 transcription factors belonging to 35 families are involved in the regulation of the biosynthesis of catechins, caffeine, and L-theanine, which determine the quality of the resulting plant production of C. sin-ensis [57]. It is important to note the presence of 67 common transcription factors in the regulatory networks for catechin and caffeine biosynthesis [57]. This indicates a positive correlation between their accumulation [57], which is of interest both from the point of view of the fundamental mechanisms of plant secondary metabolism and for solving practical problems of breeding and optimizing crop cultivation technologies. On the contrary, only two transcription factors turned out to be common in the regulation of the expression of genes for the biosynthesis of catechins and L-theanine, which confirms the inverse relationship between their production in the plant. The fact that the activity of genes responsible for the biosynthesis of catechins, caffeine, and L-theanine is influenced by transcription factors from different families indicates a complex system of transcriptional control during

the formation of the considered biologically active secondary metabolites.

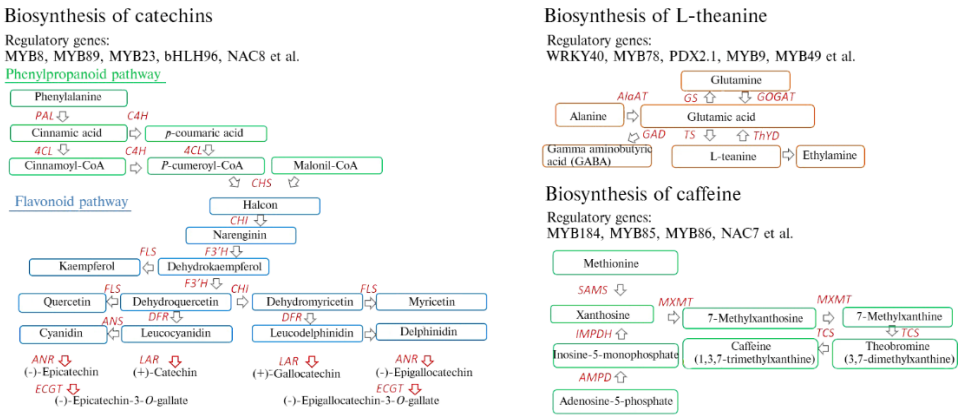
Biosynthesis of secondary metabolites under abiotic stress. Stressful environmental conditions significantly change the content of catechins, caffeine and L-theanine in tea plants [112, 113]. Transcriptomic studies have identified key transcription factors involved in the response to abiotic stress in the tea plant [44, 114-116]. It has been established that many families of transcription factors (*CBF*, *bHLH*, *WRKY*) are involved in responses to various abiotic stresses (cold, drought, salinity), that is, they are nonspecific [117-121].

The summer bud of tea plants reduced the content of catechins and suppressed the expression of the *ANS* gene [122], while the functional activity of the genes for chalcone synthase (*CHS*), flavonoid 3′-hydroxylase (*F3′H*), and dihydroflavonol-4-reductase (*DFR*,) did not change [52]. Increasing the level of illumination during the cultivation of tea calli in vitro contributed to the accumulation of catechins [123]. Short-term (30 min) exposure to ultraviolet (UV-B) irradiation of one-year vegetatively propagated seedlings of C. sinensis cvs Yulan and Fudingdabai in pot culture increased, while prolonged (360 min) exposure, on the contrary, decreased the content of catechins [124].

The accumulation of catechins also depended on the water status of plants and fertilizer application [125-127], carbon access and hormonal balance [78]. Thus, with prolonged exposure to drought, a short-term decrease and then an increase in the expression of *CHS*, *DFR*, *LAR*, *ANS*, and *ANR* genes was noted, which correlated with the accumulation of epicatechin gallate, epigallocatechin gallate, and gallocatechin gallate [128, 129]. A decrease in the content of polyphenols in tea leaves during drought has been reported [125, 130]. However, under conditions of short-term drought (2 days), the level of expression of the *FLS* and *FNS* genes increased, which was accompanied by an increase in the accumulation of compounds from the flavonoid group [128]. In the tea plant, the activity of the main identified caffeine biosynthesis genes was suppressed in response to drought [128], and the caffeine content in the 3-leaf flush decreased (by 1% on average) compared with the normal moisture content of plants [23, 30]. It was reported [128] that the content of L-theanine in the leaves of *C. sinensis* and the level of expression of the *GOGAT*, *GDH*, *ADC*, and *TS* genes decrease during drought, while the expression of the *ThYD* (L-threanine hydrolase) gene, which encodes the key enzyme of L-theanine degradation, rose.

Under nitrogen starvation, the *AlaDC* gene (annotated as a serine decarboxylase gene) was identified in two tea varieties, which may play a specific role in the accumulation of L-theanine (128, 131). Nitrogen is known to be one of the most important elements for the biosynthesis of L-theanine, caffeine and catechins [38, 132-134]. When nitrogen was deficient, tea plants accumulated various flavonoids, while the synthesis of amino acids, including L-theanine, significantly increased when nitrogen was supplied with this element [62, 135]. The total content of catechins also significantly depended on the amount and ratio of available forms of nitrogen, phosphorus and potassium in the soil [136]. At the same time, the accumulation of simple catechins (epigallocatechin, epicatechin, gallocatechin, and catechin) correlated inversely with the amount of N, P, and K introduced into the soil, while their gallic forms directly correlated with the doses of P and K [136]. It has also been reported that elevated doses of phosphorus and potassium, which led to the accumulation of catechins and carbohydrates in tea shoots, reduced the relative content of free amino acids, in particular L-theanine and glutamic acid [137].

Transcription factors and metabolic genes involved in the biosynthesis of catechins, caffeine and L-theanine in the tea plant are shown in the figure.



Transcription factors and genes for the biosynthesis of catechins, caffeine, and L-theanine in the tea plant *Camellia sinensis* (L.) Kuntze summaraised in this review.

So, in recent years, the main metabolic genes involved in the biosynthesis of catechins, caffeine, and L-theanine in the tea plant and their role in the cascade of biochemical reactions have been identified, and some transcription factors involved in the regulation of the expression of these genes have been identified. It is assumed that the identified transcription factors may be associated with the regulators of stress responses, in particular, through the response pathway mediated by abscisic acid. However, there is still insufficient knowledge about the functional role of the regulators of catechin biosynthesis, caffeine, and L-theanine in relation to the key transcription factors of stress responses. This direction seems promising for further research.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 897-910
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 634:11:631.52

doi: 10.15389/agrobiology.2022.5.897eng
doi: 10.15389/agrobiology.2022.5.897rus

RUSSIAN ADAPTIVE APPLE (*Malus* × *domestica* Borkh.) VARIETIES OF VNIISPK — CONTINUITY OF GOALS AND DEVELOPED TECHNOLOGIES

(review)

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The authors declare no conflict of interests
Received March 24, 2022

Abstract

Apple (*Malus* × *domestica* Borkh.) is one of the most economically important fruit crops with a predicted increase in global production. Apples are valued by nutritionists as an important source of sugars, ascorbic acid, other vitamins, trace elements, pectins and biologically active substances. Appearance (size, color) and aroma are the main factors of apple fruit attractiveness for the consumer. From an economic point of view, the main attention in recent decades has been paid to technological features, adaptability, productivity, keeping quality of fruits and resistance to diseases. Breeding research carried out in 1956-2021 at the oldest pomological All-Russian Research Institute of Fruit Crop Breeding (VNIISPK) which celebrated its 175th anniversary in 2020, resulted in 56 new apple cultivars, including 38 cultivars on a fundamentally new genetic basis. By the beginning of these studies in the orchards of central Russia, the main apple cultivars were landraces (Antonovka, Korichnoye Polosatoye, Osennye Polosatoye, Grushovka Moskovskaya and Papirovka) and the Michurin's cultivars Pepin Shafranny, Bellefleur Kitayka, Bessemyanka Michurinskaya, Doch Korichnogo and Kitayka Zolotaya Rannya. At the first stage of our breeding program, the main methods were re-hybridization and breeding based on geographically remote crosses and open pollination. Veteran, Orlik, Pamyat Voinu, Orlovskoye Polosatoye and a number of other cultivars were created and released. Apple breeding at the polyploid level has been carried out since 1970. Triploid cultivars are characterized by more regular fruiting over the years, large fruit size and high marketability of fruits, and increased self-fertility. We have developed a technique for creating triploid apple cultivars and obtained a series of triploid cultivars from intervalent crosses ($2n = 2\times$) × ($2n = 4\times$). To date, 18 triploid cultivars have been released of which six are immune to scab. The best are the triploid ciultivars Rozhdestvenskoye (immune to scab) and Sinap Orlovsky, derived from two diploid cultivars due to the absence of chromosome reduction in one of the parents. These cultivars have become widespread, and each of them is zoned in four regions of Russia. Breeding of cultivars immune to scab has been carried out since 1977. A technique for selecting scab-immune cultivars and seedlings under artificial infection background has been developed. Twenty-four scab-immune cultivars were created and released, including six immune and triploid cultivars and four scab immune and columnar cultivars. The best immune cultivars are Bolotovskoe, Venyaminovskoe, Imrus, and Svezhest; Alexander Boyko, Vavilovskoe, Rozhdestvenskoe, Maslovskoe and Yablochny Spas are scab immune and triploid cultivars. Breeding of columnar apple cultivars has been carried out since 1984 resulting in five columnar cultivars Vostorg, Girlanda, Priokskoye, Poeziya, and Orlovskaya Yesenia. All of them, except for Orlovskaya Yesenia, are immune to scab. Apple breeding to improve the biochemical composition of fruits has been carried out since 1970. According to long-term data, Vavilovskoye (13.0 %) and Ministr Kiselev (13.1 %) are the cultivars with a high content of sugars, Ivanovskoye (19.5 mg/100 g), Veteran (19.4 mg/100 g) and Pepin Orlovsky (15.3 mg/100 g) are enriched with vitamin C, and Kandil Orlovsky (558 mg/100 g), Orlovsky Pioner (514 mg/100 g), Pamyati Khitrovo (480 mg/100 g) and Radost' Nadezhdy (474 mg/100 g) have high content of P-active substances. In the future, we are planning to release new columnar triploid cultivars and triploid cultivars combining columnar habit and scab immunity (elite seedlings with such qualities have already been produced). Such apple hybrids have not yet existed either among cultivars or among wild forms.

Keywords: *Malus* × *domestica* Borkh., apple, breeding, repeated hybridization, polyploidy, cytoembriology, scab immunity, columnar habit, sugars, ascorbate, P-active substances

Apple (*Malus* × *domestica* Borkh.) is one of the most economically significant fruit crops [1, 2] with a projected increase in global production in 2021/2022 (due to increased production in China, Turkey, South Africa, Mexico) [3]. In 2000-2020, the main apple production was concentrated in China, the USA and Turkey; Russia ranked eighth in this list (https://www.atlasbig.com).

Apples are valued by nutritionists as an important source of sugars, ascorbic acid, other vitamins, microelements, pectins and biologically active substances, i.e., carotenoids, anthocyanins and phenolic compounds [4-6]. Such a composition of nutrients reduces the risk of chronic diseases, improves health and increases the adaptive capacity of the body [6, 7]. Appearance (size, color) and aroma are the main factors in the attractiveness of apple fruits for the consumer; from an economic point of view, the main attention, especially in recent decades, has been paid to technological features that determine, for example, the possibility of using modern methods of growing and harvesting, suitability for processing, as well as adaptability, productivity, flowering and ripening time, keeping quality of fruits, their uniformity in size, disease resistance [8-12]. Resistance to diseases, pests, abiotic factors, compact habit of the plant, marketability of fruits remain the most popular directions in the world selection of apple trees [13-17].

The selection development of the apple tree as a horticultural crop began with the use and improvement of local varieties of folk selection. For example, the Volga variety Astrakhan red (first described in 1780) under the name Roter Astrachan was the most common variety of Russian origin in Europe (in England since 1816, in Germany since 1840) and North America (https:// www.kob-bavendorf.de/sorten-detail/na-me/Roter%20Astrachan.html). In Sweden, an apple breeding program has been ongoing since the 1940s, with several varieties adapted to the Scandinavian climate developed for commercial and personal use [18, 19]. In Germany, a long-term breeding program began over 90 years ago. Its goals were fruit quality, high yield, resistance to scab, powdery mildew, bacterial burn, bacterial cancer, red spider mite, frost, new varieties are suitable, among other things, for integrated and organic fruit production [20]. The Fruit Gene bank (Research Institute For Fruit Breeding At Dresden-Pillnitz, Germany) preserves and characterizes as sources of scab resistance genes (*Rvi*) Russian cultivars Antonovka (popular selection cultivar of Russian origin, *Rvi14*, *Rvi17*), Antonovka Kamenichka (*Rvi14*, *Rvi17*), Bessemyanka Michurinskaya (*Rvi17*) (Antonovka, Antonovka Kamenichka, Bessemyanka Michurinskaв) [21]. At the University of Minnesota (USA), apple breeding has been going on since 1908, the first variety under this program, Minnehaha, bred in 1920, was obtained from seeds from free pollination collected from different regions [22]. In Japan, by the 1900s, about 300 varieties were imported from the USA, France, Canada and other Western countries, seven varieties - American Summer Pearmain, Ben Davis, Fameuse, Jonathan, Smith Cider, Ralls Janet and a variety of Russian origin Red Astrachan (Astrakhan red) became predominant. The Japanese variety Orin has been among the dominant varieties in Japan since 1952 [10].

In the All-Russian Research Institute of Fruit Crop Breeding (VNIISPK), purposeful large-scale research on apple tree breeding began in 1956. By this time, in the gardens of central Russia, the main varieties of apple trees were popular selection (Antonovka ordinary, Cinnamon striped, Autumn striped, Grushovka Moscow, Papirovka) and varieties of I.V. Michurina Pepin saffron, Bellefleur Chinese, Bessemyanka Michurinskaya, Daughter of Cinnamon, Chinese golden early. The listed varieties have played an important role in horticulture and are still officially approved for use, although they have lost their former popularity due to

increased commercial requirements. The result of 65 years of research (1956-2021) was 56 new varieties of apple trees, which are included in the State Register of Breeding Achievements approved for use, including 38 varieties on a fundamentally new genetic basis. Many of their properties are described in detail [23]. In the presented communication, the creation of these varieties is considered from the standpoint of changing breeding tasks and methodological approaches developed to solve them [24-28].

Repeated, geographically distant crossings and free pollination. Rehybridization, free pollination and crosses of geographically distant forms are generally accepted methods of traditional breeding, the application of which is associated with the first stage of the program begun in 1956 to create competitive domestic apple varieties [28]. The first varieties bred were included in the State Register in 1986. We successfully used these methods to create varieties Veteran (King, free pollination), Orlik (Mekintosh × Bessemyanka Michurinskaya), Pamyat voinu (Welsey × Antonovka obyknovennaya), Orlovskoe polosatoe (Mekintosh × Bessemyanka Michurinskaya) (Table 1).

1. Apple varieties obtained by free pollination and re-crossings (All-Russian Research Institute of Fruit Crop Breeding, Orel, 1956-2021)

A	B	C	D	E	F	G
Veteran (King — free pollination)	w	Until mid March	130	4.4/4.4	1989	3, 4, 5, 7
Desired (Mekintosh — free pollination)	ls	Until mid September	140	4.3/4.3	2002	5
European Robin (Antonovka krasnobochka × SR0523)	au	Until December	130	4.3/4.3	1999	3
Kulikovskoe (King — free pollination)	w	Until the end of March	125	4.4/4.2	1997	3, 5, 7
Morozovskoe (Antonovka vulgaris ×_Mekintosh)	w	Until the end of January	160	4.7/4.3	2011	3, 5
Olympic (Mekintosh — free pollination)	w	Until February	130	4.3/4.2	1999	8
Orlik (Mekintosh × Bessemyanka Michurinskaya)	w	Until February	120	4.4/4.5	1986	2, 3, 5
Orlinka (Stark Earliest — Pervy sdlyut)	s	Until the second decade of September	140	4.3/4.3	2001	5
Orlovim (Antonovka obyknovennaya × SR0523)	s	Until mid September	130	4.4/4.5	1999	3
Orlovskaya Zarya (Mekintosh × Bessemyanka Michurinskaya)	w	Until the end of January	135	4.6/4.5	2002	3
Orlovsky pioneer (Antonovka krasnobochka × SR0523)	au	Until the end of October	140	4.3/4.3	1999	3
Oryol polosatoe (Mekintosh ×_Bessemyanka Michurinskaya)	lau	Until the end of December	150	4.6/4.3	1986	3, 5, 7
Pamyat voinu (Welsey ×_Antonovka obyknovennaya)	w	Until the end of January	140	4.4/4.5	1997	5
Memory of Isaev (Antonovka krasnobochka ×_SR0523)	lau	Until mid-December	150	4.5/4.3	2008	3
Pepin Orlovsky (Pepin saffron — free pollination)	w	Until mid-January	140	4.5/4.3	2001	3
Radost Nadezhdy (Welsey — free pollination)	s	Until October	150	4.4/4.3	2011	5
Ranneee aloe (Melba ×_Papirovka)	s	Until mid September	130	4.5/4.4	1998	5
	w	Until the end of December	150	4.5/4.3	2008	3
$X_{average}$			138.3			
LSD_{05}			12.5			

Note. A — variety and its origin (variety, crossing varieties), B — fruit ripening period, C — keeping quality of fruits, D — fruit weight, g, E — appearance taste of fruits, score (maximum score 5.0), F — year of inclusion in the State Register, F — region of admission (2 — North-West, 3 — Central, 4 — Volga-Vyatka, 5 — Central Black Earth, 6 — North Caucasian, 7 — Middle Volga, 8 — Lower Volga); s — summer, au — autumn, w — winter, lau — late autumn, ls — late summer.

All varieties had high productivity, fruit quality in terms of sugar content, sugar acid index, content of ascorbic acid and P-active substances, and different maturation periods, therefore, met the principle of "fruit conveyor" formation to provide the consumer with fresh produce for as long as possible [23]. Of course, due to the long life cycle of the apple tree, the traditional selection is a slow process [9, 10]. Genomic [8, 10, 29, 30] and transgenic technologies [9, 10] spead up breeding, however, these techniques do not exclude, but only supplement the traditional selection and phenotypic evaluation of samples, taking into account the effects of the genotype—environment interaction [5, 6], especially under the conditions of ongoing climate change [30].

Selection at the polyploid level. At the next stage of the program, success in creating varieties was associated with the development and application

by us of a new methodological approach based on selection at the polyploid level, using data from genetic, cytological and embryological analysis. As a result of research started in 1970, we created for the first time in Russia and the world a series of triploid ($2n = 3\times$) varieties from intervalent crosses of diploids ($2n = 2\times$) with tetraploids ($2n = 4\times$).

The history of apple tree breeding at the polyploid level is associated with the work of Swedish scientists [31-33], who showed the advantages of triploid forms over diploid and tetraploid ones. For many centuries, triploid varieties that spontaneously arose in nature were distinguished by good fruit quality, increased viability and were cultivated. Thus, the Gravenstein variety, which was widely known in Denmark as early as the 17th century, is a triploid. Spontaneous triploids of American (Tompkins County King and Rhode Island Greening varieties) and British (Bramley and Ribston Pippin varieties) origin dating from the 17th-18th centuries are still grown today (https://www.apfga.org/triploid-apples/). However, spontaneous mutations of this kind rarely occur in nature. Purposeful obtaining of triploids by breeding methods is a promising way to create an "ideal variety". It was shown that by crossing diploid varieties with each other, it is possible to obtain up to 0.3% of triploid seedlings, while the yield of triploids when crossing tetraploid and diploid forms is much higher [31-35]. However, work on the mass production of triploid seedlings in order to breed varieties has not received proper development.

According to A.A. Zhuchenko, it is polyploidy that provides higher adaptability to many plant species [36]. Polyploidy is a genomic mutation that manifests itself in a spontaneous or induced increase in the diploid number of chromosome sets. Polyploidy is especially valuable when vegetative organs are used as the target product [37]. The use of polyploidy does not speed up the selection process, but increases the possibilities of selection due to the large range of hereditary variability. Polyploids serve as favorable material for natural and artificial selection [37]. Our long-term studies have shown that the yield of fruits in relation to pollinated flowers and the yield of seeds was lower with hybridization at the polyploid level (4.7 vs. 3.6%) than at the diploid level (8.6 vs. 6.4%). However, the breeding value of seedlings obtained from hybridization at the polyploid level is significantly higher than from hybridization at the diploid level. As it turned out later, with hybridization at the diploid level in comparison with the polyploid one, 4121 seedlings vs. 778 are needed to obtain one elite seedling, 86.6 thousand pollinated flowers and 16.7 thousand annual seedlings vs. 46.2 thousand flowers and 2.9 thousand seedlings are needed to create one variety. In other words, the selection efficiency increased, as the time, volume of work and, as a result, costs decreased.

Cytological and field studies allow us to establish that not all tetraploid varieties and forms can be used as donors of diploid gametes, but only homogeneous tetraploids in which all layers of somatic cells are tetraploid ($4n = 4\times$) [34, 38]. These are varieties Mekintosh tetraploid, Melba tetraploid, Alfa 68 and seedlings VNIISPK 13-6-106 (variety Suvorovets — free pollination), 25-37-45 (Orlovskaya girlyanda × Welsey tetraploid). Diploid-tetraploid chimeras of the 1st type are also suitable as donors of diploid gametes, for example, Antonovka ploskaya 2-4-4-4×, Papirovka tetraploid 2-4-4-4×, Welsey tetraploid 2-4-4-4×. The value of each specific form as a donor of diploid gametes varies and depends on the development of embryonic structures in its generative sphere. The results of cytoembryonic analysis are of decisive importance in the selection of initial forms for hybridization with the aim of mass production of triploids.

The study of the generative sphere in the Mekintosh tetraploid variety showed that the ovules and mature differentiated embryo sacs ready for fertilization develop at the same time in this form and in the diploid analogue. In both

forms, the formation of a significant number of abnormal ovules and ovaries is observed; both forms are characterized by the formation of complex ovules with two or three nucelli enclosed in a common integument. The differences between the two forms of the Mekintosh variety associated with the level of ploidy are expressed in the fact that the linear dimensions of the structures of the generative sphere of the polyploid form are traditionally somewhat larger than those of the diploid form. The formation of supernumerary nuclei in the embryo sac of the polyploid form of the variety Mekintosh could result in formation of the haploid gametes instead of diploid. Consequently, part of the hybrid progeny when using this form in intervalent crosses of the type ($2n = 4\times$) × ($2n = 2\times$) will not be triploid [35] and the Mekintosh tetraploid variety is suitable for crosses (according to the results of 12 crosses with the participation of this variety, we singled out one selected seedling, a candidate for varieties). In breeding, the tetraploid form 13-6-106 (seedling of the Suvorovets variety) is also actively used. It is also characterized by the presence of a number of features in the development of the generative sphere, which in most cases do not serve as a significant obstacle to being used as a parental form during hybridization. Since abnormal ovules average only about 25%, the ovaries always contain a sufficient number of normal ovules with normally developed embryo sacs suitable for double fertilization. With the participation of the tetraploid form 13-6-106, 30 crossings were carried out and 15 selective and six elite forms were obtained.

It has been established that crosses of the type diploid × tetraploid, and not tetraploid × diploid, are the most effective. It is much more difficult to obtain triploid offspring of an apple tree, because in the hybrid offspring from crossing diploid varieties with tetraploid varieties or forms, the proportion of triploid seedlings is from 40 to 80%. The method developed by us for obtaining triploid seedlings and varieties takes into account all these features. Crosses of the tetraploid × diploid type are possible only with castration of the maternal tetraploid form, because the latter, as a rule, has a high self-fertility [35, 38].

An assessment of the physiological and biochemical parameters and fruit quality showed that the resulting triploid apple varieties (Table 2) are characterized by a lower frequency of fruiting over the years, improved fruit marketability, increased self-fertility, high yield and fruit quality in terms of biochemical composition [23, 39].

When optimizing protocols for polyploid crosses, we relied on the results of cytoembryological analysis. In our opinion, in the selection of apple trees at the polyploid level, one cannot do without cytoembryological control. We were unable to find examples of such an approach in the specialized literature. At the beginning of our study, the use of cytological methods to determine the level of ploidy and describe mitotic and meiotic events was discussed [40], in recent years, cytogenetic characteristics are considered in connection with the phenomenon of ploidy and genome size [41].

Obviously, the resulting polyploids can acquire new traits [11, 42, 43]. At the same time, in tetra- and hexaploid forms, many physiological and morphological parameters are lower than in diploid ones [11, 42, 43]. It is assumed that plant species have optimal ploidy, and its decrease or increase reduces their growth potential, but the manifestation of a number of traits, including those of breeding value, is enhanced in tetraploids [42], and they are considered as valuable breeding material for programs for obtaining triploid plants [42]. According to some economically significant properties, the apple tree is noted to have the superiority of tetraploids over diploids, for example, drought resistance [42] and scab resistance [11].

2. Triploid varieties of apple trees obtained by selection at the polyploid level (All-Russian Research Institute of Fruit Crop Breeding, Orel, 1970-2021)

A	B	C	D	E	F	G
Augusta (Orlik × Papirovka tetraploid)	ls	Until the end of September	160	4.4/4.4	2008	5
Bezhin lug (Severny synap × Welsey tetraploid)	w	Until February	150	4.4/4.3	2010	5
Darena (Melba × Papirovka tetraploid)	ле	Until the end of September	170	4.5/4.3	2011	5
Den Pobedy (Veteran × Horcoat)	w	Until mid March	140	4.4/4.3	2020	5
Ministr Kiselev (Chistotel × Welsey tetraploid)	w	Until mid March	170	4.4/4.4	2017	5
Nizkorosloe (Skryzhapel × Pepin saffron)	w	Until the end of February	130	4.3/4.2	1997	5
Orlovsky partisan (Orlik × 13-6-106 seedling of Suvorovets variety)	w	Until mid February	190	4.4/4.4	2010	5
Osipovskoe (Mantet × Papirovka tetraploid)	s	Until mid September	130	4.4/4.4	2013	5
Pamyati Semakina (Welsey × 11-24-28 seedling of Golden Greima variety)	ew	Until the end of December	160	4.5/4.3	2001	5
Patriot [16-37-63 (Antonovka krasnobochka × SR0523) × 13-6-106 seedling of Suvorovets variety]	w	Until the beginning of February	240	4.5/4.3	2013	5
Synap Orlovsky (ceverny Sinap × Pamyati Michurina)	lw	Until the end of April	150	4.3/4.4	1989	2, 3, 5, 7
Turgenevskoe (18-53-22 × Welsey tetraploid)	w	Till March	180	4.4/4.3	2021	5
$X_{average}$			164.1			
LSD_{05}			29.6			

N o t e. A — variety and its origin (variety, crossing varieties), B — fruit ripening period, C — keeping quality of fruits, D — fruit weight, g, E — appearance taste of fruits, score (maximum score 5.0), F — year of inclusion in the State Register, F — region of admission (2 — North-West, 3 — Central, 4 — Volga-Vyatka, 5 — Central Black Earth, 6 — North Caucasian, 7 — Middle Volga, 8 — Lower Volga); s — summer, w — winter, ls — late summer. lw — late winter, ew — early winter.

Creation of scab-immune apple cultivars. The problem of apple tree resistance to diseases while maintaining the high quality of fruits does not lose its relevance [44]. This is especially true for scab lesions [11, 21, 45]. Scab caused by *Venturia inaegualis* (Cke. Wint) is one of the most harmful diseases of the apple tree, and breeding for resistance to it is one of the most popular ways to improve varieties [13-17]. We have been doing this research since 1977. A great contribution to the theory and practice of selection of scab-immune seedlings was made by Vladilen V. Zhdanov, co-author of more than 20 scab-immune apple varieties, who developed a method for selecting resistant varieties and seedlings against an artificial infectious background [46].

In different countries, more than 200 scab-immune cultivars have been created on the basis of donors with the *Vf* gene (*Rvi6*) from *Malus floribunda* 821 using backcroses [16, 47-50]. Varieties Prima, Priscilla, Florina, Freedom, Redfree, Liberty, Gold Ruch were widely used in hybridization [15-17, 51, 52]. On the basis of our own hybrid fund, we created and released scab-immune varieties with the *Vf* (*Rvi6*) resistance gene (Table 3). In addition, triploid varieties immune to scab (see Table 3) and four immune columnar varieties were obtained. In our opinion, the best immune varieties are Bolotovskoye, Venyaminovskoye, Imrus, Svezhest and Stroevskoye, while immune and triploid varieties are Alexander Boyko, Vavilovskoye, Maslovskoye, Rozhdestvenskoye, Yubilyar and Yablochny Spas, which are of interest for production.

Varieties Rozhdestvenskoye and Sinap Orlovsky already occupy large areas of gardens and are zoned in four regions of Russia. A successful example of sustainable horticulture was the experience of growing scab-immune varieties Svezhest, Afrodita, Bolotovskoye, Venyaminovskoye, Imrus, Kandil Orlovsky, Rozhdestvenskoye, Stroevskoye in the Saratov region [53]. When studying the adaptability of the scab-immune apple varieties obtained by us in the conditions of Ukraine, Aphrodite, Venyaminovskoye, Kandil Orlovsky, Orlovskoye Polesye, Rozhdestvenskoye turned out to be the best [54]. Because *Venturia inaegualis* can mutate rapidly with new races, scab resistance is most likely to be maintained when immune varieties are planted alongside non-immune varieties and a minimal

treatment cycle [55]. It is believed that the *Vf* (*R*vi6) gene is still capable of providing resistance against the pathogen [10, 45], but a long-term protective effect is more reliably provided by multiple resistance and pyramiding of several genes [8, 21, 45, 56]. The genes *Rvi5*, *Rvi11*, *Rvi12*, *Rvi14*, and *Rvi15* are considered promising [10, 45], as the sources of one of these genes, *Rvi14*, folk cultivars of Russian origin Antonovka obyknovennya and Antonovka-Kamenichka have been characterized [21], new sources of resistance genes to diseases [21]. All this is included in the range of tasks of proactive selection for scab resistance of apple trees [56].

3. Scab immune apple cultivars based on the *Vf* (*Rvi6*) gene donors, including polyploids (All-Russian Research Institute of Fruit Crop Breeding, Orel, 1977-2021)

A	B	C	D	E	F	G
Scab immune varieties (*Vf*)						
Aphrodite (814 — free pollination)	ew	Until the end of December	130	4.4/4.4	2006	5, 6
Bolotovskoye (Skryzhapel × 1924)	w	Until February	155	4.3/4.3	2001	3, 5
Venyaminovskoe (814 — free pollination)	w	Until the end of February	130	4.4/4.4	2008	2, 3, 5, 6
Zdorovye (Antonovka obyknovennaya × OR48T47)	w	Until mid February	140	4.3/4.3	2001	5
Ivanovskoe (Welsey × Prima)	w	Until mid February	150	4.4/4.4	2010	5
Imrus (Antonovka obyknovennaya × OR18T13)	w	Until mid February	140	4.3/4.4	1996	3, 5
Kandil Orlovsky (1924 — free pollination)	w	Until February	120	4.4/4.3	2001	3, 5, 6
Kurnakovskoe (814 × PA-29-1-1-63)	w	Until mid February	130	4.3/4.3	2002	3, 5
Oryol Polissya (814 — free pollination)	p3	Until mid-January	140	4.4/4.3	2001	3, 5
Pamyati Khitrovo (OR18T13 — free pollination)	w	Until the end of February	170	4.3/4.3	2001	5
Snezhet' (Antonovka krasnobochka × PR2T67)	lw	I'm at home	140	4.3/4.2	2001	3, 5
Solnyshko (814 — free pollination)	lau	Until December	140	4.4/4.3	2001	3, 5
Start (814 × Mekintosh tetraploid)	w	Until the end of February	140	4.3/4.3	2002	5
Stroevskoe (814 — free pollination)	w	Until the end of February	120	4.5/4.4	2001	3, 5
Yubilei Moskwy (814 — free pollination)	w	Until the end of February	120	4.3/4.3	2002	5
$X_{average}$			137,3			
LSD_{05}			21,3			
Scab immune triploid varieties						
Alexander Boyko (Prima × Welsey tetraploid)	w	Until the second decade of March	200	4.4/4.3	2013	5
Vavilovskoye [18-53-22 (Skryzhapel × OR18T13) × Welsey tetraploid]	w	Until the beginning of March	170	4.6/4.3	2015	5
Maslovskoye (Redfree × Papirovka tetraploid)	s	Until the end of September	220	4.3/4.3	2010	5
Rozhdestvenskoe (Welsey × BM 41497)	w	Until the end of January	140	4.4/4.3	2001	2, 3, 5, 6
Yubilar (814 — free pollination)	s	Until the end of September	130	4.4/4.3	2009	5
Yablochny Spas (Redfree × Papirovka tetraploid)	s	Until the end of September	200	4.4/4.3	2009	3, 5
$X_{average}$			176,6			
LSD_{05}			29,6			

N o t e. A — variety and its origin (variety, crossing varieties), B — fruit ripening period, C — keeping quality of fruits, D — fruit weight, g, E — appearance taste of fruits, score (maximum score 5.0), F — year of inclusion in the State Register, F — region of admission (2 — North-West, 3 — Central, 4 — Volga-Vyatka, 5 — Central Black Earth, 6 — North Caucasian, 7 — Middle Volga, 8 — Lower Volga); s — summer, w — winter, ls — late summer. lw — late winter, lau — late autumn, ew — early winter.

The development of scab-resistant apple varieties with acceptable consumer and commercial characteristics has proven to be a difficult task. It took almost 60 years to produce the first commercial *Vf*-generated Prima variety in the US (released in 1970) (the program started in 1914) [10, 57]. In recent decades, transgenic technologies have been used to solve these problems, but the practical application of varieties on this basis may be limited by local legislation [10]. Information about the creation of triploid and especially triploid scab-resistant apple varieties is not widespread. One such variety Sirius (UEB 3264/2) with the *Vf* gene was obtained from crossing Golden Delicious × Topaz, the trees are medium-sized, well branched, the fruits are large, juicy, with a well-balanced taste

(https://extension.psu.edu/apple-cultivars-newer-scab-resistant-selections, accessed 09/29/2022), the triploid Initial (X 6163) has conical fruits, medium to large in size, prone to shedding, they are used for making cider and feeding deer (https:// extension.msu.edu, accessed 09/29/2022).

Selection of columnar apple varieties. The compactness of the tree is one of the requirements of modern intensive fruit growing [10, 58-61]. This property is possessed by columnar varieties of apple trees. Their creation is considered as one of the leading directions in apple breeding, which we have been developing since 1984. Columnar varieties allow to reduce the pre-fruiting period by 2-3 years, make it possible to significantly increase the gross yield per hectare, as well as to reduce manual labor to a minimum when caring for the garden. Therefore, this biological form is promising for intensive and super-intensive gardens [27, 62, 63]. The pioneers of the creation of columnar varieties in Russia are V.V. Kichina [64] and M.V. Kachalkin [65].

To date, five columnar varieties have been obtained and released: Vostorg, Poeziya, Priokskoe, Orlovskaya Yesenia, and Garlyanda (Table 4). All of them, except for the Orlovskaya Yesenia variety, are immune to scab. In recent years, columnar varieties have been widely grown not only in home gardens and summer cottages, but also in large industrial gardens. Cultivation of columnar scab-resistant apple varieties does not require traditional shaping and pruning of trees and 6-8-fold fungicide spraying, which not only reduces material costs by 1.5-2 times, but also reduces the burden on the environment [66].

4. Columnar apple varieties (All-Russian Research Institute of Fruit Crop Breeding, Orel, 1984-2021)

A	B	C	D	E	F	G
Vostorg [270-124 (Mayak × KV103) × 23-17-62 (814 — free pollination)]	w	Until February	170	4.3/4.3	2016	5
Girlyanda [224-18 (SR0523 × Vazhak) × 22-34-95 (814 × PA-29-1-1-63)]	w	Until the end of February	120	4.3/4.3	2018	5
Orlovskaya Yesenia [224-18 (SR0523 × Vazhak) × 22-34-95 (814 × PA-29-1-1-63)]	w	Until February	170	4.3/4.5	2019	5
Poeziya [224-18 (SR0523 × Vazhak) — free pollination]	w	Until February	140	4.4/4.3	2015	5
Priokskoye [224-18 (SR0523 × Vazhak) — free pollination]	w	Until February	150	4.5/4.4	2014	5
*X*average.			150			
LSD05			26,7			

N o t e. A — variety and its origin (variety, crossing varieties), B — fruit ripening period, C — keeping quality of fruits, D — fruit weight, g, E — appearance taste of fruits, score (maximum score 5.0), F — year of inclusion in the State Register, F — region of admission (2 — North-West, 3 — Central, 4 — Volga-Vyatka, 5 — Central Black Earth, 6 — North Caucasian, 7 — Middle Volga, 8 — Lower Volga); w — winter.

Selection of apple trees to improve biochemical composition. The direction has been actively developing since 1970. Many of the apple cultivars we have created stand out for their fruit quality [23]. So, according to long-term data, the varieties Vavilovskoe ($2n = 3\times$, *Rvi6*) and Minister Kiselev ($2n = 3\times$) are characterized by a high sugar content - 13.0 and 13.1%, respectively, while the widely known varieties of folk selection are significantly inferior to them (Antonovka obyknovennaya 9.1%, Osennee polosatoe 9.2%, Moscow Grushovka 9.3%). The varieties Ivanovskoye (*Rvi6*), Veteran, Nizkorosloe and Pepin Orlovsky are characterized by an increased content of ascorbic acid in fruits (19.5; 19.4; 18.0 and 15.3 mg/100 g, respectively vs. 11.8 mg/100 g in the variety Antonovka obyknovennaya and 6.0 mg/100 g in varieties Osennee polosatoe and Grushovka Moskovskaya). The content of P-active substances was increased in the varieties Kandil Orlovsky (*Rvi6*) (558 mg/100 g), Orlovsky Pioneer (*Rvi5*) (514 mg/100 g), Pamyati Khitrovo (*Rvi6*) (480 mg/100 g) and Radost' Nadezhdy (474 mg/100 g) compared to previously widespread varieties Antonovka obyknovennaya (263 mg/100 g), Osennee polosatoe (415 mg/100 g), Korichnoe polosatoe (129 mg/100 g) (67). Apple breeding for a high content of ascorbic acid and P-

active substances in fruits has great prospects, since the introduction of vitamin varieties into production will increase the nutritional and therapeutic value of fruits.

The presented data show that a feature of our breeding program since its inception in 1965 has been the priority of high biochemical value, quality and yield of fruits in combination with resistance and other economically and technologically significant traits. In continuation of these studies, we have already obtained elite triploid apple seedlings that combine quality and yield indicators with scab immunity and columnar appearance. Such hybrid forms of apple trees have not yet existed among varieties and have not been identified among wild forms.

It is important to note that under the conditions of climate change, the improvement of varieties in terms of yield size and quality, stability and adaptability, resistance to abiotic and biotic stresses becomes even more relevant and requires an in-depth and comprehensive study of the physiological, genetic, cytoebriological characteristics of crops [6, 12, 60, 68]. Breeders around the world continue to develop new varieties with improved characteristics. Their arsenal has been replenished with modern methods — whole genome sequencing [8, 29], molecular labeling [10, 21, 29], QTL mapping [10], marker-assisted selection (MAS) and genomic (GS) selection [10, 30], genomic editing technologies (10). However, there is still a significant gap between genomics and breeding [29]. While facilitating and in some cases speeding up the process, modern methods do not reduce the value of classical selection.

Thus, the result of the 65-year breeding program was 56 state-registered apple varieties, including 38 varieties based on a fundamentally new genetic basis. For the first time in Russia and in the world, 12 triploid varieties were obtained from intervalent crossings of diploids and tetraploids, for the first time in Russia, 15 varieties immune to scab were created, as well as six triploid varieties with immunity to scab, and five columnar varieties, of which four are immune to scab. A series of varieties with improved biochemical composition of fruits has been created. To address breeding problems, a technique was developed for intervalent crossings of diploids and tetraploids using cytoembryological analysis. The issues of identifying spontaneous and creating (through hybridization) new tetraploid initial forms, the donors of diploid gametes, as well as obtaining unreduced pollen when meiosis is affected by physical and chemical agents remain topical. The next task is to create new columnar triploid varieties of apple trees, as well as triploid varieties combining columnar and scab immunity (elite seedlings with such qualities have already been obtained). Such hybrid forms of apple trees have not yet existed either among varieties or among wild-growing forms.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 911-920
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Physical research methods

UDC 633.11:631.53.011:57.084.1

doi: 10.15389/agrobiology.2022.5.911eng
doi: 10.15389/agrobiology.2022.5.911rus

EVALUATION OF HETEROGENEITY AND HIDDEN DEFECTS OF WHEAT (*Triticum aestivum* L.) SEEDS BY INSTRUMENTAL PHYSICAL METHODS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Ministry of Science and Higher Education of the Russian Federation (Agreement with the Ministry of Education and Science of Russia No. 075-15-2020-805 dated 10/02/2020)
Received July 29, 2022

Abstract

For quality control of seed material, there are a number of standard tests adopted by ISTA (International Seed Testing Association, Switzerland) as well as promising instrumental methods evaluating the characteristics of seed surface, structural integrity and integral electrophysical parameters. The aim of the study was to evaluate the efficiency of instrumental physical methods in detection of latent defects of ecologically heterogeneous wheat seeds of various genetic origin. Diversity and latent defectiveness of wheat seeds (*Triticum aestivum* L.) were evaluated using optical imaging, microfocus radiography, and electrophotography. It was found that the optical imaging method combined with automatic analysis of digital scanned images is statistically reliable to distinguish wheat seeds of different varieties and genetic lines by color characteristics of the RGB (red, green, blue) model, e.g., Hue and Saturation. Differences were also found between the seeds of the same variety and genetic line grown under field and regulated conditions. E.g., the Hue values varied from 0.081±0.0005 to 0.090±0.0006 for regulated conditions (the phytopolygon of the Agrophysical Research Institute) and from 0.084±0.0005 to 0.088±0.0005 for field conditions, the Saturation values — from 0.326±0.0005 to 0.419±0.0006 and from 0.371±0.0005 to 0.444±0.0005, respectively. With an increase in the number of cracks in the X-ray projections of wheat grains, their sowing qualities decrease. Microfocus radiography combined with automatic analysis of digital X-ray images successfully detects the damage to wheat seeds by the corn bug, and with the increase of the damage score the sowing quality of seeds in general decreases. Parameters of the digital X-ray images of seeds (Average Intensity, Shape Coefficient, and Entropy) differed between wheat varieties. The Average Intensity varied from 53.30±1.00 to 60.60±1.17, the Form coefficient from 6.67±0.35 to 8.28±0.48, and Entropy from 1.84±0.06 to 1.98±0.03. The research data indicate the effectiveness of the approaches we propose based on instrumental physical methods in the assessment of different quality and latent defectiveness of wheat seeds. Our findings make a background for the functional non-invasive diagnosis of seed quality based on the complex evaluation of external and internal anomalies and defects, significantly affecting both the biological quality of seeds and their economic suitability. This is a methodologically new tool to be used in breeding and controlled seed production.

Keywords: *Triticum aestivum* L., wheat, seed quality, optical imaging, microfocus X-ray imaging, electrophotography, image analysis

A necessary condition for obtaining a high yield of wheat and improving its quality is the use of fully formed and healthy grain for sowing [1-3]. It should be noted that at present in the Russian Federation the share of substandard seeds of grain crops can reach up to 40% [4]. To control the quality of seed material, there are a number of standard tests regulated by the ISTA (International Seed

Testing Association, Switzerland), as well as promising instrumental methods [5-8].

Progress in seed science is closely related to technical innovations and their availability for application. In addition, new methods for assessing seed quality for testing are being developed based on technological advances [9].

Instrumental methods potentially suitable for non-invasive evaluation of seed quality can be divided into three groups. The group of optical methods includes digital optical imaging [10], multispectral imaging [11], chlorophyll fluorescence measurement [12]; introscopic methods include microfocus radiography [13, 14], computed microtomography [15] and magnetic resonance imaging [16]; the third group consists of electrophysical methods (electrophotography) [17]. The method of digital optical imaging based on scanning images of seeds with obtaining various numerical characteristics, including color, is used to determine the varietal purity of rice seeds [18], describe and classify seeds of flax varieties [19]. The method of soft-beam microfocus radiography has been successfully used for many years both in Russia [13, 20] and abroad [21-24] and is included in international [25] and Russian [26] standards. The method of electrophotography [17], which makes it possible to register and quantify the glow that occurs near the surface of an object (seed) placed in a high-intensity electromagnetic field, has a certain prospect, supplementing the generally accepted methods of instrumental evaluation of seed quality.

In addition, with the advent of modern technical and software tools in seed science, computer analysis of seed images is actively used all over the world [27, 28].

Of these methods, only microfocus radiography has been standardized in Russia. Since 2022, it has the status of the current national standard GOST R 59603-2021 (developed by the Agrophysical Institute).

In the presented work, for the first time, using three non-invasive instrumental physical methods (optical imaging, microfocus X-ray diffraction and electrophotography), the characteristics of seeds were obtained and their significant differences were revealed in lines and varieties of wheat (genetic heterogeneity) when grown in field and controlled conditions (ecological heterogeneity) and post-harvest technological part-time job. It has also been shown that damage to wheat seeds by the turtle bug can be successfully detected using microfocus radiography in combination with automatic analysis of digital x-ray images.

The purpose of the study was to evaluate the effectiveness of instrumental physical methods in the study of latent defectiveness in ecologically different quality wheat seeds of different genetic origin.

Materials and methods. Seeds of common wheat (*Triticum aestivum* L.) spring varieties Zlata, Radmira, Yubileinaya 58 and hybrid lines API 1 (Agata × ITMI29) F_5, API 2 (Agata × ITMI47) F_5, API 7 (Liza × ITMI10) F_4, h2788 (Zlata × Moskovskaya) F_{12}, h3021 (Biora × Zlata) F_4 were obtained under strictly controlled growing conditions (agricultural biopolygon AFI, Leningrad Province, 2022) and in the field (FRC Nemchinovka, Moscow Province, 2020) (seeds from the AFI collection were served for sowing). The sample sizes are 100 seeds of each sample. Seeds obtained from crops were analyzed by optical digital scanning.

We also used seeds of winter common wheat cv. Svetoch, damaged to varying degrees by a harmful bug (provided by A.V. Kapustkina, Laboratory of Agricultural Entomology, VIZR, St. Petersburg; the degree of damage was assessed visually on a 4-point scale from 0 to 4 points) [29] and six samples of wheat seeds from industrial crops subjected to mechanical post-harvest processing (provided by N.I. Zhukov, OOO Strong Semen, Krasnodar Territory, harvest of 2020). The sample sizes are 100 seeds. The samples were analyzed by microfocus X-ray diffraction.

In addition, five samples of seeds of spring wheat varieties MiS, Zlata, Lyubava, Esther, Liza were received from the collection of the Federal Research Center Nemchinovka (provided by Prof. B.I. Sandukhadze, Laboratory of Breeding and Primary Seed Production of Winter Wheat). Sample volumes were 50 seeds of each variety. The obtained samples were analyzed by electrophotography.

Scanned images of seeds were obtained using a digital flatbed scanner HP ScanJet 200 (Hewlett-Packard, USA), the format of the saved file is *.tiff. Software processing of digital scanned images of seeds was carried out using the VideoTesT-Morphology program (OOO ArgusSoft, St. Petersburg, Russia). Parameters of digital scanned images, the color ratio according to the RGB model (red, green, blue, Brightness, units of brightness); Hue (relative units); Saturation (relative units) were analyzed. The technique for obtaining and processing scanned images has been described previously [30].

To control the quality of seeds, microfocus shooting of seeds was performed (a hardware-software complex of a mobile X-ray diagnostic unit PRDU-02, developed by the AFI and Ulyanov-Lenin St. Petersburg State Electrotechnical University LETI, manuvactured by ELTECH-Med, St. Petersburg, Russia). The X-ray image magnification factor was 3.0×. Processing of digital X-ray images of wheat seeds was carried out in the VideoTesT-Morphology program. Two analyzed image parameters were the seed projection area (mm^2) and the calculated average brightness normalized to the projection area (relative units). The technique for obtaining and processing digital X-ray images has been described previously [31]. Electrophotographic (gas-discharge) images of wheat seeds were obtained using the GDV-Camera Pro software and hardware complex (developed and manufactured by OOO Biotechprogress, St. Petersburg, Russia). The analyzed parameters were Average intensity (brightness units), Shape coefficient (relative units), Entropy (relative units). The technique for obtaining and processing images has been described previously [32].

The sowing quality of seeds was assessed according to GOST-12038-84 (Moscow, 2011), including seedling length (mm) and growth vigor (proportion of strong seedlings, %) as additional parameters [33].

Statistical processing, including the construction of regression models, was performed using the MS Excel program (Microsoft Corp., USA). The text and tables show the arithmetic mean values of the parameters (M) and their confidence intervals at the 95% probability level according to Student's t-test ($t_{0.05}$ × SEM).

Results. The results of automatic analysis of digital scanned images of wheat seeds are shown in Figures 1 and 2.

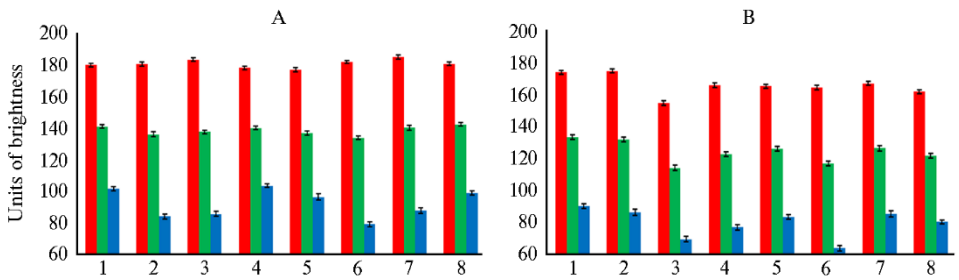

Fig. 1. Color models RGB (red, green, blue) **of seeds in wheat (*Triticum aestivum* L.) varieties grown under regulated conditions (agro-biopolygon AFI, Leningrad Provine, 2022)** (A) **and in field (FRC Nemchinovka, Moscow Province, 2020)** (B)**:** 1 — API 1 (Agata × ITMI29) F_5, 2 — API 2 (Agata × ITMI47) F_5, **3** — API 7 (Liza × ITMI10) F_4, 4 — Zlata, 5 — Radmira, 6 — Yubileinaya 58, 7— h2788 (Zlata × Moskovskaya) F_{12}, 8 — h3021 (Biora × Zlata) F_4 (n = 100 eaach). Means and confidence intervals are shown, $M\pm(t_{0.05}$ × SEM). HP ScanJet 200 digital flatbed scanner (Hewlett-Packard, USA); software for processing digital scanned images VideoTesT-Morphology (OOO ArgusSoft, St. Petersburg, Russia).

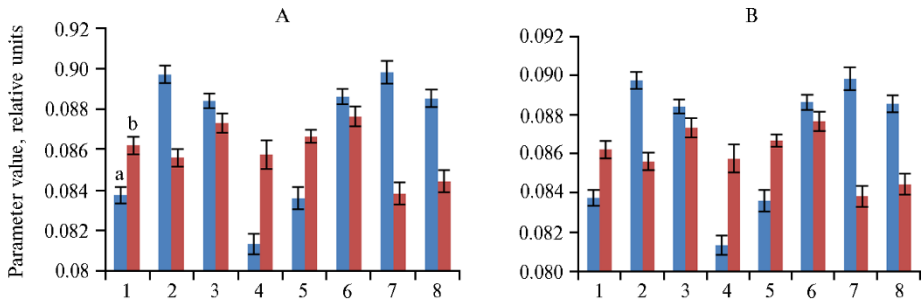

Fig. 2. Hue (A) **and Saturation** (B) **of RGB models of seeds in wheat (*Triticum aestivum* L.) varieties grown under regulated conditions (agro-biopolygon AFI, Leningrad Provine, 2022)** (A) **and in field (FRC Nemchinovka, Moscow Province, 2020)** (B)**:** 1 — API 1 (Agata × ITMI29) F_5, 2 — API 2 (Agata × ITMI47) F_5, **3** — API 7 (Liza × ITMI10) F_4, 4 — Zlata, 5 — Radmira, 6 — Yubileinaya 58, 7— h2788 (Zlata × Moskovskaya) F_{12}, 8 — h3021 (Biora × Zlata) F_4 (n = 100 eaach). Means and confidence intervals are shown, $M \pm (t0.05 \times SEM)$. HP ScanJet 200 digital flatbed scanner (Hewlett-Packard, USA); software for processing digital scanned images VideoTesT-Morphology (OOO ArgusSoft, St. Petersburg, Russia).

It was found that the seeds of varieties and varieties of wheat differ among themselves according to the RGB model - in the ratio of colors (Fig. 1, A, B), tone, and color Saturation (Fig. 2, A, B). In addition, we found that the seeds of the same varieties and varieties of wheat, obtained under field and controlled conditions, also differ significantly in the same parameters of the RGB model (color ratio, Hue, and Saturation) (see Fig. 1, A, B , Fig. 2, A, B).

The presented data suggest that the differences in the color characteristics of the surface of the seeds are associated with their varietal characteristics and the genetic origin of the studied material. In addition, it is important to note that growing conditions also affect the color characteristics of the resulting seeds (see Fig. 1, 2).

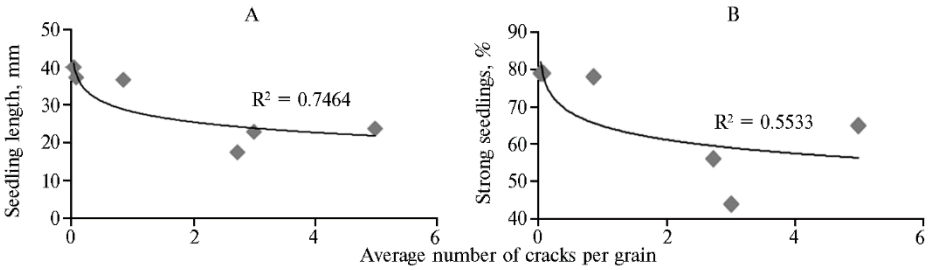

Fig. 3. Sowing quality indicators for six seed samples from industrial wheat (*Triticum aestivum* L.) crops (LLC Strong Seeds, Krasnodar Territory, harvest of 2020, for each sample n = 100**) depending on the number of cracks detected on the X-ray projection of the grain.** Device-software complex based on a mobile X-ray diagnostic unit PRDU-02 (ELTECH-Med, St. Petersburg, Russia), software for processing digital scanned images VideoTesT-Morphology (OOO ArgusSoft, St. Petersburg, Russia).

When visually analyzing digital X-ray images of six seed samples obtained in industrial wheat crops in the Krasnodar Territory (Fig. 3, A, B), we found that an increase in the number of cracks that are detected on the x-ray projection of the grain reduces the sowing quality of seeds. We attribute this to an increase in the probability of penetration of fungal and bacterial infection into the grain with an increase in the number of cracks in the endosperm. In addition, the presence of cracks near the embryo disrupts the supply of nutrients to it from the endosperm. Such a seed may either be unsimilar or have underestimated growth rates.

Evaluation of the characteristics of digital X-ray images of wheat seeds damaged by the pest showed that the projection area of digital X-ray images significantly ($p < 0.05$) decreased compared to the control and depended on the

degree of damage, assessed visually in points (Fig. 4, A). The calculated average brightness of grain digital radiographs, normalized to the projection area, increased significantly ($p < 0.05$) compared to the control at any degree of damage by the harmful turtle. The maximum increase in this indicator was noted with a 3-point lesion (see Fig. 4, B). When evaluating the growth parameters, it was found that the length of the sprout on the 7th day decreased when the grain was damaged by a harmful turtle, while the maximum decrease was also noted with a 3-point damage to the grain (see Fig. 4, C). Thus, bug damage by a harmful turtle was detected using automatic analysis of digital X-ray images of caryopses. At the same time, as it was established by our studies, the sowing qualities of seeds decreased with an increase in the damage score.

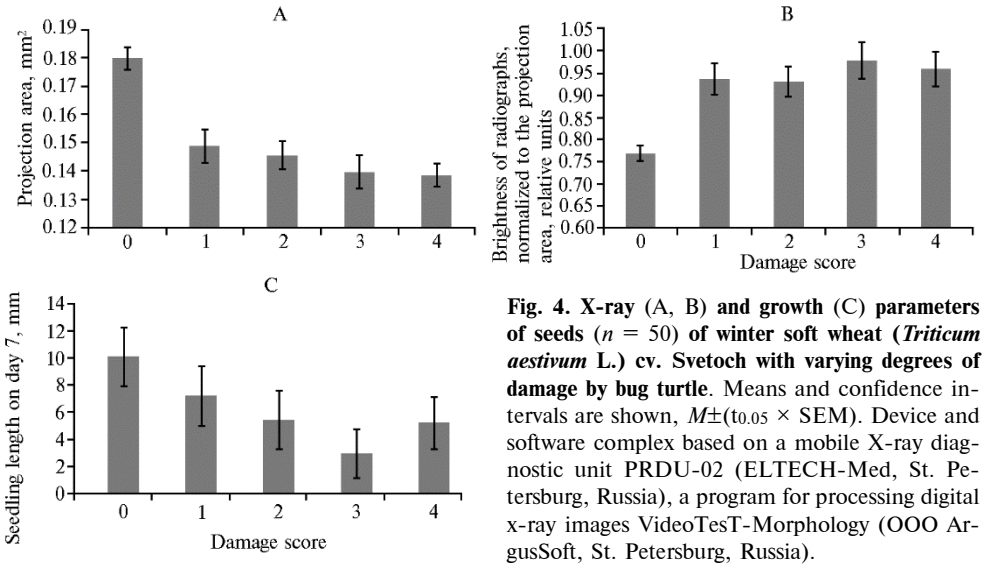

Fig. 4. X-ray (A, B) **and growth** (C) **parameters of seeds** ($n = 50$) **of winter soft wheat (*Triticum aestivum* L.) cv. Svetoch with varying degrees of damage by bug turtle**. Means and confidence intervals are shown, $M \pm (t_{0.05} \times SEM)$. Device and software complex based on a mobile X-ray diagnostic unit PRDU-02 (ELTECH-Med, St. Petersburg, Russia), a program for processing digital x-ray images VideoTesT-Morphology (OOO ArgusSoft, St. Petersburg, Russia).

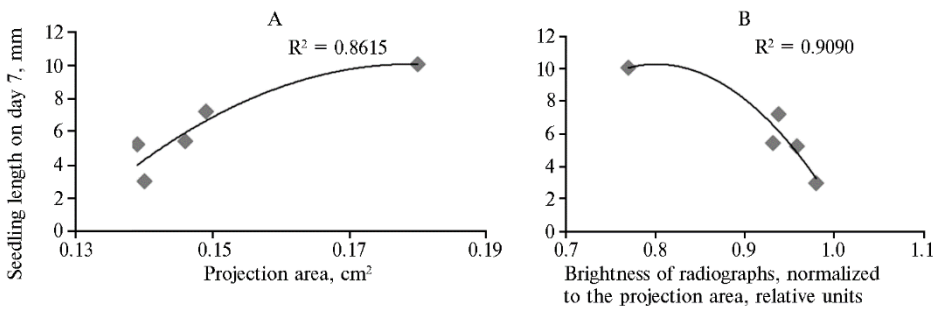

Fig. 5. Quality of seeds ($n = 50$) **of winter soft wheat (*Triticum aestivum* L.) cv. Svetoch with varying degrees of damage by bug turtle** depending on the **X-ray projection area of the grain** (A) and brightness of the radiographs, normalized to the grain projection area (the points on the graphs correspond to damage scores). Device and software complex based on a mobile X-ray diagnostic unit PRDU-02 (ELTECH-Med, St. Petersburg, Russia), a program for processing digital x-ray images VideoTesT-Morphology (OOO ArgusSoft, St. Petersburg, Russia).

The results of the electrophotographic analysis of digital gas-discharge images of seeds in five wheat varieties of different genetic origin (Table) showed that the Zlata variety was characterized by the minimum average image intensity, the Liza variety has the maximum intensity. The Liza variety has the minimum Shape coefficient value, the MiS variety the maximum Shape coefficient, MiS has the minimum Entropy of digital gas-discharge images, Lyubava the maximum Entropy. These differences, apparently, reflect the unequal genetic origin of the studied accessions, and not the influence of ecological and geographical factors (the

seeds were obtained in the crops of the Nemchinovka in the Moscow region in 2015). The reasons for the revealed differences in the characteristics of gas-discharge luminescence in wheat seeds of different varieties require further study.

Electrophotographic Analysis of digital gas discharge images of seeds in different varieties of spring wheat (*Triticum aestivum* L.) (samples from the FRC Nemchinovka collection, $n = 50$ each)

Parameter	MiS	Zlata	Lyubava	Ester	Liza
Average intensity, units of brightness	56,26±0,73	53,30±1,00	55,74±0,97	57,75±0,84	60,60±1,17
Shape coefficient, rel. units	8,28±0,48	8,25±0,38	7,78±0,44	7,12±0,30	6,67±0,35
Entropy, rel. units	1,84±0,06	1,94±0,04	1,87±0,04	1,98±0,03	1,97±0,02

N o t e. Mean values and confidence intervals are presented, $M \pm (t_{0,05} \times \mathrm{SEM})$.

Previously, we revealed a relationship between the electrophotographic characteristics of seeds and their viability [34], as well as plant bioproductivity [35]. With the data obtained [32], the Average intensity of digital gas-discharge images (a measure of brightness) negatively correlates with the length of the sprout and the area of the flag leaf. The Shape coefficient (a measure of the irregularity of the image contour) has a positive correlation with the weight of 1000 seeds (the indicator may reflect grain frailty caused by enzyme depletion).

Figure 6 shows an examples of images of wheat seeds obtained using various instrumental methods.

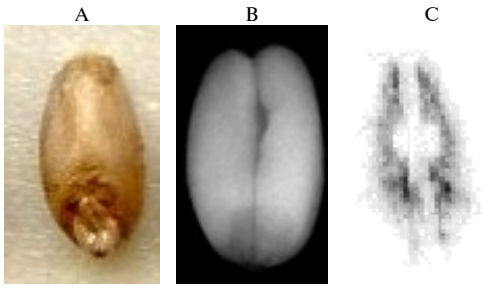

Рис. 6. Examples of digital images of wheat (*Triticum aestivum* L.) seeds obtained with various instrumental methods: A — digital scanned image (HP ScanJet 200, Hewlett-Packard, USA), B — digital X-ray image (mobile X-ray diagnostic unit PRDU-02, Russia), B — digital gas-discharge image (hardware-software complex GDV-Camera Pro, Russia).

When analyzing results, obtained using non-invasive techniques and reflect external features and internal structural disorders in caryopses associated with morphophysiological parameters of seedlings, it should be noted that the available publications on the problem are mainly devoted to purely methodological aspects [14, 15]. These papers concern the features of the analysis of caryopsis images depending on the topography of the defect, the degree of its severity, as well as the creation of adequate methods for digital processing of caryopter images for assessment. Therefore, we can compare our findings only with those obtained in our previous studies of the relationship between the X-ray images of the seed and the seed growth potential. In the world literature on seed science and seed production, there is practically no information about the effects of biotic and abiotic factors (plant growing conditions) in breeding and production sowings of grain crops.

The results we submit are pioneering in terms of assessing the influence of ecogenic and technogenic factors on the indicators of the grain structural integrity, both under the conditions of precision experiments and in mass crops. Here, we propose the principles of a new functional non-invasive diagnostics of seed quality. From a practical point of view, the method we propose provides an assessment of the economic suitability of commercial lots of seeds, in contrast to the characteriяфешщт of the biological quality of an individual grain. Currently, the approach has no analogues in the world. It opens up wide opportunities in studying the fundamental aspects of seed control and in developing fundamentally new ways to determine the commercial value of seeds based on a comprehensive non-invasive assessment. In general, this technique can bring seed quality control in

Russia to the highest level in the world. Further fundamental research will be devoted to a detailed analysis of the relationship between physical and growth parameters. In addition, in the near future, we plan to submit the worlds's first data on the relationship of the grain physical characteristics instrumentally measured by a non-invasive method with a plant reproductive function, quantitative and qualitative traits and the seed lot yield and quality.

Thus, the color characteristics of the seed surface, estimated using the digital scanned images, differ statistically significantly between different wheat varieties and genetic lines and in one variety grown under different (field or regulated) environmental conditions. The Hue values of seed images in different wheat varieties varied under controlled growing (phytopolygon of the Agrophysical Institute) from 0.081±0.0005 to 0.090±0.0006, in field from 0.084±0.0005 to 0.088±0.0005, the Saturation values from 0.326±0.0005 to 0.419±0.0006 and from 0.371±0.0005 to 0.444±0.0005, respectively. This may serve both to distinguish between batches of seeds and to determine seed maturity. Hidden defects, e.g., cracked endosperm assessed by the microfocus radiography method with visual interpretation, reduces seed sowing qualities. The severity of the defect affects germination and further growth parameters. It has been confirmed that damage to grain by the corn bug can be effectively detected using automatic analysis of digital X-ray images. The sowing quality of seeds decreases with increasing damage score. Statistically significant ($p < 0.05$) differences in the characteristics of gas-discharge luminescence of seed images were revealed between wheat varieties of different genetic origin. E.g., the Average intensity of luminescence varied from 53.30±1.00 to 60.60±1.17, the Shape coefficient from 6.67±0.35 to 8.28±0.48, Entropy from 1.84 ±0.06 to 1.98±0.03. Our approach to assessing the heterogeneity and latent defectiveness of seeds based on non-invasive physical methods currently has no analogues in the world and allows us to advance from biological characterization of an individual grain to assessment of the economic suitability of seed lots in industrial seed production.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 921-932
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 631.524.5:57.084.1

doi: 10.15389/agrobiology.2022.5.921eng
doi: 10.15389/agrobiology.2022.5.921rus

ACCURACY ASSESSMENT OF *Syringa vulgaris* L. MORPHOLOGICAL PHENOTYPING WITH A LASER 3D SCANNER PlantEye F500 DEPENDING ON PLANT LOCATION ON THE SCANNED SURFACE

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The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Ministry of Science and Higher Education of the Russian Federation (grant No. FZWG-2021-0018 as part of the state assignment)
Received May 11, 2022

Abstract

Since the methodological methods of direct genetics are applicable only for monogenic traits, the created breeding material, line or variety must be tested in the field, since the presence of the desired gene in the genome, confirmed by molecular methods, does not always lead to the formation of a trait valuable for selection. Systems based on 3D imaging technologies make it possible to obtain a plant model, as well as information on morphological parameters. However, very little attention is paid to the preparation of protocols for phenoscreening. The purpose of this study was a comparative assessment of the accuracy of determining the morphological characteristics of lilac plants by traditional methods and using machine vision technology, depending on the plant location on the scanned surface. Microclones of lilac (*Syringa vulgaris* L.) cv. Microclones are morphologically homogenous and small in size, which allows measurements of sufficiently large sets of samples and makes it easier to compare the research results by their normalization to average values. The measurements were made after the plant complete adaptation and cultivation for 1 month in a greenhouse. With traditional morphometry, in 10 microclones, the height was measured with a measuring ruler, and the leaf area was measured using the contour method. When scanning (PlantEye F500 3D scanner, Phenospex B.V., Netherlands), each of 10 selected plants was placed at five different positions of the scanned surface, and at least five repeated scans were performed in the same position. When using machine vision technology, 3D leaf area, projected leaf area, digital biomass, height, maximum height, leaf tilt, leaf tilt angle, light penetration depth were determined. It has been established that in order to obtain objective and comparable data from using a 3D scanner, it is optimal to place plants in the center of the scanned surface in the same position. The following parameters can be recommended to identify varieties and assess plant growth rate: the leaf area, projected leaf area, height, and leaf inclination angle. For each plant species, it is necessary to preliminarily study particular morphological traits and to compare the obtained data with the scan results in order to introduce correction factors/ This will confirm the information content of the feature set used, thereby increasing the accuracy of machine vision technology data.

Keywords: phenotyping, morphology, *Syringa vulgaris* L., machine vision technology, 3D canning

Modern genetic research is focused on the genome structure [1, 2] in order to identify determinants for economically valuable traits and mechanisms of gene acttivity [3-5], to assess population variability [6], to identify varieties at the early stages of plant development [7, 8], to reveal patterns of genome organization and evolution [9, 10].

The widely used approaches of direct classical genetics in which genes are

identified by the traits they encode are being replaced by reverse genetics methods when not the phenotype and its genetic control are analyzed but the DNA sequence itself and its phenotypic effects are revealed [11-14]. The paradigm shift is due to the fact that the methodological methods of direct genetics are applicable only for monogenic traits. However, in most cases, the properties of biological objects are polygenic and are formed as a result of the combined action of several genes, or phenotypic expression may be the result of mutations in different genes [15]. Therefore, the traits of the obtained breeding material, lines or varieties should be checked in the field, since the presence of the desired gene in the genome confirmed by molecular methods does not always lead to the formation of a trait valuable for breeding [16-18]. In addition, when analyzing qualitative and quantitative morphological features, it is necessary to recognize the modification variability that occurred due to various environmental factors [19].

The morphological characterization of plants is an obligatory stage of selection and genetic studies [15, 17]. Modern phenotyping methods based on machine vision technologies are highly productive and allow obtaining real-time data on several morphological parameters [20, 21]. Automation of phenotyping processes significantly speeds up the analysis and increases its accuracy, eliminating the human factor as a source of subjective evaluation of the results, and provides with parameters that were not used in traditional morphometric measurements [22].

The most important morphological features in plant phenotyping include plant size, type of leaf arrangement, shape and area of the leaf blade. There are automated platforms that allow identification of plant species from photographs, such as INaturalist (https://www.inaturalist.org/) and PlantNet (https://plantnet.org/). However, the accuracy of phenotyping depends on the accumulated photographic material (the number and quality of photographs at different stages of plant vegetation), the frequency of occurrence of the species in the study area, and the actual confirmation of its identification during field observations [23, 24]. Thus, when using automated platforms, it is possible to determine plant species with sufficient content of the database, but it is not possible to assess the modification variability of morphological characters, as well as to determine varieties.

Systems based on 3D imaging technologies provide a plant model as well as information on morphological parameters [25]. In this case, the image processing software plays a major role, and not the resolution of the scanner [26]. As a result, current research on phenotyping is mainly devoted to software development, improvement of the camera positioning system [27]. However, very little attention has been paid to the development of protocols for phenoscreening [28-30]. There is no doubt that the automation of phenotyping processes, carried out both in laboratory and in the field, will not only significantly speed up the evaluation of breeding material, but will also increase the homogeneity of selected plants when working with annual crops [31-34]. Despite many publications on the use of 3D scanners for assessing morphological parameters, the literature covers rather superficially the issues of accuracy of morphological characteristics in plant phenotyping depending on their location on the scanned surface [35-37].

In this paper, we compared the results of direct morphometric measurements carried out by personnel and indirect measurements based on machine vision technology, and identified conditions that, if not observed during phenoscreening, can lead to unreliable results.

The purpose of our study was to comparatively assess the accuracy of determining morphological traits of lilac plants by traditional methods and by machine vision technology, depending on the location of the object on the scanned

surface.

Materials and methods. Lilac plants (*Syringa vulgaris* L.) cv. Mercy were obtained by the in vitro method after adaptation. Accounts were made after the completion of the stage of adaptation and cultivation of plants for 1 month in greenhouses.

With traditional morphometry, the sample consisted of 10 microclones, in which plant height was measured with a ruler, and the surface area of each leaf was measured by the contour method.

Scanning was performed on a PlantEye F500 multispectral 3D unit (Phenospex B.V., the Netherlands) (equipment of UNU Botanical Garden of Belgorod State National Research University, https://ckp-rf.ru/usu/200997/). Each of the 10 selected plants was scanned at five different points on the scanned surface, and at least five repeated scans were performed in the same position. Using the PlantEye F500 setup, the values of the following morphometric parameters were analyzed: 3D Leaf Area, cm^2; Projected Leaf Area, cm^2; Digital Biomass, cm^3; Height, mm; Height Max, mm; Leaf Inclination, cm^2/cm^2; Leaf Angle, °; Light Penetration Depth, mm. For processing the obtained data, the PlantEye F500 HortControl software was used.

Arithmetic mean values (M) and confidence intervals (±CI) were calculated at a confidence level $p = 0.05$, and correlation analysis was performed.

Results. The choice of microclones as an object is due to a high degree of morphological uniformity and small plant sizes, which allows measurements and comparison of the data obtained in sufficiently large samples, normalizing them to average values.

At the first stage of the study, we carried out morphometric measurements of plant height (22.7±2.3 cm) and leaf surface area (388.3±12.3 cm^2). Digital biomass (product of plant height and leaf surface area) was 8814.41±325 cm^3.

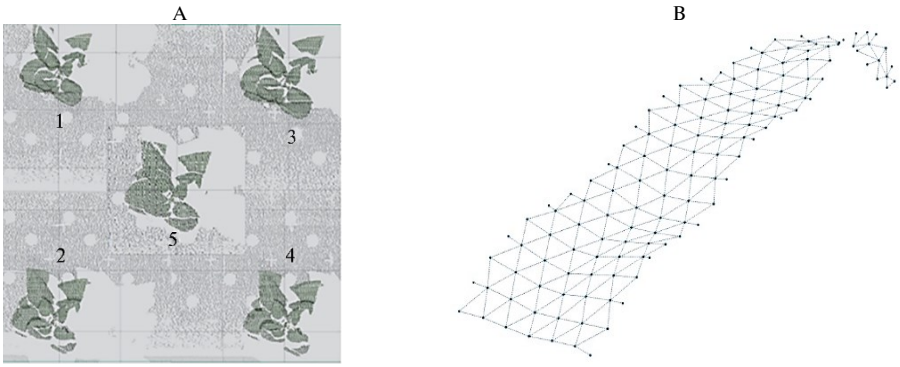

Fig. 1. Positioning of lilac (*Syringa vulgaris* L.) cv. Mercy plants (A) **and triangulation of points to generate a 3D cloud** (B) **for phenotyping by 3D scanning** (PlantEye F500, Phenospex B.V., the Netherlands).

The position of each plant during 3D scanning is shown in Figure 1, A. When using the PlantEye F500 3D scanner to measure leaf area, points are created in the point cloud that belong to the same array, which are triangulated (connected into triangles). Since an uneven distribution of points in space is allowed, the size of the triangles can vary (see Fig. 1, B).

A group of triangles forming a uniform surface represents a domain and corresponds to one sheet. Then the total area of 3D scanning of plant leaves is calculated as the sum of the areas of elementary triangles of all scanned leaves of one plant (Fig. 2).

The presented data show that plant locations significantly affected the result. At points 2 and 4, the smallest values of the leaf area were obtained, at points

3 and 5, the largest, and the location of the plant at point 1 corresponded to the average value obtained at all five points. It should also be noted that the confidence interval for five repeated scans in 10 plants turned out to be the largest at point 1.

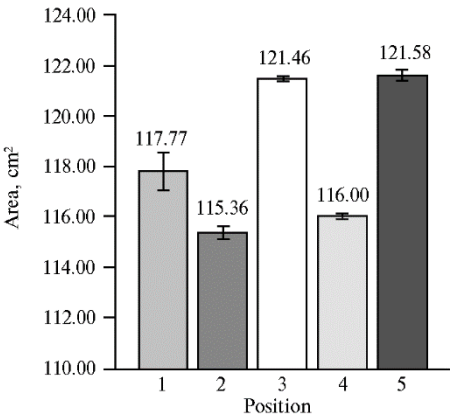

Fig. 2. Leaf area depending on posiooning of of lilac (*Syringa vulgaris* L.) cv. Mercy plants (n = 10, M±CI, p = 0.05; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

That is, when conducting automated measurements, the data obtained are affected by the position of the plant relative to the scanning area. Even with a static location of the object and the absence of external changing factors (changing the illumination did not affect the scan results) at the scan point closest to the beginning of the scan area, a high instability of the obtained data occurred.

The total leaf area per plant when using the contour method was 3.2 times higher than that in 3D scanning. This significant discrepancy is due to the fact that some leaves overlap each other, which underestimates the figure. Therefore, the use of a 3D scanner to estimate leaf area requires the introduction of a correction factor calculated on the basis of a comparison of data obtained by different methods (in our case, the contour method and as a result of scanning with the PlantEye F500).

The projected leaf area is defined as the projection area of all elementary triangles onto the X-Y plane. However, it is equivalent to a value that can be measured with a conventional 2D camera. PlantEye F500 measures the projection area of the plant on the X-Y plane and turns the 3D object into a flat 2D object (Fig. 3).

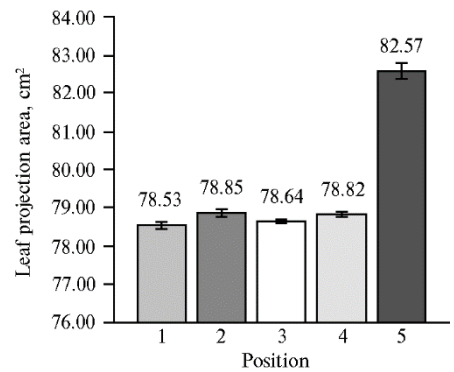

Fig. 3. Leaf projection area depending on posiooning of of lilac (*Syringa vulgaris* L.) cv. Mercy plants (n = 10, M±CI, p = 0.05; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

It can be seen from the histogram that the location of the plant at points 1, 2, 3 and 4 did not significantly affect the obtained data, while the location in the center of the scanned surface (point 5) led to both a significant increase in the leaf area in the projection and an increase in the confidence interval. The average values of the area of all leaves for 10 plants and the projected area of the leaves obtained by 3D scanning are correlated (r = 0.55, p < 0.05). At the same time, the value of the projected leaf area is much less than the leaf area, since in 3D scanning, leaves with the same position in the X-Y plane, but located at different heights above the ground, are not counted twice. That is, the projected leaf area serves as an analogue of the projective cover, which determines the relative leaf projection area on the underlying surface.

With the PlantEye F500 instrument, digital biomass is calculated as the

product of height and leaf area values, provided that the plant has a shoot structure, the volume of which can be calculated from height and length (Fig. 4).

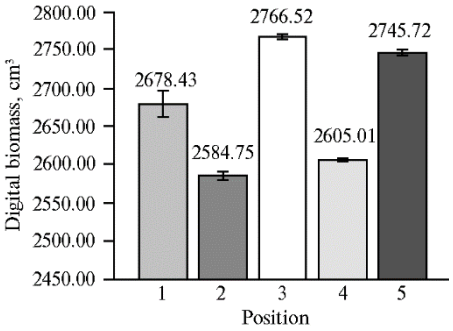

Fig. 4. Digital leaf biomass depending on positioning of of lilac (*Syringa vulgaris* L.) cv. Mercy plants (n = 10, M±CI, p = 0.05; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

Similarly, the location of the plant influenced the r assessment of both digital biomass and leaf area (r = 0.98, p < 0.05). Points 2 and 4 showed the smallest digital biomass, points 3 and 5 showed the largest one, and point 1 corresponded to the average value from all five points, at point 1 the value of the confidence interval was also the largest, as in the case of leaf area (see Fig. 2).

To calculate plant height, PlantEye F500 uses the distribution of elementary triangles along the Z axis. To do this, a histogram along the Z axis is first calculated, which reflects the number of elementary triangles at different heights above the ground. Next, the top 10% of the plant height is averaged, and the height itself is calculated as the distance from the height of the pot to the part for which the averaging was performed (Fig. 5).

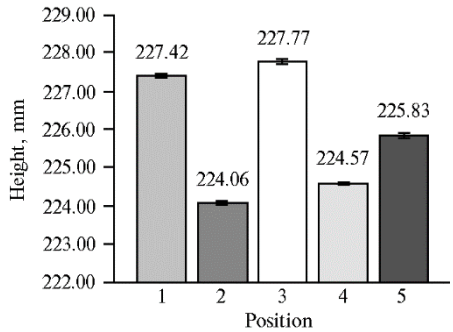

Fig. 5. Plant height depending on positioning of lilac (*Syringa vulgaris* L.) cv. Mercy plants (n = 10, M±CI, p = 0.05; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

It can be seen from the histogram that the location of the plant at points 1 and 3 gave the maximum height values, at points 2 and 4 the minimum, while the location of the plant in the center of the scanned surface (point 5) corresponded to the average value for all five points. It should be borne in mind that the error in the obtained values is determined by how deep the plant is located relative to the edge of the pot, and can be from 1 to 5 cm.

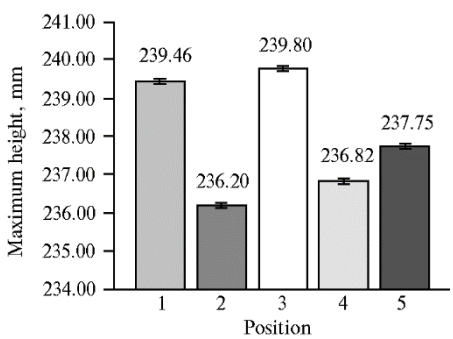

Fig. 6. Maximun plant height depending on positioning of lilac (*Syringa vulgaris* L.) cv. Mercy plants (n = 10, M±CI, p = 0.05; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

The maximum height is designed to define the absolute highest point of the plant in millimeters. This indicator does not replace the current height setting, but complements it. The current height focuses on averages rather than measurement accuracy, minimizing the effect of external artifacts or daily plant movements. To calculate the maximum height, PlantEye F500 finds the highest area (a group of

points in a 3D file) that contains the required number of points and is close enough to other areas. In this domain, the highest point is then given as the maximum height (Fig. 6).

In general, for the height and maximum height of plants during 3D scanning, the same dependence of the change in indicators for different positions of the plant on the scanned surface can be traced (for the correlation between these two parameters, $r = 0.99$, $p < 0.05$). The difference between the height and maximum height values in our experiment was about 12 mm at all points.

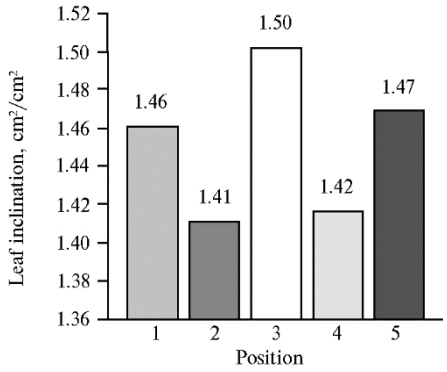

Fig. 7. Leaf inclination depending on positioning of lilac (*Syringa vulgaris* L.) cv. Mercy plants ($n = 10$, $M \pm CI$, $p = 0.05$; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

The leaf slope reflects information about how high the leaves are on the plant and is calculated as the total leaf area divided by the sum of the projections of each elementary triangle onto the X-Y plane (Fig. 7). The confidence interval for the obtained values is so small that it can be neglected with five repeated measurements in 10 plants. The maximum leaf slope values were obtained at point 3, the minimum values were obtained at points 2 and 4.

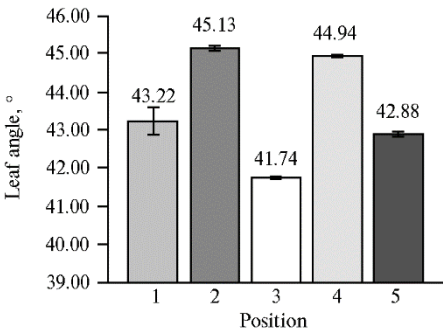

Fig. 8. Leaf angle depending on positioning of lilac (*Syringa vulgaris* L.) cv. Mercy plants ($n = 10$, $M \pm CI$, $p = 0.05$; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position.

The leaf angle is the arithmetic mean of all the angles of each facet based on their normal (Fig. 8).

The presented histogram shows that the location of the plant on the scanned surface significantly affects the obtained values. Thus, the location at points 2 and 4 gives the maximum values of the leaf angles, at point 3 the minimum, at points 1 and 5 the values are closest to the average for all five points. The slope angle was inversely proportional to the slope of the leaves ($r = -0.99$, $p < 0.05$) and leaf area ($r = -0.92$, $p < 0.05$) in 3D scanning. The higher the leaves are above the ground and more rotated relative to the scanning element, the greater the total leaf area will be (without changing the predicted area), and as a result, the slope of the leaves will increase.

The depth of penetration of light reflects the distance that the laser beam can penetrate through the leaf surface of the plant (Fig. 9). From the presented data, it can be seen that the location of plants at points 1, 2 and 3 did not significantly affect the depth of light penetration, at point 4 there was a slight decrease in the indicator, and at point 5 we noted the lowest degree of penetration of the laser beam. Thus, we can state with confidence that the location at point 5 is the most informative. The value of the projected leaf area in different locations of the plant on the scanned surface is inversely proportional to the depth of light

penetration (r = –0.95, p < 0.05).

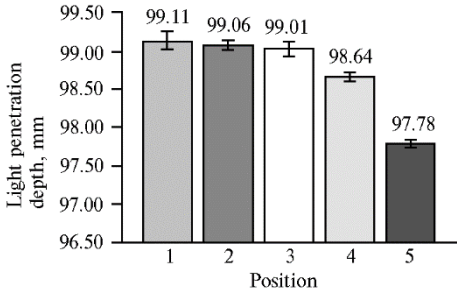

Fig. 9. Light penetration depth depending on positioning of lilac (*Syringa vulgaris* L.) cv. Mercy plants (n = 10, M±CI, p = 0.05; 3D scanning, PlantEye F500, Phenospex B.V., the Netherlands). Each plant was placed at 5 points, and at least 5 repeated scans were performed in the same position..

Thus, our study showed that the location of the plant on the scanned surface significantly affects the values of morphological parameters measured using the PlantEye F500 3D laser scanner. Of the eight morphological parameters, two (plant height and maximum plant height) duplicate each other. When analyzing plant size, plant height is more preferable, since the confidence interval was smaller with five height measurements. The value of leaf area and digital biomass at different points of plant location on the scanned surface correlate (r = 0.98). Digital biomass is a less informative indicator for 3D scanning. It has a large confidence interval and depends on the plant architectonics. A necessary condition for determining this parameter is the ability to calculate the volume given the height and the plant length. Two indicators with an inverse relationship (r = –0.95) are the leaf projected area and the depth of penetration of the laser beam. The depth of penetration of light could be an interesting indicator of the density of shoots and leaves. Howevere, with small plants, as it was in our study since we used plants grown in vitro, these parameters are not significant. The value of the angle of inclination is inversely proportional to the inclination of the leaves (r = –0.99). However, the leaf tilt angle is a more informative indicator in 3D scanning, allowing a better understanding of the architectonics of the plant, despite the fact that the confidence interval for leaf tilt is almost zero. In any case, both of these values are calculated based on the average of all leaf slopes and slope angles, therefore, a decrease in shoot turgor can significantly affect the results obtained.

The analysis of publications and the results obtained by us of the practical application of machine vision technologies in assessing the morphological parameters of lilac plants of the Mercy variety using the PlantEye F500 3D scanner made it possible to identify the following advantages and disadvantages of the automated approach. The advantages include the fact that phenotyping platforms allow one scan to determine from 5 to 15 morphological characteristics on one or several plants at once [38-40]. Carrying out measurements of morphological parameters by traditional methods requires the use of various types of equipment, as well as significant labor costs. The accuracy of the obtained values of various morphological parameters is characterized by a high degree of convergence (see Fig. 2-9) even despite the existing measurement errors. The obtained data are loaded into a computer, and it is possible to assess the dynamics of changes in morphological parameters over time. The lack of protocols for phenoscreening of morphological parameters for different crops should be considered as a disadvantage [41]. It should be taken into account that when several plants are studied simultaneously on the scanned surface, the probability of measurement error increases, as indicated by our experimental data. The impossibility of using the installation in the field during experiments, external factors (wind) prevent the plant from remaining in a static position and, as a result, affect the accuracy of the data obtained [36]. Most of the morphometric parameters studied by 3D scanning are in strong positive or negative interdependence and duplicate each other, for example, height and maximum height, digital biomass and leaf area, projected leaf

area and light penetration depth. That is, software developers need to focus not on the number of output parameters, but on their informativeness in assessing the state of plants and the possibility of determining the dynamics of growth processes. when using various forms of drugs. Based on the fact that when changing the position of the same plant on the scanned surface (moving along five points), the recorded morphological parameters differed significantly, it can be confidently expected that the location of several plants on the scanned surface will lead to significant differences in the data obtained.

Thus, our finndings show that, when using the PlantEye F500 3D scanner, it is optimal to place plants in the center of the scanned surface in the same position in order to obtain objective and comparable results. As morphological parameters for identifying varieties and fixing growth, we can recommend using the leaf area parameters, projected leaf area, plant height, and leaf angle. For each plant species, it is necessary to conduct primary morphological studies using traditional methods, and then compare the obtained data with the scan results to calculate the correction factor and confirm the information content of the trait set used, thereby increasing the accuracy of the data provided by machine vision technology.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 933-944
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 633.15:577.336

doi: 10.15389/agrobiology.2022.5.933eng
doi: 10.15389/agrobiology.2022.5.933rus

USE OF INTERNAL REFLECTION SPECTROSCOPY FOR MAIZE (*Zea mays* L.) GRAIN DIAGNOSIS

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The authors declare no conflict of interests

Acknowledgements:

In Serbia, research was funded by the Zemun Pole Maize Research Institute (Belgrade) and the Faculty of Physical Chemistry of the University of Belgrade, as well as the Ministry of Education, Science and Technological Development of Serbia (Projects 03E211, 03E22, TR-20014, Nos. 31028, 31037). In the Russian Federation, research is being conducted under the guidance of Professor G.V. Maksimov and funded by the RSF Project No. 19-79-30062. The study was also supported by the Interdisciplinary Scientific and Educational School of Lomonosov Moscow State University "Molecular technologies of living systems and synthetic biology".

Received June 16, 2022

Abstract

Infrared (IR) spectroscopy and Raman spectroscopy (RS) are modern methods on the basis of which biotechnological approaches are being successfully developed that allow genetic and functional analysis of individual plant organs and tissues at the molecular level. In the present work, using surface internal reflection spectroscopy (SIR), which is a modification of IR spectroscopy, differences in the content and conformation of biomolecules in grain homogenates and its components (endosperm, pericarp, germ) of ZP 735 maize hybrids were first recorded and revealed. Our goal was to develop a methodology for surface SIR for the identification of organic molecules, their content and conformation in corn seed and its tissues. The grains of the corn hybrid (*Zea mays* L.) ZP 735 (originator Maize Research Institute, Zemun Polje, Belgrade, Serbia) served as the object of the study. Thirty grains were selected by random sampling. To obtain SIR spectra, samples of grains and their parts (endosperm, pericarp, and embryo) were crushed, homogenized, and placed in a special cell of the device. A spectrometer (FT-IR spectrophotometer, Thermo Scientific, USA) with diamond (diamond ATR crystal, Thermo Scientific, USA) was used to record the SIR spectra, and a software package (Thermo Scientific™) was used to analyze the spectra. The SIR spectra were compared in the data library of the Advanced ATR correction Algorithm program. The SIR spectroscopy method is based on the reflection of a light beam at the interface between two phases: the phase of a crystal with a high refractive index, which is part of the SIR device, and the phase of the sample under study with a lower refractive index. During the measurement, the light beam penetrates to a small depth into the phase of the sample, where it is partially absorbed. During subsequent irradiation of the sample, this phenomenon is repeated, and as a result, the SIR spectrum is recorded. It has been proven that in the range from 400 cm^{-1} to 4000 cm^{-1} ATR spectra of whole grain, endosperm, pericarp and embryo of the ZP 735 hybrid are similar to the previously obtained IR spectra of grain, which indicates the

possibility of using a new method for molecular breeding technologies. The location of the bands of the SIR spectrum characterizes various forms of vibrations of the valence bonds of the functional groups of the organic molecules of the seed, which makes it possible to identify not only the presence of certain molecules, but also their conformation. It has been proved that by analyzing the amplitude of the SIR spectrum bands (maximum intensity amplitude and high intensity amplitude), it is possible to control changes in the content of a number of organic compounds in seed tissues: proteins, lipids, sugars, esters, amides, ketones, aldehydes, carboxylic acids, simple ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkenes, alkanes and alkynes. The obtained results are important for testing the presence of the necessary organic compounds in the grain or changes in their conformation during the selection process. The important advantages of SIR spectroscopy compared to IR spectroscopy include, on the one hand, the preservation of the nativeness of the object (the possibility of conducting research without fixing or staining the object) and simple sample preparation, on the other hand, an effective assessment of the content and conformation of molecules with high sensitivity (resolution about 1.0 cm^{-1}). The implementation of the developed methodology for the formation of molecular breeding technology will increase the profitability of cultivation and the efficiency of breeding not only corn, but also other agricultural plants.

Keywords: *Zea mays* L., hybrid, grain, endosperm, pericarp, embryo, surface internal reflection spectroscopy

At present, considerable attention is paid to the development of methodological techniques that allow one to control the content and conformation of various molecules in plant cells and tissues. Infrared (IR) spectroscopy and Raman spectroscopy (RS) are modern methods on the basis of which biotechnological approaches are successfully formed that allow genetic and functional analysis of plant organs and tissues at the molecular level, which is important for crop breeding [1-4]. Rapid molecular monitoring makes it possible to effectively evaluate the results of diagnostics and selection not only in the laboratory, but also in the field. In addition, these methods can serve as the basis for new technologies for prompt and qualified quality control of incoming raw materials for the manufacturing industry [5-7].

Previously, with the IR and Raman spectroscopy, important characteristics were obtained not only of the structure of individual molecules, but also of changes in their conformation (change in the proportion of characteristic vibrations of chemical bonds in molecules) [8-11]. For example, using Raman spectroscopy, we revealed changes in the content and conformation of carotenoid molecules in the chloroplasts of maize hybrids [7]. Using IR spectroscopy (range 3500-3000 cm^{-1}), it was found that in chloroplast molecules (water, carbohydrates, proteins) the proportion of vibrations of OH groups and intra- and intermolecular H-bonds was maximum in the ZPPL 186 line, and the proportion of vibrations The N−H groups of amides (proteins) turned out to be minimal in the ZPPL 225 line. ZPPL 186 chloroplasts were characterized by the maximum proportion of stretching vibrations from molecules of alkanes, carboxylic acids (range 2920-2860 cm^{-1}) and bending vibrations of aromatic structures (1000 cm^{-1}), and for the line M1-3-3-sdms, the fraction of stretching vibrations of O=C=O bonds (2300 cm^{-1}). Using Raman spectroscopy (regions 1250-500 cm^{-1} and 1535-1400 cm^{-1}) it was found that differences in chloroplasts in maize lines are associated with changes in the conformation of carotenoid molecules, and not cellulose. In all samples, except for ZPPL 225, carotenoid molecules were in the 15-trans form with different conformations of the polyene chain. It is important that the conformation of carotenoid molecules of the ZPPL 186 line is characterized by the minimum rotation of the molecular axis outside the plane of the polyene chain and more pronounced vibrations of the $C-CH_3$ side methyl group. It is assumed that carotenoids from the chloroplasts of the leaves of various maize lines do not interact with the aromatic amino acids of proteins [7].

The introduction of these methods for analyzing the conformation of molecules in plant tissues, along with molecular genetic technologies, contributes to

the formation of molecular breeding methods in agriculture [7].

In the present work, using surface internal reflection spectroscopy (ATR-IR), which is a modification of IR spectroscopy, for the first time, differences in the content and conformation of biomolecules in the grain, endosperm, pericarp, and germ of ZP 735 maize hybrids were recorded and revealed.

Our goal was to develop a methodology for surface internal reflection spectroscopy to identify the content and conformation of organic molecules in whole maize seed and its tissues.

Materials and methods. The grains of the corn hybrid (*Zea mays* L.) ZP 735 (originator Maize Research Institute, Zemun Polje, Belgrade, Serbia) served as the object of the study. Endosperm, pericarp, and grain germ were separated according to the described method [12]. Agronomic, morphological and chemical-physiological properties of the ZP 735 corn hybrid, including breeding, seed production and technological characteristics, are described in detail in M. Radosavljević et al. [13].

Thirty grains were randomly selected. To obtain internal IR-reflexion spectra (IRS), samples of whole grains and their parts (endosperm, pericarp, and embryo) were crushed, homogenized, and placed in a special cell of the instrument. The TER spectra were recorded using a spectrometer (Nicolet™ iS20 FTIR Spectrometer, Thermo Scientific, USA) with a diamond (diamond ATR crystal, Thermo Scientific, USA), and a software package (Thermo Scientific, USA) was used to analyze the spectra). The characteristics of the device were as follows: spectral range 3800-375 cm^{-1}, resolution about 1.0 cm^{-1}, signal-to-noise ratio more than 20000:1, ordinate linearity 0.07% T, wavenumber accuracy 0.01 cm^{-1}, maximum speed 40 scans/s. The spectra were recorded in 32-fold repetitions and identified using data libraries (more than 1500 compounds). The .ATR-IR spectra were compared in the data library of the Advanced ATR correction Algorithm program (Thermo Scientific, USA).

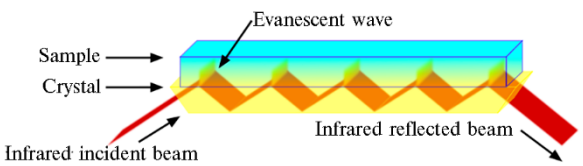

Fig. 1. Optical path of infrared beams in a crystal of IR total internal reflection (ATR-IR) **spectroscopy.**

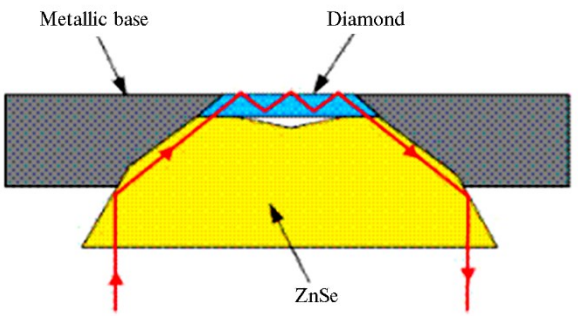

Fig. 2. Scheme of sample holder for IR total internal reflection (ATR-IR) **spectrometer.**

Results. In traditional IR spectroscopy, the spectrum of the emission of light transmitted through the sample is analyzed, in .ATR-IR spectroscopy, infrared radiation reflected from the surface of the sample is recorded. The ATR-IR spectroscopy method is based on the reflection of a light beam at the interface between two phases: the phase of a crystal with a high refractive index, which is part of the ATR-IR device, and the phase (homogeneous surface) of the sample under study with a lower refractive index. During the measurement, the light beam penetrates the sample to a shallow depth, where it is partially absorbed. During subsequent irradiations, this phenomenon is repeated, and as a result, the ATR-IR spectrum is recorded.

When analyzing the composition of a sample using ATR-IR spectroscopy,

the substance or object was placed on the surface of the crystal in the attachment of the ATR-IR spectrometer (Fig. 1). Further, IR radiation was directed through the crystal at a specially selected angle, the intensity of which was then recorded at the exit of the light beam from the crystal. It is important that the material of the crystal used for ATR-IR spectroscopy has a high refractive index (crystals of diamond and zinc selenide) (Fig. 2).

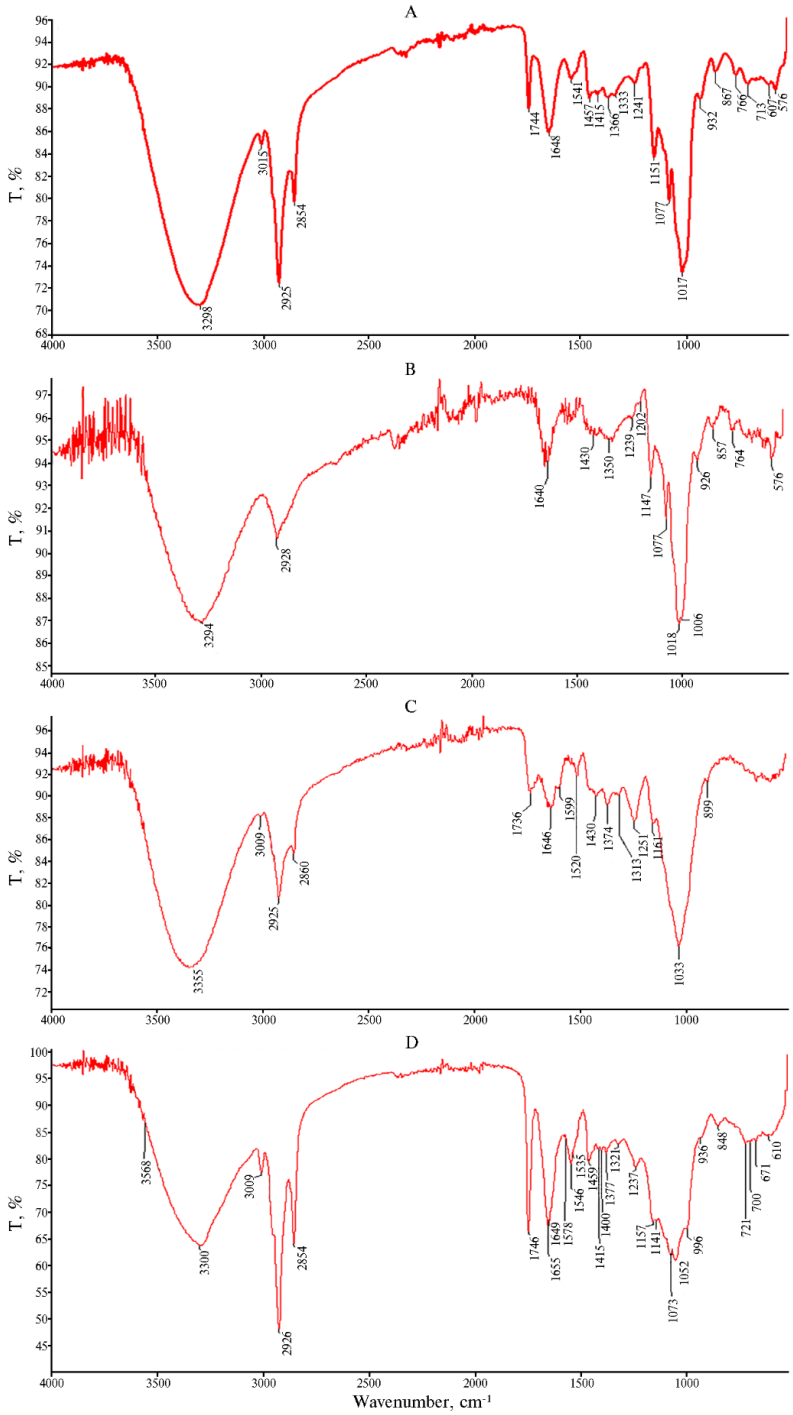

Fig. 3. ATR-IR spectra of whole grain homogenate (A)**, endosperm** (B)**, pericarp** (C)**, germ** (D) **of corn (*Zea mays* L.) hybrid ZP 735. T — transmittance (the ratio of the intensity of light passing through a**

sample to the intensity of light falling on the sample, i.e., the fraction of the incident light that passes through the test sample).

A significant advantage of ATR-IR spectroscopy is that it allows one to study opaque native samples, as well as to do without lengthy sample preparation of the object and to carry out analysis directly in the field. The proposed method makes it possible to assess the state (conformation) and the content of various biomolecules in a whole tissue (for example, a leaf, a root, etc.).

The grain of the hybrid maize ZP 735 is 84.75% endosperm, 5.30% pericarp, 9.95% germ [13]. The air defense spectrum of the ZP 735 hybrid maize grain homogenate (in the range from 400 cm^{-1} to 4000 cm^{-1}) (Fig. 3) was characterized by 20 bands, which differed from each other in intensity amplitude and frequency. All bands of the ATR spectrum both for the homogenate of the whole grain and for the homogenates of the endosperm, pericarp, and embryo were divided into four groups according to the amplitude of the maxima (T, %).

1. Characterization of ATR-IR spectrum bands of the homogenate of whole grain, endosperm, pericarp and embryo of the maize (*Zea mays* L.) hybrid ZP 735

Order of registration of spectral bands	Intensity, %	Wavenumber, cm^{-1}	Spectral bands of maximum and high intensity
		Whole grain	
1	20.8	3298	Proteins, lipids, carboxylic acids, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	12.5	2925	
3	2.9	2854	
4	7.0	1744	
5	6.1	1648	
6	1.9	1541	
7	3.6	1457	
8	1.8	1413	
9	0.8	1366	
10	0.2	1333	
11	0.4	1241	
12	8.0	1151	
13	6.0	1077	
14	9.2	1017	
15	0.2	932	
16	1.8	867	
17	1.4	766	
18	0.8	713	
19	0.2	607	
20	0.2	576	
		Endosperm	
1	17.9	3294	Carboxylic acids, lipids, proteins, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, esters, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	4.0	2928	
3	6.0	1640	
4	2.6	1430	
5	0.8	1350	
6	1.1	1239	
7	1.2	1202	
8	6.6	1147	
9	6.0	1077	
10	12.0	1018	
11	11.8	1006	
12	0.4	926	
13	0.4	857	
14	1.8	764	
15	1.6	576	
		Pericarp	
1	18.0	3355	Proteins, lipids, carboxylic acids, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	0.2	3009	
3	7.0	2925	
4	0.2	2860	
5	5.0	1736	
6	2.4	1646	
7	0.2	1599	
8	1.8	1520	
9	2.0	1430	
10	2.0	1374	
11	2.5	1313	
12	4.0	1251	
13	10.0	1162	
14	11.0	1033	
15	0.2	899	

Continued Table 1

		Embryo	
1	5.0	3568	Proteins, lipids, carboxylic acids, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	10.0	3300	
3	2.0	3009	
4	14.0	2926	
5	7.6	2854	
6	13.4	2746	
7	9.1	2655	
8	0.2	1648	
9	2.0	1578	
10	1.6	1546	
11	3.2	1538	
12	0.2	1459	
13	0.2	1415	
14	0.2	1400	
15	0.2	1377	
16	0.2	1321	
17	2.2	1237	
18	4.5	1157	
19	1.6	1141	
20	2.0	1073	
21	2.1	1052	
22	7.4	996	
23	2.0	936	
24	0.4	848	
25	0.2	721	
26	0.2	700	
27	0.2	671	
28	0.2	610	

Note. For all homogenates, the following vibrations of atoms in the molecule were revealed: symmetric and antisymmetric stretching (stretching); deformation planar scissor (scissoring); deformation planar pendulum (rocking); deformation out-of-plane fan (wagging); torsional out-of-plane deformation (twisting); trembling, valence bonds and molecular structures (trembling). The interpretation of the bands of the spectra obtained by us was carried out according to the published data [14-17].

In the whole grain, the maximum intensity amplitude (20% > T > 6%) was noted at 3298, 2925, 1744, and 1151 cm^{-1}, high (6% > T > 3%) at 1648, 1457 и 1077 $см^{-1}$, low (3.0% > T > 1.0%) at f2854, 1541, 1413, 867 and 766 cm^{-1}, and very low (1.0% > T > 0.2%) at 1366, 1333, 1241, 932, 607 and 576 cm^{-1} (Tables 1, 2).

The recorded ATR-IR spectra of the endosperm homogenate, pericarp, and embryo differed from the ATR-IR spectra of the grain homogenate both in intensity and in the frequency of specific bands. It was established that the amplitude of the intensity of the bands of the endosperm air defense spectrum was maximum at 3294, 1147, 1018 and 1006 cm^{-1}, high at 2928, 1640, 1147, 1077, 1018 and 1006 cm^{-1}, low at of 1430, 1239, 1202, 764 and 576 cm^{-1} and very low at of 1350, 926 and 857 cm^{-1} (see Fig. 3, B, Tables 1, 2). The intensity of the bands in the ATR-IR spectrum of the pericarp homogenate was maximum at of 3355, 2925, 1162, and 1033 cm^{-1}, high at of 1736 and 1251 cm^{-1}, low at of 1646, 1520, 1430, 1374, and 1313 cm^{-1}, and very low at 3009, 2860, 1599, and 899 cm^{-1} (see Fig. 3, C, Tables 1, 2). The intensity of the bands of the air defense spectrum of the embryos of the hybrid maize ZP 735 was maximum at 3300, 2926, 2854, 2746, 2655, and 996 cm^{-1}, high at 3568, 1538, 1157, and 996 cm^{-1}, low at 1578, 1538, 1073, 1052, and 936 cm^{-1}, and minimum at 1648, 1459, 1415, 1400, 1377, 1321, 848, 721, 700, 671 и 610 cm^{-1} (see Fig. 3, D, Tables 1, 2).

It is important that earlier in our works, vibrations of the valence bonds of the functional groups of organic molecules were also revealed in the classical IR spectra of whole grains [18, 19].

Thus, we have established that with the help of ATR-IR spectroscopy based not on absorption, but on the reflection of a light beam at the interface between two phases (crystal and biological object), it is possible to record spectra that allow not only to analyze the conformation of molecules of various substances, but and to determine their presence and concentration in seed tissues. For the first

time, the structure (a set of bands) of the ATR-IR spectrum of the grain homogenate of the hybrid maize ZP 735 and its constituent tissues was described in terms of the amplitude of the characteristic bands. These data are proposed to be used to study the content and conformation of various molecules of substances in the tissues of seeds according to the ATR-IR spectra. Such results are important for determining the presence of the necessary organic compounds in the grain or assessing changes in their conformation during the selection process.

2. Vibrations of the valence bonds of organic molecules in the homogenate of whole grain, endosperm, pericarp and embryo of the corn (*Zea mays* L.) hybrid ZP 735 revealed by ATR-IR spectroscopy

Wavenumber, cm^{-1}				Forms of vibrations of valence bonds of functional groups of organic molecules
whole grain,	endosperm	pericarp	embryo	
				Alcohols ((O—H)
				Amines (N—H), (C—H)
				Alkynes (C≡N), (C≡C)
				Ketones (=C=O)
				Alkenes (C=C)
				Esters (O—CH_2—)
				Lipids (C=O)
				Carbonyl groups (C=O) (esters)
				Amides (N—CH_2—), (—CO—N=)
				Amino groups (—NH—)
				Primary amines (—$CONH_2$)
				Carboxyl groups (—CO_2H)
				Imides (—CO—N—CO—)
				Acid chlorides (—COCl)
				Nitrite (—C≡N)
				Amides (N—CH_2—)
				Carbonyl groups (C=O) (amides)
				Aliphatic carbon-hydrogen bonds
				Aldehydes (—CHO)
3298	3294	3355	3568	
2925	2928	3009	3300	
2854	1640	2925	3009	
1744	1430	2860	2926	
1648	1350	1736	2854	
1541	1239	1646	1746	
1457	1202	1599	1655	
1415	1147	1520	1648	
1366	1077	1430	1578	
1333	1018	1374	1546	
1241	1006	1313	1535	
1151	926	1251	1459	
1077	857	1161	1415	
1017	764	1033	1400	
932	576	899	1377	
867			1321	
766			1237	
713			1157	
607			1141	
576			1073	
			1052	
			996	
			936	
			848	
			721	
			700	
			671	
			610	

N o t e. The interpretation of the bands of the obtained spectra was carried out according to the published data [14, 16, 17].

The data presented by us indicate that with the ATR-IR spectroscopy it is possible to differentiate the state and content of molecules of substances in tissues not only in the laboratory, but also in field tests. The analysis of the ATR-IR spectra by two parameters — the maximum and high amplitude of the bands, makes it possible to found out changes in the content and conformation of various molecules in the tissues of the seeds, while the bands with low and very low

intensity amplitudes, which we noted in the ATR-IR spectra of grain of ZP 735, probably allow only detection of the presence of molecules present in seed tissues in low concentrations (trace amounts).

It is obvious that the new method for studying seeds proposed by us requires a simpler sample preparation compared to IR spectroscopy and, at the same time, allows us to study changes in the conformation and content of individual biomolecules in the whole tissue with high sensitivity. Previously, studies of the semen homogenate were carried out using IR spectroscopy [18, 19]. We proved that the bands of the IR spectra of seeds of corn hybrids are similar to the set of bands of the IR spectra of pure lines of corn: the vibrations of the valence C−H bonds of alkenes and saturated hydrocarbons correspond to the set of bands at 2852 cm^{-1}, 2926 cm^{-1} and 995 cm^{-1}, valence O−H bonds to a set of bands 1161 cm^{-1} and 1082 cm^{-1} of secondary and tertiary alcohols, and vibrations of valence C=O bonds of proteins amide I and amide II to a set of bands 1651 cm^{-1} and 1541 cm^{-1} [20, 21]. In this work, it was found that the bands of the ATR-IR spectra of ZP 735 corn seed homogenates are similar to the set of IR bands: the vibrations of the valence C−H bonds of alkenes and saturated hydrocarbons corresponded to the set of bands at 2854 cm^{-1}, 2925 cm^{-1}, and 932 cm^{-1}, vibrations of valence O−H bonds to a set of bands at 1151 cm^{-1} and 1077 cm^{-1} of secondary and tertiary alcohols, and vibrations of valence C=O bonds of amide I and amide II of proteins — to a set of bands at 1648 cm^{-1} and 1541 cm^{-1}. Using another method, vibronic spectroscopy (Raman spectroscopy), additional signals were detected in the Raman spectra of corn seeds, namely, bands characteristic of the carotenoid molecule (960, 1006, 1156, and 1520 cm^{-1}) corresponding to C−C stretching vibrations. bonds and delocalization of π-electrons in a molecule. It was proved that the structure of carotenoid molecules in hybrids is not the same. The minimum length of the polyene chain of seed carotenoids was found in ZP 335. In other hybrids, this parameter is almost identical: in seeds of pure lines, the I_{1520}/I_{1156} ratio varied from 1.5 (ZP 186, ZP 225) to 1.9 (M1-3-3-sdms). Using Raman spectroscopy, it was found that the carotenoids of whole seeds ZP 341 have the lowest I_{960}/I_{1006} ratio among hybrids, and it is similar to that for seeds of lines ZPPL 186 and ZPPL 225. The ratio I_{1156}/I_{1190} in the Raman spectrum of carotenoids was similar in of all the hybrids studied, and the maximum value of I_{1120}/I_{1190} was revealed in the seeds of ZP 434. The latter probably indicates a high proportion of carotenoids associated with chlorophylls in the seed [18]. Note that all these studies did not provide data on the composition and conformation of metabolites in different tissues of the seed, which is important for breeding and genetics.

Next, we compare some of our results on the band amplitudes for different maize seed tissues using IR and ATR-IR spectroscopy. Using IR spectroscopy, it was found that for the seeds of the ZP 735 hybrid, the amplitude of the 1017 cm^{-1} band is maximum in the endosperm homogenate and minimum in the embryo homogenate. Probably, in this region of the IR spectrum (1017-1054 cm^{-1}), the band maxima are due to aromatic CH-planar bending vibrations [20, 21]. In this work, it was also found that the amplitude of the 1018-1052 cm^{-1} band of the ATR-IR spectrum is maximum in the endosperm and minimum in the embryo homogenate. The amplitude of the band at 1648 cm^{-1} in the IR spectrum was maximum in the endosperm and minimum in the embryo homogenate. Probably, these bands are due to C=O stretching vibrations in amides, N−H and C−N bending vibrations in secondary amides of proteins, peptides, and free amino acids, and also characterize vibrations of the OH group of crystalline cellulose water [20-

22]. In the present study, the amplitude of the band at 1640 cm^{-1} of the ATR-IR spectrum was also maximal in the endosperm and minimal in the embryo homogenate.

As is known, their functional activity is associated with the conformational state of photosynthetic pigments [23, 24], which is important, in particular, for selection. Of the spectroscopy methods, IR spectroscopy is most often used to identify and study organic compounds in biological objects [25]. In addition, it should be taken into account that IR exposure has become one of the most common methods for processing plant materials [26, 27], which requires an in-depth study of its physicochemical effects.

It should be noted that in the spectroscopy of surface internal reflection, it is not the absorption of IR radiation of light that is recorded, but its reflection by the sample. Therefore, one of the advantages of ATR-IR spectroscopy is that, unlike IR spectroscopy in which light must pass through the sample, for surface internal reflection spectroscopy, the thickness of the sample does not matter. In addition, in IR spectroscopy, additional preparation is usually needed to obtain a useful spectrum: homogenized grain samples are rolled into a tablet with potassium bromide (KBr, in a ratio of components 1:100), while this is not required in ATR-IR spectroscopy. The proposed approach and the original method will provide a screening study program not only for corn, but also for other plants during selection, diseases, and also when analyzing the impact of extreme environmental factors on plants.

Thus, in the wavenumber range from 400 to 4000 cm^{-1}, all known bands obtained by infrared (IR) spectroscopy were revealed in the spectra of surface internal reflection (SIR) of the whole grain, endosperm, pericarp, and embryo of the ZP 735 hybrid corn. By detecting the bands of the ATR-IR spectrum with maximum and high intensity amplitude, it is possible to control changes in the content of a number of organic compounds (proteins, lipids, sugars, esters, amides, ketones, aldehydes, carboxylic acids, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkenes, alkanes and alkynes) in the tissues of the seed. Important advantages of the ATR-IR spectroscopy method compared to classical IR spectroscopy include, on the one hand, simple sample preparation, which does not affect the physicochemical properties of the sample, and, on the other hand, an effective assessment of the content and conformation of molecules with high sensitivity (resolution of the order of 1.0 cm^{-1}. The implementation of the developed methodology for the formation of molecular breeding technology will increase the profitability of cultivation and the efficiency of breeding not only corn, but also other agricultural plants.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 945-953
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Genetics and genomics

UDC 633.15:577.2

doi: 10.15389/agrobiology.2022.5.945eng
doi: 10.15389/agrobiology.2022.5.945rus

EXPRESSION OF THE LYCOPENE-ε-CYCLASE *LcyE* GENE CORRELATES WITH THE CONTENT OF β-CAROTENE AND CHLOROPHYLLS IN MAIZE VEGETATIVE TISSUE

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Federal Scientific and Technical Program for the Development of Agriculture of the Russian Federation (subprogram "Agrarian Science — a Step into the Future Development of the Agro-Industrial Complex").
Received July 15, 2022

Abstract

Maize (*Zea mays* L.) is an important world crop. One of the valuable traits of this plant is the biosynthesis of vitamin A precursors in kernel (dietary nutrition) and photosynthetic tissue (protection of the plant from stress; silage with increased dietary value). The amount of synthesized provitamin A in kernel depends on the level of expression of the lycopene-ε-cyclase *LcyE* gene, which catalyzes the formation of α-carotene and is involved in the regulation of the ratio of β-β and β-ε fluxes of carotenoid metabolism. The aim of the study was to analyze the correlation between the content of the sum of carotenoids, β-carotene, and chlorophylls a and b with the expression of the *LcyE* gene in the leaves of inbred maize lines of domestic selection. To achieve the goal, four inbred maize lines were used in the study: three white-grained (6097-1, MBK and Shumny's Tetraploid) and one (5580-1) with yellow grain color. Expression of the *LcyE* gene in leaves was determined by quantitative real-time PCR. Quantitative determination of the amount of carotenoids, chlorophylls a and b, and β-carotene in leaves was carried out spectrophotometrically using the Folch method. Correlations between pigment content and *LcyE* gene expression were evaluated using statistical methods. As a result, we assessed a possible correlation between the activity of the *LcyE* gene and the content of carotenoids and chlorophylls in the photosynthetic tissue of four maize lines: three white-grained (6097-1, MBC and Shumnoy Tetraploid) and one (5580-1) with yellow-colored kernel. Quantification of carotenoids revealed the highest content of these pigments in the leaves of the Tetraploid Shumny line. The accessions of the remaining three lines synthesized a smaller amount of carotenoids and were similar to each other in this parameter. At the same time, β-carotene, as well as chlorophylls a and b, were most of all contained in the leaves of line 6097-1 — approximately 2 times more than in other analyzed lines, where the pigment content did not differ significantly. Thus, the absence of associations between the color of the kernel and the content of the β-carotene and sum of carotenoids in maize leaves was confirmed. On the other hand, the obtained data suggest a positive relationship between the amount of β-carotene and chlorophylls (a and b). It is possible to assume an increased rate of photosynthesis in the photosynthetic tissues of line 6097-1 in comparison with other analyzed maize lines. Accordingly, line 6097-1 may have an increased resistance to oxidative stress, as well as be a donor of a trait with an increased content of provitamin A (as a silage crop). The expression of the lycopene-ε-cyclase *LycE* gene was determined in the same leaf tissues. It was shown that the *LycE* gene was expressed ~ 4-5 times higher in the leaves of accessions of lines 5580-1 and Tetraploid Shumny than

in the leaves of accessions of lines MBK and 6097-1. Correlation analysis showed an inverse relationship between the content of β-carotene and chlorophylls (a and b) and the level of *LycE* gene expression. Thus, in this study, for the first time, we assessed a possible correlation between the activity of the *LcyE* gene and the content of carotenoids and chlorophylls in the photosynthetic tissue of white and yellow grain maize lines of domestic selection. No associations were found between grain color and the content of total carotenoids and β-carotene in maize leaves. A positive relationship was found between the amount of β-carotene and chlorophylls a and b. For the first time, an inverse relationship between the content of β-carotene and chlorophylls a and b and the level of *LycE* gene expression was determined. The possibility of using data on the expression of the *LycE* gene in the leaf as an expression molecular marker of the amount of provitamin A synthesized in the leaves, as well as the degree of plant resistance to photooxidative stress, was demonstrated. The data obtained can be used in maize breeding to search for donors of the trait of increased content of provitamin A in the leaves.

Keywords: *Zea mays* L., maize, lycopene-ε-cyclase, LycE, carotenoids, chlorophylls, gene expression

Photosynthesis is accompanied by the formation of reactive oxygen species (ROS) which have a pronounced reactivity [1]. The action of ROS is aimed at the metabolic modification of proteins, nucleic acids, and lipids; however, an excess of ROS causes increased oxidative degradation of chemical compounds in cells.

Protection against oxidative stress is achieved by regulating the amount of ROS and leveling the damage they cause, including through chlorophylls and carotenoids [2]. Carotenoids absorb light energy and carry out singlet-singlet transfer of excitation energy to chlorophyll molecules. In turn, chlorophylls give excess energy to carotenoids through triplet-triplet transfer. The return of carotenoids from the triplet state to its original state occurs due to the dissipation of energy in the form of heat [1].

Therefore, carotenoids play the role of antioxidants associated with the quenching of triplet chlorophyll and singlet oxygen [1]. We are talking about carotenoids of the xanthophyll cycle [3] which is mainly involved in the regulation of the redistribution of light energy between violaxanthin, zeaxanthin and chlorophyll a. In response to light stress, violaxanthin is converted to zeaxanthin via antheraxanthin, which acts as a lipid-protective antioxidant and stimulates non-photochemical quenching in the light-harvesting chlorophyll a/b-protein complex [3]. In low light, violoxanthin acts as a light-harvesting compound, serving as an energy donor for chlorophyll. In addition to the main one, higher plants have an additional second type of xanthophyll cycle, the lutein-5,6-epoxide type which is based on the reversible transformation of lutein into lutein-5,6-epoxide [3, 4].

The biosynthesis of carotenoids begins with the formation of the precursor of all carotenoids, phytoin, under the action of PSY phytoin synthase. In subsequent reactions, lycopene is synthesized. Further, the metabolic pathway is divided into branches β-β and β-ε leading to the formation of xanthophylls of the main (zeaxanthin, antheraxanthin, violaxanthin) and additional (lutein) types of the xanthophyll cycle. In the cycle of the first type, xanthophylls are derivatives of the β-β branch of the carotenoid biosynthesis metabolic pathway, when, under the action of lycopene-β-cyclase (LcyB), β-ionone rings (β-carotene; hydroxylation products of β-carotene zeaxanthin, antheraxanthin and violaxanthin) appear [5, 6]. In the second type of cycle, xanthophylls are derivatives of the β-ε branch of the carotenoid biosynthesis pathway. Their synthesis begins with α-carotene which is a molecule with a β-ionone ring at one end and a ε-ionone ring at the other end of the isoprenoid chain, resulting from the combined action of LcyB and lycopene-ε-cyclase (LcyE) (the end product of the branch β-ε is lutein) [5, 6].

Interestingly, β-cryptoxanthin (xanthophyll of the β-β branch), like α- and β-carotenes, not only performs a photoprotective function in the host plant, but also serves as a precursor of deficient vitamin A. For this, β-carotene is the most significant, since its structure has two β-ionone rings, as a result, the oxidative

cleavage of β-carotene leads to the formation of two vitamin A molecules [7-9].

Maize *(Zea mays* L.) plays an increasing role in the economy of the Russian Federation as a source of food and technical raw materials, as well as a silage crop. In connection with the wide use of corn plants, it is important that vitamin A precursors be contained in an increased amount not only in grain (dietary nutrition), but also in photosynthetic tissue (plant protection from stress, silage with increased fodder value).

Silage includes the above-ground part of the plant (cobs, leaves, stems) and in the feeding of farm animals provides about 50% of the dry matter of the main feed [10-12]. The use of photosynthetic tissue (especially leaves) of maize plants in animal husbandry may be more economically advantageous compared to grain. In the grain of traditional maize varieties and lines, carotenoids make up only 0.5-2.5 μg/g ww (13-15), while xanthophylls in leaves are about 200 rg/g ww, which is about 100 times more (16).

In maize, both lycopene cyclases, LycE and LycB, have been identified and characterized, including the expression of genes encoding them in grain [17-19]. However, there are no data on the activity of LycE and LycB in maize photosynthetic tissue. Polymorphisms in the sequence of the *LycE* gene make it one of the molecular markers that determine the amount of provitamin A in the tissue [13, 15, 20, 21]. Donors of mutant *lcyE* alleles are actively used in breeding maize lines that produce grain enriched with provitamin A [14, 15, 22]. There is an inverse relationship between thr *LycE* gene expression and provitamin A content [13]. This correlation is conservative in higher plants, as demonstrated by the example of the leaves of the model species *Arabidopsis thaliana* L. (23).

This research, for the first time, showed the absence of associations between grain color and the total carotenoids and β-carotene levels in corn leaves, a positive relationship between the amount of β-carotene and chlorophylls a and b, an inverse relationship of the contents of β-carotene and chlorophylls a and b with the *LycE* gene expression. The *LycE* gene expression in leaves can serve as a molecular marker associated with provitamin A synthesis in leaves and the degree of plant resistance to photooxidative stress.

The aim of the work was to analyze the correlation between the content of the sum of carotenoids, β-carotene, and chlorophylls a and b with the *LcyE* gene expression in the leaves of Russian inbred maize lines.

Materials and methods. Four inbred maize lines were used in the work: three white-grained (6097-1, MBK, Shumnoy's tetraploid) and one (5580-1) with yellow grain color. The seed material was kindly provided by the Institute of Agriculture, a branch of the Kabardino-Balkarian Scientific Center RAS. The grains were germinated in moist soil at 23/25 °C and 16/8 h (day/night) under the conditions of the EUIK artificial climate experimental facility (Federal Research Center of Biotechnology RAS) until the 4th true leaf appeared.

Total RNA was extracted from 50-100 mg of leaf tissue (RNeasy Plant Mini Kit, QIAGEN, Germany), purified from DNA impurities (RNase-free DNasy set, QIAGEN, Germany) and used for cDNA synthesis (GoScript™ Reverse Transcription System, Promega, USA) according to manufacturer's protocols. The quality of RNA was checked by electrophoresis in 1.5% agarose gel. RNA and cDNA concentrations were determined on a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA) using appropriate reagents (Qubit RNA HS Assay Kit and Qubit DS DNA HS Assay Kit, Invitrogen, USA).

Expression of the *LcyE* gene in leaves was determined by quantitative real-time PCR (RT-PCR) with normalization using the reference gene *Zea mays polyubiquitin* (NM_001329666.1; primers ZmUBI-rtF: 5'-ATCGTGGTTGTGG-CTTCGTTG-3', ZmUBI-rtR: 5' -GCTGCAGAAGAGTTTTGGGTACA-3′).

3 ng cDNA template, cDNA-specific primers (ZmLcyE-F: 5'-TTTACGTG-CAAATGCAGTCAA-3', ZmLcyE-R: 5'-TGACTCTGAAGCTAGAGAAAG-3'), kit Reaction mixture for RT-PCR in presence of SYBR GreenI and ROX (OOO Synthol, Russia) and thermal cycler CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Reactions were carried out in three technical and two biological repetitions. The program for RT-PCR was as follows: 5 min at 95 °C (initial denaturation); 15 s at 95 °C (denaturation), 40 s at 60 °C (annealing and alongation) (40 cycles).

Quantification (mg/g fresh weight) of the total carotenoids, chlorophylls a and b, and β-carotene in leaves was carried out in three technical and two biological replicates [24-26]. Leaves (0.2 g) were homogenized in Folch solution (chloroform:methanol, 2:1 v/v) with trace amount of Mg_2CO_3, allowed for 1 h at 4 °C in an ice-water bath, and centrifuged for 10 min at 4000 rpm and 4 °C (Eppendorf 5418 R centrifuge, Eppendorf, Germany). In the chloroform phase, lycopene, β-carotene, total carotenoids, chlorophylls a and b were quantified. Absorption spectra were recorded on Eppendorf BioSpectrometer® basic (Eppendorf, Germany) and Cary 50 (Agilent Technology, USA) spectrophotometers. The amount of pigments was calculated [24, 25].

The results were processed using GraphPad Prism v.8 (GraphPad Software Inc., USA; https://www.graphpad.com/scientific-software/prism/). Data were expressed as means (*M*) with standard deviations (±SD). Unequal variance Welch's *t*-test was used to assess the significance of differences in gene expression or pigment content between maize lines (at $p < 0.05$, the differences are statistically significant). The correlations between pigment content and *LcyE* gene expression in the leaves of maize lines were also evaluated using GraphPad Prism v.8. The correlation was unambiguously present at $R^2 > 0.7$, highly probable at $R^2 = 0.4$-0.7, and absent at $R^2 < 0.4$.

Results. Different shades of corn grain color (from yellow to orange) depend on the composition and quantitative ratio of carotenoids [27]. Therefore, the white-grain lines 6097-1, MBK, and Shumny's tetraploid were taken as samples with presumably impaired synthesis of lycopene, carotenes, and xanthophylls. The yellow grain line 5580-1 with preserved biosynthesis of colored carotenoids served as a control. We also investigated presumed correspondences between the peculiarities of carotenoid biosynthesis in grain and in photosynthetic tissue. The dark green color of the leaves in all four analyzed lines indicated successful photosynthesis and photoprotection, i.e. xanthophylls were biosynthesised in the leaves [3].

The highest content of carotenoids in leaves was characteristic of the line Shumnoy's tetraploid (Fig. 1, A). The rest lines synthesized a smaller amount of carotenoids and were similar to each other in this trait. In the leaves of line 6097-1, there was approximately 2 times more β-carotene than in other analyzed samples (see Fig. 1, B). For chlorophylls a and b, a quantitative profile was similar to that of β-carotene (see Fig. 1, C, D).

Therefore, the absence of associations between grain color and the content of total carotenoids and β-carotene in maize leaves was confirmed. We suggest that this may be due to the activity of other genes in the carotenoid biosynthetic pathway, for example, the *PSY* gene for phytoin synthase which catalyzes the synthesis of phytoin, the precursor of all carotenoids. The maize genome contains three *PSY* paralogs. *PSY1* triggers carotenoid synthesis in grain endosperm, while carotenogenesis in leaves depends primarily on *PSY2* activity [16].

The analysis data also suggest a positive relationship between the amount of β-carotene and chlorophylls a and b. Since β-carotene is a precursor of xanthophylls in the main xanthophyll cycle of plant photoprotection [3], one can

speak of an increased rate of photosynthesis in the photosynthetic tissues of line 6097-1 compared to other lines. Accordingly, line 6097-1 may have an increased resistance to oxidative stress, as well as be a donor of the trait of an increased content of provitamin A as a silage crop.

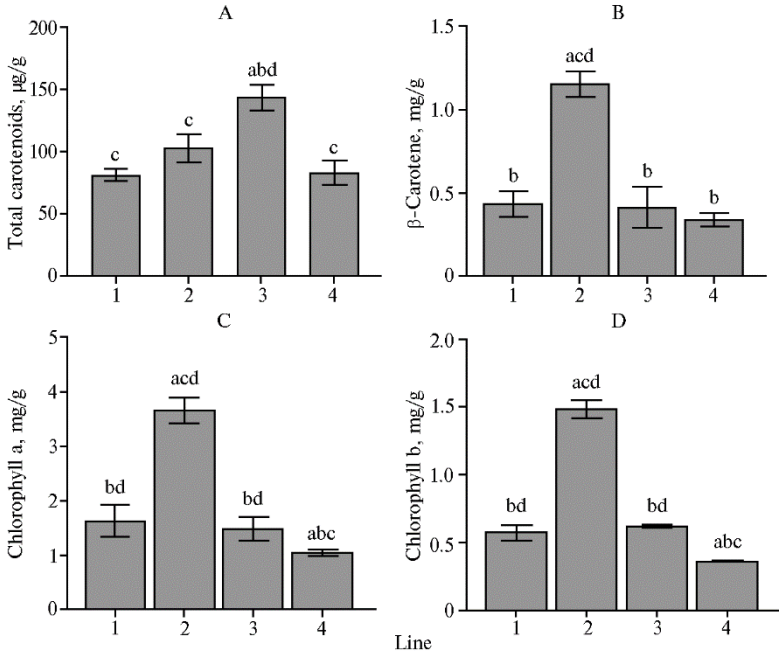

Fig. 1. Total carotenoids (А)**, β-carotene** (Б)**, chlorophyll a** (В) **and chlorophyll b** (Г) **accumulation in leaves of inbred corn (*Zea mays* L.) lines:** 1 — MBK, 2 — 6097-1, 3 — Shumnoy's tetraploid, 4 — 5580-1 (lab test, $n = 3$, $N = 2$).
[a, b, c, d] Differences between samples are stistically significant at $p < 0.001$.

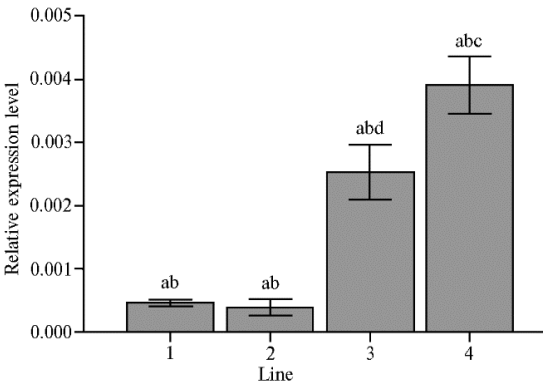

Fig. 2. Relative expression of the gene *LcyE* in leaves of inbred corn (*Zea mays* L.) lines: 1 — MBK, 2 — 6097-1, 3 — Shumnoy's tetraploid, 4 — 5580-1 (lab test, $n = 3$, $N = 2$).
[a, b, c, d] Differences between samples are stistically significant at $p < 0.001$.

It is known that the type of carotenes and xanthophylls is determined by the ratio of the β-ε and β-β branches of the carotenoid biosynthesis pathway, which depends on the expression levels of the *LcyE* and lycopene-β-cyclase *LcyB* genes. In addition, the accumulation of β-carotene is affected by the activity of the β-carotene hydroxylase 1 gene (*β-CH*, or *crtRB1*) [28].

In the same leaf tissues, we determined the expression of the lycopene-ε-cyclase *LycE* gene (Fig. 2). The expression was the highest in the leaves of line 5580-1 and slightly lower in the line Shumny's Tetraploid. In the lines MBK and 6097-1, the gene transcription in leaves was ~ 4-5 times lower (see Fig. 2). This intersample profile is consistent with the previously shown inverse relationship between *LycE* expression and provitamin A production [13, 22]. High *LycE* expression presumably means a shift in carotenoid biosynthesis towards the β-ε branch with the production of α-carotene and xanthophylls of the minor (second) type of the xanthophyll cycle with the formation of lutein and its derivatives.

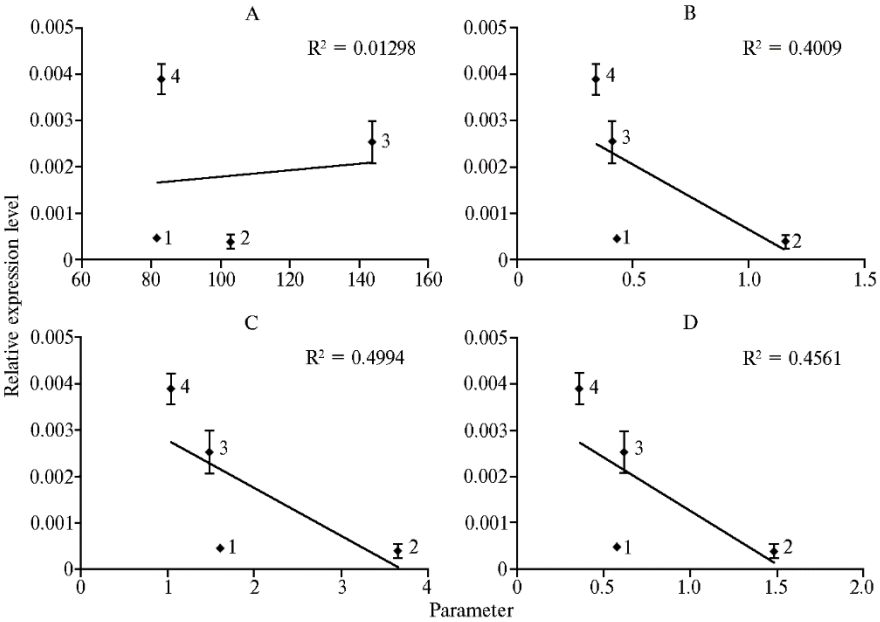

Fig. 3. Correlations between the level of relative expression of the gene *LcyE* and total carotenoids (μg/g) (A)**, β-carotene (mg/g)** (B)**, chlorophyll a (mg/g)** (C) **and chlorophyll b (mg/g)** (D) **concentrations in leaves of inbred corn (*Zea mays* L.) lines:** 1 — MBK, 2 — 6097-1, 3 — Shumnoy's tetraploid, 4 — 5580-1 (lab test, $n = 3$, $N = 2$).

Correlation analysis confirmed our assumptions. While there was no relationship between the *LycE* gene expression and the total carotenoids (Fig. 3, A), an inverse correlation between the *LycE* gene expression and the amount of β-carotene, chlorophylls a and b (Fig. 3, B-D) was predicted with high probability. This is consistent with previously obtained data for the photosynthetic tissue of *A. thaliana* [23].

Thus, the color of the maize grain does not correlate with the sum of carotenoids and β-carotene in the leaves. There is a positive relationship in the leaves between the concentration of β-carotene and chlorophylls a and b. In addition, we revealed an inverse relationship between the content of β-carotene and chlorophylls a and b and the level of *LycE* gene expression. Based on the findings, we suggest that the *LycE* gene expression level can be an expression molecular marker for provitamin A synthesized in maize leaves and also in assessment of plant resistance to photooxidative stress.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 954-964
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UD 635.64:631.52:577.21

doi: 10.15389/agrobiology.2022.5.954eng
doi: 10.15389/agrobiology.2022.5.954rus

FEATURES OF THE PRIMARY STRUCTURE OF THE *Ph-3* GENE, REVEALED BY DEVELOPMENT OF A NEW GENE-BASED MARKER OF LATE BLIGHT RESISTANCE IN TOMATO

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The authors declare no conflict of interests
Acknowledgements:
The work was carried out within the framework of State Assignment No. 0431-2022-0004.
Received July 1, 2022

Abstract

Late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is one of the most harmful diseases of tomatoes. Late blight control remains challenging due to the high genetic variability and complex racial composition of *P. infestans*. Therefore, the most promising method of combating late blight is the breeding of resistant varieties of tomato. When creating resistant varieties, the introgression of resistance genes from wild-growing related species is widely used. In particular, several late blight resistance genes identified in the wild tomato species *Solanum pimpinellifolium* have been introgressed into tomato cultivars. Among these genes, the *Ph-3* gene is considered to be the strongest late blight resistance gene, as it provides resistance to a variety of *P. infestans* isolates. Therefore, considerable efforts of scientific groups around the world are directed to the study of this gene in order to include it in breeding programs and introduce it into new commercial varieties and lines of tomato. To date, DNA markers associated with this gene are known. However, homologues of this gene were found in the tomato genome, which do not have functional activity. Analysis of the multiple alignment of the nucleotide sequences of the *Ph-3* gene and its homologues showed that the primers used in the known markers for amplification of this gene are in the conservative regions of these sequences, and it is impossible to specifically amplify the *Ph-3* gene with them. Therefore, the aim of this work was to design a new highly specific marker of the *Ph-3* gene and compare it with already known markers by analyzing the collection of tomato varieties of the Federal Scientific Center for Vegetable Growing for the presence of known and new markers and assessing the linkage of these markers with resistance to late blight disease in the studied varieties. To this end specific primers were designed (5′-AATATTGAAAATAGCTGCACTGA-3′/5′-CGAGATTTGGAGGGAATGTAA-3′) that discern the *Ph-3* gene from its homologues and amplify a 412 bp gene fragment (the Ph3-412 marker). Using these primers, 24 tomato (*Solanum lycopersicum* L.) varieties bred at the Federal Scientific and Technical Center and tested for late blight field resistance (Federal Scientific and Technical Center, Moscow Province, 2021) were analyzed. Also, these varieties were analyzed with known marker NC-LB-9-6678. To determine the nucleotide sequence of the new marker, we cloned the amplified product obtained from the studied varieties into pAL-TA vector and sequenced the resulting clones. In addition, we cloned and sequenced 601 and 907 bp fragments obtained with a known marker. We compared the nucleotide sequences of all three fragments with the sequences of the prototype gene and its known homologues. As a result, we confirmed that the fragment amplified using primers designed by us belongs to the *Ph-3* gene, while the 601 bp fragment obtained with the known primers corresponds to the *SlRGA4* homologue, and the 907 bp fragment obtained with the same primers is homologous to the *Ph-3* gene but it contains an insertion of the LTR retrotransposon of the Ty1-*copy* family with a size of 306 bp. Thus, the gene containing such insertion is most likely inactive. We also showed that in all analyzed varieties, in which the *Ph-3* gene was found, this gene contains the above-mentioned insertion. The presence of such insertion can lead to a loss of functional activity; this must be taken into account when marking the *Ph-3* gene. For the breeding programs it is necessary to identify plants in which the *Ph-3* gene does not have this retrotransposon insertion.

Keywords: tomatoes, late blight disease, *Ph-3* gene, DNA markers, resistance genes

Late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is one of the most harmful tomato diseases that can destroy up to 100% of the crop [1]. Late blight control remains challenging due to the high genetic variability and complex racial composition of *P. infestans*. The use of fungicides, in particular metalaxyl, is not effective enough, since pathogen races quickly mutate and acquire resistance to this drug [2-6]. In addition, fungicide treatment is expensive, and fungicides themselves are harmful to the environment and dangerous to human health. The most promising method of combating late blight is the breeding of resistant varieties of tomato.

When creating resistant varieties, the introgression of genetic material from wild-growing related species is widely used in order to pyramid their resistance genes [7-9]. In particular, several late blight resistance genes identified in the wild tomato species Solanum pimpinellifolium have been introgressed into cultivars [10, 11]. These are the *Ph-1*, *Ph-2* and *Ph-3* genes. The *Ph-1* gene, mapped on chromosome 7, confers resistance to the T0 race of *P. infestans* [12]. The *Ph-2* gene, originally identified in *S. pimpinellifolium* West Virginia 700 (WV700) in chromosome 10, provides resistance to the T0 race and partial resistance to the T1 race [13]. However, the resistance determined by these genes is quickly overcome by new races of *P. infestans*. The *Ph-3* gene was identified in the L3708 sample of S. pimpinellifolium and mapped to the long arm of the chromosome 9. It provides resistance to many races of *P. infestans* that overcome the resistance conferred by the *Ph-1* and *Ph-2* genes [14]. *Ph-2* and *Ph-3* have also been shown to act synergistically and together confer resistance to a broader range of pathogen isolates than either gene alone [15].

Currently, the *Ph-3* gene is considered to be the strongest gene for resistance to tomato late blight. Considerable efforts have been directed to its study in order to include it in breeding programs and introduce it into new commercial varieties and lines [16]. The *Ph-3* gene was cloned and characterized at the molecular level from the L3708 specimen of *S. pimpinellifolium*. *Ph-3* has been found to encode a protein containing a supercoil domain, a nucleotide-binding domain, and leucine-rich repeats (CC-NBS-LRR). When transgenic, it can confer late blight resistance in susceptible tomato varieties [17]. Four structural homologues of this gene, the *SlRGA1*, *SlRGA2*, *SlRGA3*, and *SlRGA4*, were found in the locus corresponding to the *Ph-3* locus of *S. pimpinellifolium* in the genome of the tomato cv. Heinz1706.

Efforts are also being made to mark the *Ph-3* gene for more efficient transfer to material of interest by marker assistant selection (MAS) and pyramiding with other late blight resistance genes. To date, several DNA markers are known to be somehow associated with this gene, including three SCAR (sequence characterized amplified region) markers [18-20] and one CAPS (cleaved amplified polymorphic sequences) marker [21]. However, these markers have a number of disadvantages.

In particular, with the help of SCAR markers described by Y. Park et al. [18], homologues of the *Ph-3* gene can be distinguished, but not the gene itself. In addition, the analysis is proposed to be carried out using a set of three pairs of primers, which increases labor and time costs and complicates the interpretation of the results. The marker described by H.T.H. Truong et al. [19], was derived from a RAPD marker, and its relationship to the *Ph-3* gene sequence is unknown. Multiple alignment analysis of the nucleotide sequences of the *Ph-3* gene and its known homologues showed that the primers used by D.R. Panthee et al. [20] for amplification of this gene, are located in the conserved regions of the mentioned sequences and it is not possible to specifically amplify the gene with their help. CAPS marker described by Y.-Y. Wang et al. [21], involves the use of restriction

endonucleases and the separation of restriction products in a polyacrylamide gel, which makes this analysis relatively expensive and time consuming. In addition, in the case of CAPS markers, there may be problems with the reproducibility of results, since the restriction efficiency is affected by the activity of the enzyme and the amount of DNA, and these parameters are difficult to accurately control. In addition, the validation of all these markers was carried out on segregating populations obtained by crossing susceptible and resistant parental forms.

In the present work, it was shown for the first time that in the varieties of tomato of domestic selection in the presence of the *Ph-3* gene, its other homologues are absent. It was also established for the first time that a retrotransposon insertion is present in the *Ph-3* gene sequence, which can lead to the loss of the genome's functional activity.

Our goal was to design an easy-to-use, highly specific DNA marker for the *Ph-3* gene to be used to distinguish *Ph-3* from its structural homologues. In addition, we aimed at validation of this marker in comparison with already known markers based on the analysis of the collection of domestic varieties and lines of tomato and the assessment of the relationship of markers with field resistance to late blight.

Materials and methods. The study was performed on 24 samples of tomato (*Solanum lycopersicum* L.) bred by the Federal Scientific Center for Vegetable Growing (FNTSO) and included in the State Register of Breeding Achievements approved for use in the Russian Federation, These are Ottawa 30 (late blight resistance standard), Talalikhin (susceptibility standard to late blight), Fitilek, Primorets, Grot, Charovnitsa, Lotus, Rosinka, Pos'yet, Toptyzhka, Odyssei, Patrocl, Blagodatny, Viking, Dubok, Revansh, Talisman, Monakh, Perst, Kameya, Severyanka and Voskhod VNIISSOKa, lines l-DVot30- 2/19 and l-Ft5/20. Thirty plants of each variety and line were grown under laboratory conditions up to 4 weeks of age at 23-25 °C, air humidity of 70-80% and 16 hours of artificial lighting (from 7.00 to 23.00), after which they were transplanted into open ground.

The experiments were carried out in 2021 on the experimental field of the Federal Scientific Center for Vegetable Growing (Moscow Province, Odintsovo District) against a provocative infectious background (an isolated area with a monoculture of tomato). Seedlings were planted at 4-5 true leaves in the first ten days of June using a two-line planting according to the scheme 70×40 cm for determinant varieties, 70×35 cm for standard varieties. Agrotechnics for growing seedlings was standard for tomato culture.

For the phenotypic assessment of tomato resistance to *P. infestans*, 10 plants of each accession were planted in 3 replicates according to the scheme of randomized blocks so that each accession had the same chances of infection. To control the evenness of the infectious background and the dynamics of the development of the disease, the susceptible variety Talalikhin and the resistant variety Ottawa 30 were used, which were planted through five studied samples. Accounting for late blight lesions was carried out in dynamics every 7 days, starting from the appearance of the first symptoms (III decade of July). Plant damage was assessed visually by characteristic symptoms according to a modified ten-point scale where 0 means no symptoms; 0.1 means 1-5% affected leaf area, small lesions (<2 mm), no stem lesions; 0.5 means 6-10% affected leaf area, no damage to the stems; 1 means 11-20% affected leaf area, no damage to the stems; 1.5 means 21-30% affected leaf area, confluent leaf lesions or tiny watery stem lesions; 2 means 31-40% affected leaf area, expanding along the edges of the leaf lesion or several small stem lesions (< 5 mm); 2.5 means 41-50% affected leaf area, stem lesions (< 30 mm); 3 means 51-60% affected leaf area, drying damage to the leaves or damage to the stem with the expansion of the edges, 20% affected fruits; 3.5 means

61-70% affected leaf area, drying damage to the leaves and damage to the stem with the expansion of the edges, 40% affected fruits; 4 means 71-100% affected leaves, stems and fruits. The resistance of each sample was assessed by the lesion index (I, average score). According to the totality of all assessments, the samples were differentiated into resistance groups, R for resistant (I = 0), RS for relatively stable (0 < I ≤ 1), MS for moderately susceptible (1 < I ≤ 2), S for susceptible (2 < I ≤ 3), HS are highly susceptible (I > 3).

Total DNA was extracted from young leaves of 2-week-old plants using the Sorb-GMO-B reagent kit (Synthol, Russia) according to the manufacturer's protocol. For each variety, DNA was isolated from all 8 plants, after which the DNA preparations were combined into one common sample.

The design of primers for specific amplification of the *Ph-3* gene was based on multiple alignment of the nucleotide sequence of the *Ph-3* gene (GenBank no. KJ563933) and its structural homologues *SlRGA1*, *SlRGA2*, *SlRGA3*, and *SlRGA4*, the nucleotide sequences of which were taken from the nucleotide sequence of the tomato chromosome 9 registered in the GenBank NCBI database (GenBank no. EF647605.1). Forward (5′-AATATTGAAAATAGCTGCACTGA-3′) and reverse (5′-CGAGATTTGGAGGGAATGTAA-3′) primers were designed in which the sequences of the 3′-ends were strictly specific for the *Ph-3* gene and differed from the sequences of the gene homologues in this position. These primers were located in the LRR domain of the *Ph-3* gene, and the expected amplicon size was 412 bp. In addition, for comparative analysis, primers of the marker NC-LB-9-6678 5′-CCTTAATGCAATAGGCAAAT-3′ and 5 -ATTTGAATGTTCTG-GATTGG-3′ [11] were used the sequences of which were absolutely conserved for the *Ph-3* gene and its homologs.

The amplification program was 3 min at 94 °C; 30 s at 94 °C, 30 s at 60 °C, 1 min at 72°C (35 cycles); 5 min at 72 °C (final synthesis). The volume of the reaction mixture was 25 μl. For one reaction, 50 ng of total DNA was taken. For amplification, a GeneAmp PCR System 2700 device (Applied Biosystems, Inc., USA) was used. Electrophoretic separation of amplification products was carried out in 1% agarose gel with 1× TAE buffer. Amplification conditions with primers NC-LB-9-6678 were as described by D.R. Panthee et al. [20], 3 min at 92 °C; 30 s at 92 °C, 1 min at 52 °C, 30 s at 72 °C (35 cycles); 8 min at 72 °C (final alongation).

For nucleotide sequencing, the obtained amplicons were cloned into the pAL-TA vector (Evrogen, Russia), which was used to transform competent cells of *Escherichia coli* DH5α, and sequenced by the Sanger method using the Big Dye Terminator v.3.1 reagent kit (Applied Biosystems, Inc., USA; an ABI PRIZM 3730 automatic sequencer, Applied Biosystems, Inc., USA) according to the manufacturer's instructions.

Multiple alignment of nucleotide sequences was performed using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clust-alo/) followed by analysis of the alignment results with the GeneDoc 2.7 program (https://genedoc.software.informer.com/2.7/). The TREECON program [22] was used to construct the dendrogram. Derived amino acid sequences were obtained using the EditSeq program (https://macdownload.informer.com/editseq/download/). The BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for homologues of the obtained sequences in the NCBI database.

Results. During PCR amplification of total DNA preparations isolated from 24 tomato samples with primers specific for the *Ph-3* gene, the Ph3-412 marker we created was found in all analyzed samples, except for three (l-Ft5-19, Viking and Revansh) (Fig. 1, A). According to the results of analysis with primers to the marker NC-LB-9-6678, three of these samples had a marker, which in the

work of H.L. Merk et al. [11] was associated with resistance to late blight of tomato (600 bp), and the rest were a marker associated with susceptibility (900 bp) (see Fig. 1, B). In other words, the Ph3-412 marker was absent in varieties in which the previously known marker associated with resistance was found, and was present in varieties in which a 900 bp fragment associated with susceptibility to late blight was detected. That is, an analysis with a new and previously known marker gave diametrically opposite results.

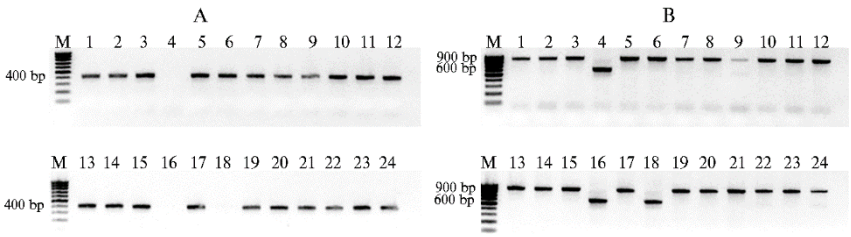

Fig. 1. Electrophoregram of PCR amplification products of total DNA of tomato (*Solanum lycopersicum* L.) samples with primers of markers Ph3-412 (A) and NC-LB-9-6678 (B), specific for the late blight resistance gene *Ph-3*: M — molecular weight marker, 1 — Ottawa 30, 2 — l-DVot30-2/19, 3 — Fitilek, 4 — l-Ft5/20, 5 — Primorets, 6 — Grot, 7 — Charovnitsa, 8 — Lotus, 9 — Talalikhin, 10 — Rosinka, 11 — Pos'yet, 12 — Toptyzhka, 13 — Odyssei, 14 — Patrocl, 15 — Blagodatny, 16 — Viking, 17 — Dubok, 18 — Revansh, 19 — Talisman, 20 — Monakh, 21 — Perst, 22 — Kameya, 23 — Severyanka, 24 — Voskhod VNIISSOKa

Comparison of the results of molecular analysis using DNA markers of the late blight resistance gene *Ph-3* with the data of phenotypic assessment of field resistance to late blight in tomato (*Solanum lycopersicum* L.) samples

Variety	Resistance group by phenotype	Marker		
		Ph3-412	NC-LB-9-6678 600 bp	NC-LB-9-6678 900 bp
Ottawa 30 (resilience standard)	SR	+	–	+
Fitilek	MS	+	–	+
Lotus	MS	+	–	+
Patrocl	MS	+	–	+
Primorets	MS	+	–	+
Blagodatny	MS	+	–	+
Talisman	MS	+	–	+
Monakh	MS	+	–	+
L-DWot30-2/19	MS	+	–	+
Revansh	MS	–	+	–
Viking	MS	–	+	–
l-Ft5/20	S	–	+	–
Talalikhin (susceptibility standard)	S	+	–	+
Rosinka	S	+	–	+
Charovnitsa	S	+	–	+
Toptyzhka	S	+	–	+
Dubok	S	+	–	+
Perst	S	+	–	+
Kameya	S	+	–	+
Voskhod VNIISSOKa	S	+	–	+
Pos'yet	HS	+	–	+
Grot	HS	+	–	+
Odysseus	HS	+	–	+
Severyanka	HS	+	–	+

N o t e. SR — semiresistant, MS — medium susceptible, S — susceptible, HS — higly susceptible; «+» — present, «–» — absent.

The weather conditions of 2021 were characterized by a hot and dry growing season: precipitation for the entire period was 29.4 mm less than the long-term average, the air temperature was 2.5 °C above the climatic norm. Accounting for the damage of varieties-differentiators by the oomycete *P. infestans* showed the presence of the T1 race, which is characterized by high aggressiveness and virulence in open ground conditions in 2021.

When comparing the results of molecular analysis with the data of phenotypic assessment of field resistance to late blight, it was found that the Ph3-412 marker was present in the Ottawa 30 variety resistance standard, and only a 900 bp fragment was noted for the NC-LB-9-6678 marker, while a 600 bp fragment associated with resistance was absent (Table). Of the nine moderately susceptible samples, the Ph3-412 marker was found in seven samples, the 600 bp resistance marker NC-LB-9-6678 in two samples. Among susceptible samples, the Ph3-412 marker was detected in 13 samples, and a known resistance marker was detected in one sample. Consequently, none of the markers showed an unambiguous association with field stability.

To find out what may cause such ambiguity, we cloned and sequenced 412 bp PCR products obtained with primers Ph3-412, 600 bp and 900 bp PCR products obtained with primers NC-LB-9-6678. The obtained nucleotide sequences were compared with the sequence of the Ph-3 prototype gene and its structural homologues SlRGA1, SlRGA2, SlRGA3, and SlRGA4. The comparison results are presented in the form of dendrograms (Fig. 2).

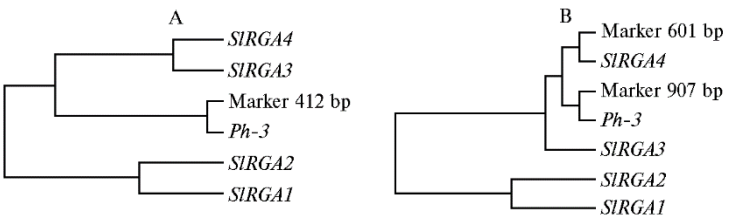

Fig. 2. Dendrograms based on comparing the nucleotide sequences of the tomato (*Solanum lycopersicum* L.) late blight resistance gene *Ph-3* and its homologues *SlRGA1*, *SlRGA2*, *SlRGA3*, *SlRGA4* with the nucleotide sequence of the 412 bp PCR product generated with the primers of the Ph3-412 marker (A)**, and nucleotide sequences of 601 bp and 907 bp PCR products generated with the primers of the NC-LB-9-6678 marker** (B)**.**

The fragment amplified with the Ph3-412 primers clustered together with the *Ph-3* prototype gene, and its nucleotide sequence was 99.3% homologous to the *Ph-3* sequence (see Fig. 2, A). That is, we can confidently state that the primers we created specifically amplify the *Ph-3* gene and not its homologues. The exact sizes of the fragments amplified with primers NC-LB-9-6678 were 601 bp. and 907 bp. It turned out that the 601 bp fragment belongs to the *SlRGA4* homologue, on the dendrogram, it clustered together with *SlRGA4* with 99.5% sequence homology (see Fig. 2, B).

The most interesting results were obtained by analyzing the nucleotide sequence of the 907 bp fragment. It turned out that the difference in length between the fragments obtained with primers NC-LB-9-6678 is due to an insert of the LTR fragment of the retrotransposon of the Ty1-*copia* family, the size of which was 306 bp (Fig. 3). At the same time, the rest of the sequence of the fragment with a size of 907 bp. was 99.7% homologous to the *Ph-3* gene and clustered with it on the dendrogram (see Fig. 3, B). That is, a 907 bp fragment. belongs to the *Ph-3* gene, but with a retrotransposon insertion that disrupts the reading frame and translation of the functional protein. Thus, we found for the first time that the *Ph-3* gene in the tomato genome could have a retrotransposon insertion, which, apparently, renders this gene nonfunctional.

The obtained results confirms the high specificity of the *Ph-3* gene marker Ph3-412 that we created. Most of the analyzed tomato samples (21 out of 24) had the *Ph-3* gene, which was indicated by the presence of the Ph3-412 marker and a 907 bp fragment obtained with primers NC-LB-9-6678. These samples most likely

did not have *Ph-3* homologues, since they lacked a 601 bp fragment, which, according to our data, belongs to the *SlRGA4* homologue, and none of the samples simultaneously had markers 412 bp/907 bp and 601 b.p. Apparently, with the introgression of the *S. pimpinellifolium* genetic material into the tomato genome, the locus containing *Ph-3* replaced the locus containing all homologues of this gene in the *S. lycopersicum* genome via homologous recombination.

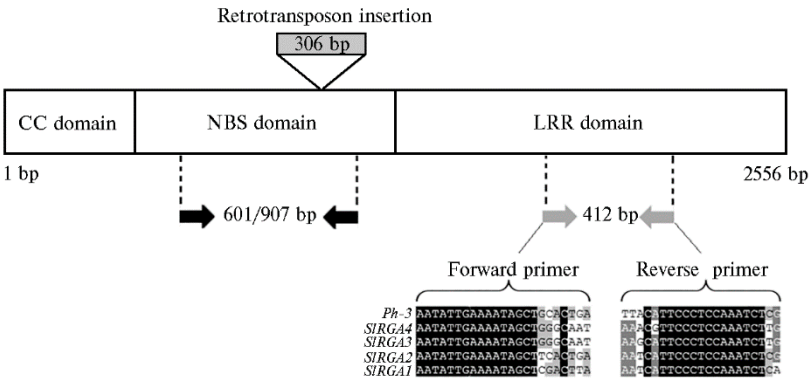

FIg. 3. The tomato (*Solanum lycopersicum* L.) late blight resistance gene *Ph-3*, primer arrangement and insertion of a retrotransposon. CC-domain, NBS-domain and LRR-domain designate the regions of the gene encoding the corresponding domains of the Ph-3 protein. Gray arrows indicate the location of the primers of the Ph3-412 marker, their nucleotide sequences are compared with the nucleotide sequences of the corresponding regions of the gene homologs, black arrows indicate the location of the primers of the marker NC-LB-9-6678; the numbers 1 and 2556 indicate the size of the *Ph-3* gene in bp.

However, samples bearing the Ph3-412 marker appeared to contain an inactive *Ph-3* gene with an insertion of a Ty1-*copia* family retrotransposon. Retrotransposons of this type are very common in the tomato genome (23). The presence of a retrotransposon insert can explain the fact that the Ph3-412 marker we created did not show a clear relationship with the field resistance of the analyzed samples to late blight, they all contained an inactivated form of the *Ph-3* gene.

The resistance of accessions to late blight was due to other genetic factors. For example, the resistant variety Ottawa 30 which, according to our data, has the *Ph-3* gene in an inactive form, contains the resistance genes *Ph-1* and P*h-2* [24, 25]. Since *S. pimpinellifolium* also served as the source of these genes, it can be assumed that in the donor forms included in the selection process, the *Ph-3* gene initially had a retrotransposon insertion. Subsequently, the genetic material of these donors, and not sample L3708, was widely distributed among domestic tomato varieties.

According to our results, for the same reason, the marker NC-LB-9-6678 did not show a connection with field resistance to late blight, since the authors carried out its verification in splitting populations of tomato plants obtained by crossing with the initial donor of the *Ph-3* gene, sample L3708 *S. pimpinellifolium* [20]. As a result, despite its nonspecificity, the NC-LB-9-6678 marker allowed the authors to distinguish the inactive form (907 bp fragment) containing the insert in susceptible genotypes from the functional *Ph-3* form of resistant samples, which upon PCR amplification gives a 601 bp fragment, like other homologues of this gene. Since in the work of D.R. Panthee et al. [20] the L3708 sample which did not contain other homologues acted as a donor of the *Ph-3* gene; in the obtained stable forms, such homologues were also absent and a fragment of 601 bp in size. corresponded to the functional *Ph-3* gene.

Interestingly, D.R. Panthee et al. (20) also noted the presence of stable

forms which, according to the results of the analysis, had both 907 bp and 601 bp fragments, and, along with the *Ph-3* gene, beared the *Ph-2* gene. That is, the inactive form of the *Ph-3* gene can enter the tomato genome when other active *S. pimpinellifolium* resistance genes are introduced into it, since these genes are located in different chromosomes, are inherited unlinked, and are selected independently. It can be assumed that domestic breeders could use forms of *S. pimpinellifolium* containing the *Ph-3* gene with an insert as donors of resistance to late blight; therefore, in our experiments, the marker NC-LB-9-6678 did not work as a marker of resistance to late blight.

Thus, we have created a highly specific marker Ph3-412 of the tomato late blight resistance gene *Ph-3*. We also showed that in the tomato varieties of domestic breeding in the presence of the *Ph-3* gene, there are no other homologues of this gene. In the samples we analyzed, in which the *Ph-3* gene was found, there was a retrotransposon insert. The presence of such an insert can lead to a loss of functional activity, which must be taken into account when marking the *Ph-3* gene during marker-mediated selection for late blight resistance. Tomato forms in which the *Ph-3* gene does not have a retrotransposon insertion should be used as late blight resistance donors. The Ph3-412 marker we developed may be used both in identification of such donors when applied together with the NC-LB-9-6678 marker and in breeding programs.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 965-980
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Genetics and breeding

UDC 635.656:631.52:575.167

doi: 10.15389/agrobiology.2022.5.965eng
doi: 10.15389/agrobiology.2022.5.965rus

GENOTYPE-ENVIRONMENT INTERACTION AND STABILITY OF QUANTITATIVE TRAITS IN GARDEN PEA (*Pisum sativum* L.)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially from the National Science Foundation of Bulgaria (grant KP-06-N26/12)
Received May 4, 2022

Abstract

Peas are among the most common and widely cultivated annual legumes. Productivity potential of most modern pea varieties is high but limited by their low homeostasis and sensitivity to abiotic stress, i.e., the varieties tend to reduce adaptability. Therefore, one of the main challenge in pea breeding is to create an optimal genotype capable of realizing the biological potential and adequately responding to changes in growing conditions. Therefore, environmental testing remains relevant. This paper is the first assessment of the breeding samples of the pea working collection (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria) with respect to their ability to form economically significant quantitative traits. Three sources of variability (genotype, environment, and genotype-environment interaction) were found to be statistically significant for the total number of pods, the number of productive nodes with two pods per plant, pod weight, and grain weight. In 2018-2020, the phenotypic stability of ten pea (*Pisum sativum* L.) genotypes was assessed, including four perspective lines (22/16-af, 22/16-n, B4/34-n, and 1/17-n) and six varieties (Kazino-af, Plovdiv-n, Marsy-n, Echo-af, Shugar dwarf-n, and Vecherniza-n). The main examined quantitative traits were the number of pods per plant, the number of fertile nodes with one pod per plant, the number of fertile nodes with two pods per plant, pod length, pod width, pod weight per plant, and grain weight per plant. The effect of all factors of variation on the number of pods per plant, number of fertile nodes with two pods per plant, weight of pods per plant, and grain weight per plant is statistically significant. The strongest was the effect of the environmental factor on the manifestation of the number of pods per plant (52.20 %) and the number of fertile nodes with two pods per plant (59.00 %). The genotype factor has the largest contribution to the total variability of the weight of pods per plant (64.10 %) and grain weight per plant (67.40 %). Therefore, an effective breeding should be focusing on these traits regardless of the environmental conditions. The number of pods per plant and pod length requires more trials to give a more accurate estimate due to the superiority of the genotype×environment interaction variance over the genotype variance. Our findings indicate that the varieties Marsy-n and Echo-af are the most valuable genotypes for the number of pods per plant. The varieties Kazino-af, Plovdiv-n and the line 1/17-ob are highly variable and form fewer pods. For pod weight, all genotypes showed good responsiveness, especially Plovdiv-n (b_i = 2.68), 1/17-ob (b_i = 2.63), and Marsy-n (b_i = 2.18), all three having a higher pod weight, and the Echo-af variety shows better stability (b_i = 1.39; S_i^2 = 1.91). For the grain weight per plant, the Marsy-n (b_i = 3.08), 1/17-ob (b_i = 2.62), and Plovdiv-n (b_i = 4.02) are highly productive but also the most variable.

Keywords: phenotypic stability, genotype, environment, yield stability, ecological plasticity

Peas are among the most widespread and cultivated annual legumes. At the same time, legumes are almost the only source of vegetable protein, which

represents about 23-25% of the dry weight of pea seeds [1].

The problem of evaluating the adaptive properties of breeding material is usually solved by experiments that dissect the interaction between the genotype and the environment. As the degree of phenotypic manifestation of the genotype depends on the environment of development, conducting experimental ecological tests, both in time and space, is a real necessity. Data on the productivity of the samples are a reflection of the influence of agro-climatic conditions. These conditions can be much more contrasting and their effect on productivity is much greater than the behavior of the corresponding trait in the conditions of classical testing of varieties [2].

The varietal potential of agricultural crops is one of the main factors for the effective functioning of crop production. Most modern varieties of peas have a high productive potential, the realization of which is limited due to their low homeostasis and sensitivity to abiotic stress [3]. Modern pulse varieties of peas under biotic and abiotic stress (severe drought, excessive moisture, damage from enemies) form 55-72% lower seed weight compared to favorable conditions. In the breeding process there is a tendency to decreasing the adaptive properties of plants to environmental factors, which could become the main reason for reducing the cultivation of this crop [4-6]. In this regard, one of the main tasks facing breeders of this crop is to create an optimal genotype capable of realizing its biological potential and at the same time adequately responding to changes in growing conditions [7)]. The terms “plasticity” and “stability” are used to characterize the potential for modification and genotypic variability of individual traits and plant species. Plasticity, which reflects the variability of traits under different environmental conditions, as well as stability, are considered to be the main adaptive properties of living organisms [8]. The ecological plasticity of the genotype is the ability to stably reach the highest values of the heritable traits under examination in a wide area with sufficiently diverse meteorological conditions [9]. When evaluating varieties of cultivated plants for plasticity and stability of the trait some authors believe that genotypes with medium plasticity and high average trait under different environmental conditions are the best]10]. Others believe that the most promising are the most adaptive genotypes, which are least dependent on the environment and have a high stability of the trait. The third view is that the optimal variety should have a high overall adaptive potential, ensuring maximum yield in both favorable and unfavorable environments [11, 12].

This report presents for the first time the results of studying the ability to form economically significant quantitative traits in breeding accessions from the pea working collection (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria). Three statistically significant sources of variability, the genotype, environment, and genotype-environment interaction were identified for the total number of pods, the number of productive nodes with two pods per plant, the mass of pods, and the mass of grains per plant.

The aim of the study was to evaluate the phenotypic stability of quantitative traits related to productivity in pea genotypes.

Materials and methods. The study was conducted with a garden pea during two consecutive years 2018-2020 at Maritsa Vegetable Crop Research Institute - Plovdiv, Bulgaria. Ten garden pea genotypes from the collection of Maritsa Vegetable Crop Research Institute (Plovdiv, Bulgaria) were chosen as objectives of the present study. Three of them (line 2-22/16-af, 3-Kazino-af. and 5-Echo-af) had afila leaf type, while the other seven (line 1-22/16-n, 4-Plovdiv-n, 6-Marsy-n, 7-Shugar dwarf-n, 8-line B4/34-n, 9-line 1/17-n and 10-Vecherniza-n) possessed normal leaf type. Line 1-22/16-n and line 2-22/16-af were F_{10} generation of the cross Plovdiv × Kazino. 4-Plovdiv-n, 6-Marsy-n, 9-line 1/17-n and

10-Vecherniza-n were varieties developed at the Maritsa Vegetable Crop Research Institute, while 3-Kazino-a., 5-Echo-af, 7-Shugar dwarf-n and 8-line B4/34-n were received through non-cash exchange from the Institute of Plant Genetic Reassures in Sadovo, Bulgaria.

Seeds of the ten genotypes were sown in the field in the second half of March on a high flatbed by scheme 80 + 20 + 40 + 20/4-5 cm (high 4-row bed, 160 cm width). The seeds were planted in two couples of double rows 40 cm apart. The distance between the seeds in the row was 4-5 cm, and the distance between the rows in the couple was 20 cm. The experiments were laid out in a randomized complete block design with three replicates. Plot size was 1.6×4.0 m with 20 seeds in a metre in a row.

The following quantitative features were considered: number of pods per plant (NPP); number of fertile nodes with one pod per plant (NFN-1); number of fertile nodes with 2 pods per plant (NFN-2); pod length (PL), cm; pod width (PW), cm; total weight of pods per plant (WPP), g; grain weight per plant (WGP), g.

The data obtained were processed by two-way analysis of variance ((two-way ANOVA) for each trait to determine the effects of genotypes (G), environment (E) and the genotype-environment interaction (G×E). The assessment of the ecological stability was performed by applying regression analysis according to S.A. Eberhart and W.A. Russel [10] and G.C.C. Tai [13], in which the regression coefficient (b_i, a_i) and the variance of the regression deviations (Sd^2_i, λ_i) were calculated. The stability parameter (Di) of W.D. Hanson [14] was calculated, which uses the minimum slope of the regression line by the method of K.W. Finlay and G.N. Wilkinson [12]. Analysis of variance was applied to assess average dispersion component (θi) according to R.I. Plaisted and L.C. Peterson [15]; ecovalence (W_2) was estimated by G. Wricke [16] and P. Annicchiarico [17] methodw. The P. Annicchiarico method offers a reliability index (Wi), which estimates the probability that a genotype (variety) will perform lower than the average for the environment or below any standard used. In the nonparametric analysis, the parameter Pi according to the model of C.S. Lin и M.R. Binn [18] and ranking (R) of the samples by adaptability (A) according to the methods of M. Nascimento et al. [19] and M. Huehn [20, 21]. A GGE biplot model was fitted, which uses singular value decomposition of first two principal components [22]. All experimental data were statistically processed using the computer software GENES 2009.7.0 for Windows XP as described [23]. Means (*M*) and standard deviations (±SD) are shown. Differences between the means were assessed by Student's *t*-test and considered statistically significant at $p < 0.05$

Results. Table 1 submeets the main characteristics of the studied genotypes.

1. Basic information about the pea (*Pisum sativum* L.) samples included in the experiment
(*M*±SD, Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020)

Genotype	NPP	NFN-1	NFN-2	PL	PW	WPP	WGP
22/16-ob	11,59±1,30^{ab}	2,45±0,55^{ab}	4,56±0,43^{bc}	7,30±0,19^{de}	1,17±0,21^{bc}	36,66±0,70^{a}	14,23±0,97^{a}
22/16-af	10,00±1,12^{ab}	2,92±0,66^{ab}	3,61±0,51^{ab}	6,69±0,22^{bc}	1,01±0,25^{a}	26,23±0,84^{a}	10,36±0,97^{a}
Kazino-af	11,00±1,23^{ab}	2,79±0,6^{ab} 3	4,24±0,49^{abc}	6,56±0,21^{bc}	1,03±0,24^{ab}	30,89±0,80^{a}	12,72±0,93^{a}
Plovdiv-n	10,93±1,23^{ab}	3,62±0,82^{b}	3,65±0,64^{ab}	6,053±0,27^{a}	1,03±0,31^{ab}	32,20±1,04^{a}	15,32±1,20^{a}
Echo-af	12,00±1,35^{ab}	2,24±0,51^{a}	4,94±0,39^{bc}	6,09±0,17^{ab}	0,93±0,19^{a}	25,10±0,64^{a}	12,33±0,74^{a}
Marsy-n	13,69±1,54^{b}	2,95±0,67^{ab}	5,40±0,52^{c}	7,78±0,22^{e}	1,05±0,25^{ab}	56,48±0,85^{b}	26,27±0,98^{b}
Shugar dwarf-n	12,00±1,35^{ab}	3,62±0,82^{b}	4,42±0,64^{abc}	6,66±0,27^{abc}	1,22±0,31^{c}	31,49±1,04^{a}	13,14±1,20^{a}
B4-34-n	12,00±1,35^{ab}	2,59±0,59^{ab}	4,67±0,46^{bc}	6,88±0,20^{cd}	1,17±0,22^{bc}	30,59±0,74^{a}	10,60±0,86^{a}
1/17-ob	11,00±1,23^{ab}	1,78±0,40^{a}	3,01±0,31^{a}	6,74±0,13^{d}	1,05±0,15^{ab}	30,64±0,51^{a}	14,83±0,59^{a}
Vechernitza-n	9,00±1,01^{a}	2,05±0,46^{a}	3,55±0,36^{ab}	6,61±0,16^{abc}	0,97±0,17^{a}	23,66±0,59^{a}	10,53±0,68^{a}
Mean±SD	11,32±1,27	2,70±0,61	4,20±0,74	6,74±0,51	1,06±0,09	32,39±9,28	14,03±4,66

Note. NPP means the number of pods per plant; NFN-1 means the number of fertile nodes with one pod per plant; NFN-2 means the number of fertile nodes with 2 pods per plant; PL means pod length, cm; PW means pod width, cm; WPP means total weight of pods per plant, g; WGP means grain weight per plant, g.
^{abcd} Different letters mean statistically significant differences at $p < 0.05$.

Agrometeorological conditions for the study period are represented by the average daily air temperature and the amount of precipitation (Table 2). The average amount of precipitation is characterized by a pronounced maximum in April 2020 (76.0 l/m^2) and especially in the third ten days of June 2018, as well as the first ten days of June 2019 (125 and 108 l/m^2, respectively). The average daily temperature in March, April, May and June ranges from 4.6 °C to 24.8 °C. The lowest air temperature was recorded in March 2018 and in April and May 2020. The month of May is characterized by lower temperatures in 2019 and 2020, when stronger deviations are observed, while in 2018 they are relatively constant. The average values of meteorological factors have shown a favorable combination with each other for 2020, which had a positive effect on plant development.

2. Characteristics of meteorological elements in different months during the vegetation period (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria)

Decade/10-day period/month	Average temperature for ten days, °C			Rainfall for ten days, l/m^2		
	2018	2019	2020	2018	2019	2020
I/1-10/03	4.62	11.48	10.02	8.00	1.00	21.00
II/11-20/03	10.71	11.49	9.57	15.00	8.00	21.00
III/21-30/03	6.75	10.53	8.22	42.00	0.00	61.00
I/1-10/04	14.04	10.89	7.88	18.00	45.00	76.00
II/11-20/04	16.05	11.86	13.97	2.00	35.00	16.00
III/21-30/04	18.32	14.67	12.75	1.00	8.00	25.00
I/1-10/05	18.92	15.26	15.54	2.00	2.00	14.00
II/11-20/05	19.64	17.71	21.97	9.00	9.00	0.00
III/21-30/05	19.35	20.99	15.66	21.00	59.00	24.00
I/1-10/06	23.80	20.86	20.02	3.00	108.00	15.00
II/11-20/06	24.10	24.83	21.04	10.00	11.00	34.00
III/21-30/06	21.00	24.03	23.29	125.00	41.00	2.00

3. Mean squares (MS) **from the two-way analysis of the variance of 10 samples of peas (*Pisum sativum* L.) for seven traits** (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020)

Source of variation	df	MS						
		NPP	NFN-1	NFN-2	PL	PW	WPP	WGP
Environment (E)	2	33.3969***	14.3263***	13.3668**	1.9813	0.2212	241.9320***	43.0120***
Genotypt (G)	9	13.3116***	3.3788*	4.8911**	2.379*	0.0786	775.2542***	195.0155***
G×E	18	17.2714***	1.6588	4.39401*	6.8851	0.3845	192.5514***	51.3245***
E/G	20	18.884***	2.9256*	3.5337	0.5424	0.0413	197.4895***	50.4933***
E/G-1	2	6.3693*	2.2789	7.8776*	0.6962	0.0728	167.8477***	58.0156***
E/G-2	2	14.3731***	0.9139	6.365	1.8486	0.0152	73.3264***	5.7927**
E/G-3	2	43.9108***	0.5971	1.73642**	0.0648	0.0386	315.4953***	48.1701***
E/G-4	2	47.0667***	3.5308	1.44234**	2.1878	0.0294	550.1775***	192.2619***
E/G-5	2	3.6688*	5.7229	8.5688*	0.0338	0.0224	50.2644***	22.8852***
E/G-6	2	3.0963	3.4624	3.7374	2.2838	0.1226	118.9497***	56.0016***
E/G-7	2	6.1129*	2.0293	2.0486	2.8334	0.1568	221.9331***	49.0267***
E/G-8	2	10.9084***	3.1261	4.2422	0.2178	0.3042	160.1757***	33.3229***
E/G-9	2	50.1025***	3.7525	5.1368	0.5432	0.0234	237.2773***	33.8647***
E/G-10	2	3.2311	3.8416	0.9098	0.1382	0.0416	79.4469***	5.5911**
Residual	29							

N p t e. G-1 — 22/16-ob, G-2 — 22/16-af, G-3 — Kazino-af, G-4 — Plovdiv-n, G-5 — Echo-af, G-6 — Marsy-n, G-7 — Shugar dwarf-n, G-8 — B4-34-n, G-9 — 1/17-ob, G-10 — Vechernitza-n; NPP means the number of pods per plant; NFN-1 means the number of fertile nodes with one pod per plant; NFN-2 means the number of fertile nodes with 2 pods per plant; PL means pod length; PW means pod width; WPP means total weight of pods per plant; WGP means grain weight per plant.

*, **, *** The influence of the factor is statistically significant at p = 0.1, p = 0.05 and p = 0.01, respectively,

A N O V A. The results of the two-factor analysis of variance (Table 3) show that there are significant differences between the pea samples on almost all traits except pod width (PW). The genotypic differences were found insignificant for this triat. The influence of the environment was statistically reliable for the following parameters: total number of pods is reliable; number of fertile nodes

with one pod per plant; number of fertile nodes with 2 pods per plant; pod weight and grain weight per plant. According to the analysis of the variance, the factor genotype×environment interaction has a significant influence on the total number of pods, number of fertile nodes with 2 pods per plant, pod weight and grain weight per plant.

The values of the sum of the squares (SS) of the traits analysis were used to determine the contribution of each source of variation in the total variability. The variation of the indicators total number of pods (52.20%) and number of fertile nodes with 2 pods per plant (59.00%) is mostly due to the environment, and the influence of genotype and genotype-environment interaction is significantly less (Fig. 1). The largest contribution of the total variability of the traits pod weight (64.10%), grain weight (67.40%) is due to the genotype factor. Therefore, an effective breeding can be done for these traits, regardless of the environmental conditions. The part of the total variation due to the genotype-environment interaction is greater than that resulting from the influence of the genotype factor on the traits total number of pods and pod length (PL), taking into account the insignificance of the genotype-environment factor for the second indicator. The obtained results show that longer-term studies are needed to establish the ecological stability of these traits. Statistically significant effect of all three factors of variation of such traits as total number of pods; number of fertile nodes with 2 pods per plant; pod weight and grain weight is a prerequisite for determining their stability during the study period.

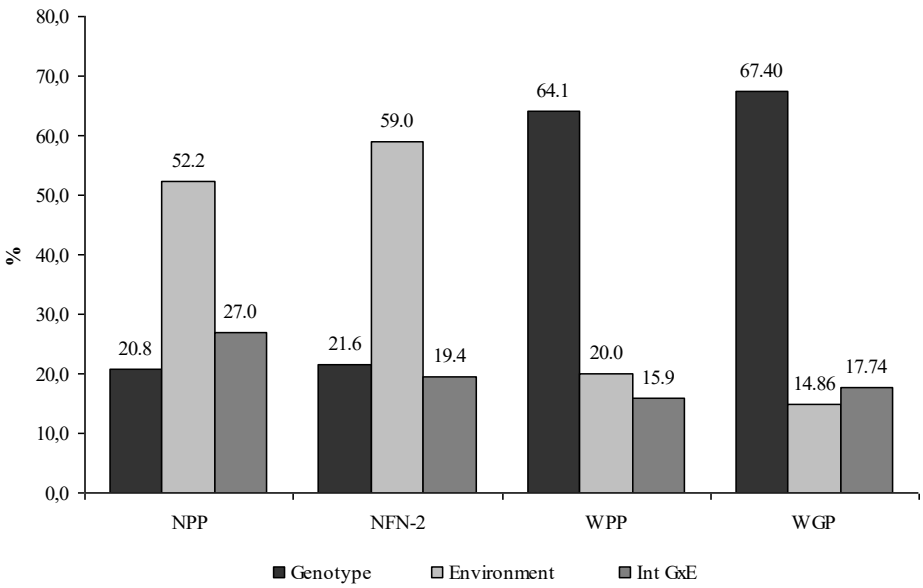

Fig. 1. Percentage impact of the factors genotype, environment and genotype×environment interaction on the general variation of the studied traits in 10 samples of peas (*Pisum sativum* L.). NPP means the number of pods per plant; NFN-2 means the number of fertile nodes with 2 pods per plant; WPP means total weight of pods per plant; WGP means grain weight per plant (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020). For a list of samples, see the Materials and methods section.

Stability parameters. The indicators of S.A. Eberhart и W.A. Russel [10] и G.C.C. Tai [13] for ecological plasticity and stability allow assessing the adaptability of the samples to the specific conditions of the growing environment. The plasticity of the genotypes is calculated by the coefficients bi and ai, and their stability according to the variance of the stability of the trait (S_i^2 and λ_i).

4. Phenotypic stability of the main productivity traits in the studied pea (*Pisum sativum* L.) samples based on regression analysus (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020)

Sample	S.A. Eberhart и W.A. Russel [10]		G.C.C. Tai [13]		S.A. Eberhart и W.A. Russel [10]		G.C.C. Tai [13]		S.A. Eberhart и W.A. Russel [10]		G.C.C. Tai [13]		S.A. Eberhart и W.A. Russel [10]		G.C.C. Tai [13]	
	b_i	S_i^2	a_i	λ_i	b_i	S_i^2	a_i	λ_i	b_i	S_i^2	a_i	λ_i	b_i	S_i^2	a_i	λ_i
	NPP				NFN-2				WPP				WGP			
22/16-ob	1.25	0.44	1.26	1.28	1.64	–0.10	1.69	0.33	0.61	105.52**	0.61	176.41	0.46	37.72***	0.45	63.40
22/16-af	2 .068	0.27	2.10	–0.03	1.47	–0.13	1.50	0.32	1.61*	6.51**	1 .61	11.38	1.03	0.49	1.03	1.37
Kazino-af	1.13	26.10**	1.13	44.06	0.74	4.96**	0.72	8.82	1.82**	156.14**	1.83	260.73	1.40	26.16***	1.41	44.14
Plovdiv-n	2.96***	11.44**	3.03	19.19	2.05	0.71	2.14	1.61	2.68**	250.25**	2.69	417.33	4.02***	81.33***	4.10	135.07
Echo-af	0.52	1.52*	0.50	3.06	1.32	0.96	1.35	2.14	1.39	1.91*	1.39	3.73	1.23	10.58***	1.24	18.19
Marsy-n	0.17	1.66*	0.15	3.25	0.96	0.09	0.96	0.71	2.18**	1.76*	2. 19	3.34	3.08***	9.69***	3.14	16.21
Shugar dwarf-n	–1.02	1.43*	–1.08	2.48	–0.84	–0.28	–0.99	–0.32	–1.29**	120.61**	–1.30	200.99	–2.49***	14.47***	–2.58	23.28
B4-34-n	0.40	6.58**	0.38	11.49	0.88	0.39	0.87	1.21	–2.44**	9.98**	–2.46	15.87	–2.45***	4.62**	–2.54	6.91
1/17-ob	2.51	19.05**	2.55	32.06	1.35	–0.25	1.38	0.12	2.63**	46.06**	2.64	77.04	2.62**	2.41**	2.67	4.28
Vechernitza-n	0.01	1.82*	–0.02	3.48	0.42	–0.19	0.38	0.20	0.79	42.58**	0.79	71.52	1.08	0.02	1.09	0.59

N o t e. NPP means the number of pods per plant; NFN-1 means the number of fertile nodes with one pod per plant; NFN-2 means the number of fertile nodes with 2 pods per plant; PL means pod length; PW means pod width; WPP means total weight of pods per plant; WGP means grain weight per plant.

*, ** Coefficients are statistically significant at p = 0.05 and p = 0.01, respectively.

5. Phenotypic stability of the main productivity traits in the studied pea (*Pisum sativum* L.) samples based on dispersion analysus (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020)

Образец	R.I. Plaisted и L.C. Peterson [15]	G. Wricke [16]	P. Annic-chiarico [17]	R.I. Plaisted и L.C. Peterson [15]	G. Wricke [16]	P. Annic-chiarico [17]	R.I. Plaisted и L.C. Peterson [15]	G. Wricke [16]	P. Annic-chiarico [17]	R.I. Plaisted и L.C. Peterson [15]	G. Wricke [16)]	P. Annic-chiarico [17]
	PP	W^2	W_i	PP	W^2	W_i	PP	W^2	W_i	PP	W^2	W_i
	NPP			NFN-2			WPP			WGP		
22/16-ob	2.80	2.73	97.85	0.24	1.78	99.45	61.84	324.86	98.72	19.02	116.66	81.39
22/16-af	3.27	7.80	80.63	0.18	1.20	75.83	35.35	38.81	74.40	8.45	2.47	70.22
Kazino-af	9.90	79.42	78.41	1.56	16.07	77.17	78.29	502.56	76.65	15.71	80.86	73.38
Plovdiv-n	8.21	61.18	76.09	0.64	6.11	67.37	114.07	888.99	73.53	38.21	323.84	73.52
Echo-af	3.21	7.13	100.43	0.46	4.16	105.03	33.07	14.18	72.81	11.30	33.21	76.72
Marsy-n	3.52	10.58	111.79	0.19	1.27	120.89	38.66	74.56	170.71	14.47	67.51	173.84
Shugar dwarf-n	5.56	32.51	93.14	0.93	9.22	85.76	88.93	617.48	77.17	22.07	149.62	71.21
B4-34-n	4.69	23.20	94.28	0.28	2.22	102.07	87.81	605.40	74.72	19.10	117.45	56.30
1/17-ob	9.34	73.38	70.46	0.13	0.58	62.26	56.59	268.17	79.66	11.10	31.09	93.85
Vechernitza-n	3.75	12.96	74.94	0.20	1.31	78.71	43.88	130.90	63.76	8.33	1.13	72.07

N 0 t e. NPP means the number of pods per plant; NFN-2 means the number of fertile nodes with 2 pods per plant; WPP means total weight of pods per plant; WGP means grain weight per plant.

According to the data in Table 1, the variety Marsy-n forms the largest number of pods per plant, approx. 13-14. According to the methods of S.A. Eberhart и W.A. Russel [10] и G.C.C. Tai (13), it is difficult to interpret the plasticity of the number of pods, given the significance of the regression coefficient only for the variety Plovdiv (b_i = 2.96) (Table 4). The low values of the S_i^2 parameter for Shugar dwarf, Echo-af and Marsy-n characterized them as ecologically stable. Three varieties (Vechernitza (S_i^2 = 1.82). Kazino-af, 1/17-ob and Plovdiv), which form a number of pods below the average for the test group, can be referred to as the group of highly variable and unstable genotypes. Most of the variance-based parameters such as PP [15] W^2 [16] define 22/16-ob as the genotype with the highest ecological stability of the number of pods, followed by Echo-af (Table 5).

The situation is similar for the values of the parameters based on the regression analysis for the number of fertile nodes with 2 pods per plant (see Table 4). Therefore, other methodological approaches may be applied to characterize the stability of this trait. E.g., the stability parameters of R.I. Plaisted and L.C. Peterson [15] and G. Wrike [16) give preference to the 1/17-ob line (PP = 0.13; W^2 = 0.58), which forms a negligible number of fertile nodes with 2 pods per plant. However, the G. Annicchiarico' index [17] rated 22/16-ob (W_i = 99.45), Echo-af (W_i = 105.03) and B4-34 (W_i = 102.07) as the highest (Table 5). The PP and W^2 stability parameters confirm the approximate conclusion that can be drawn from the model of S.A. Eberhart and W.A. Russel [10]. In most cases, the coefficients bi of linear regression are positive, but in others they may have negative values due to causes of another nature (such as disease and pest infestation, a significant percentage of lodged plants). These reduce the coefficient values for the respective trait and lead to incorrectly formulated conclusions. The Shugar duarf variety is in such a situation for all four traits iassessed for the ecological stability.

The results obtained (see Table 4) for the reaction of samples of peas on the basis of the pods weight when changing the growing environment showed that a significant part of them had a very good responsiveness. In a favorable environment, these plants can be expected to form heavier pods. The varieties Plovdiv (b_i = 2.68), 1/17-ob (b_i = 2.63) and Marsy-n (b_i = 2.18) are the most plastic, which is characterized by the heaviest pods (56.48 g) (see Table 1) with statistically significant difference. The stability criterion S_i^2 indicates that Echo-af, Marsy-n and 22/16-af are relatively more stable than the other samples.

In the studied collection of pea specimens, according to the criteria presented in Table 5 afila type line 22/16-af and variety Echo-af have the smallest dispersion. The W_i [17] categorically ranks Marsy-n as the most unstable (W_i = 170.71), which from a breeding point of view is of interest due to the greater pods weight compared to other genotypes.

In terms of plant grain weight, it is noteworthy that high grain yields were characterized by the regression coefficient as the most variable with values of b_i = 2.62 (1/17-ob), b_i = 3.08 (Marsy-n), and b_i =4.02 (Plovdiv-n) (see Table 4). It can be assumed that Vechernitza-n and 22/16-af are close in stability and responsiveness to the ideal genotype with a b_i coefficient close to 1 and with a lower variance of the regression deviations. These specimens cannot take advantage of this because they occupy the lowest positions with respect to seed weight. Their parameters of stability and plasticity are nonsignificant and therefore their reaction to different environmental limits is unpredictable. The stability parameters PP and W^2 (see Table 5) showed that in the varieties Plovdiv-n and Shugar dwarf-n, the grain weight is very sensitive when the environmental conditions change. The assessment of these indicators as well as of W_i for the stability of the trait for 22/16-af and Vechernitza-n is unambiguous.

Table 6 report alternative approaches to assess the behavior of specimens

grown in different environments. The rank analysis by the method of M. Huehn [20] makes it possible to assess the stability of certain genotypes in response to changes in the environment on the basis of their classification in different growing conditions. Line 22/16-ob ranked lowest (R = 2) in total number of pods, followed by 22/16-af, Echo-af, Marsy-n, and Vechernitza-n in second position (R = 4). According to the number of productive nodes with 2 pods per plant, only the 1/17-ob line has R = 3, and it is unpromising for this trait. Of the following samples in the ranking, the variety Marsy-n is of interest. Therefore, for breeding peas for an increased weight of the pod (with a sufficiently stable manifestation of the trait), we can recommend the variety Marsy-n and the line 22/16-ob, for the increased weight of grains per plant Marsy-n and the line 1/17-ob.

6. Phenotypic stability of the main productivity traits in the studied pea (*Pisum sativum* L.) samples based on non-parametric indicators (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020)

Sample	R, M. Huehn [20]				P_i, C.S. Lin и M.R. Binns [18]			
	NPP	NFN-2	WPP	WGP	NPP	NFN-2	WPP	WGP
22/16-ob	2	5	5	8	5.79	0.94	225.91	90.25
22/16-af	4	4	4	2	11.64	2.56	458.54	129.43
Kazino-af	7	7	7	6	10.18	2.13	348.16	93.81
Plovdiv-n	8	7	8	9	11.82	2.79	345.17	85.19
Echo-af	4	7	2	5	4.05	0.61	494.05	98.81
Marsy-n	4	4	4	6	1.56	0.18	391.31	103.86
Shugar dwarf-n	7	9	7	7	5.75	1.53	360.03	101.08
B4-34-n	6	5	8	7	5.90	0.83	396.39	142.23
1/17-ob	9	3	7	4	13.05	4.09	339.09	65.87
Vechernitza-n	4	4	4	2	16.08	2.71	554.49	128.14

Note. NPP means the number of pods per plant; NFN-2 means the number of fertile nodes with 2 pods per plant; WPP means total weight of pods per plant; WGP means grain weight per plant.

The P_i parameter by C.S. Lin and M.R. Binns [18] gives preference to the genotype with the lowest index. In terms of the total number of pods and number of fertile nodes with 2 pods per plant, the Echo-af and Marsy-n varieties, both also having high values of these traits, occupy the first two places by the stability. On the number of fertile nodes with 2 pods per plant, the B4-34 and 22/16-ob lines take the leading places. The index Pi for weight of pods and weight of grains gives priority to the lines 1/17-ob and 22/16-ob, as well as the variety Plovdiv-n.

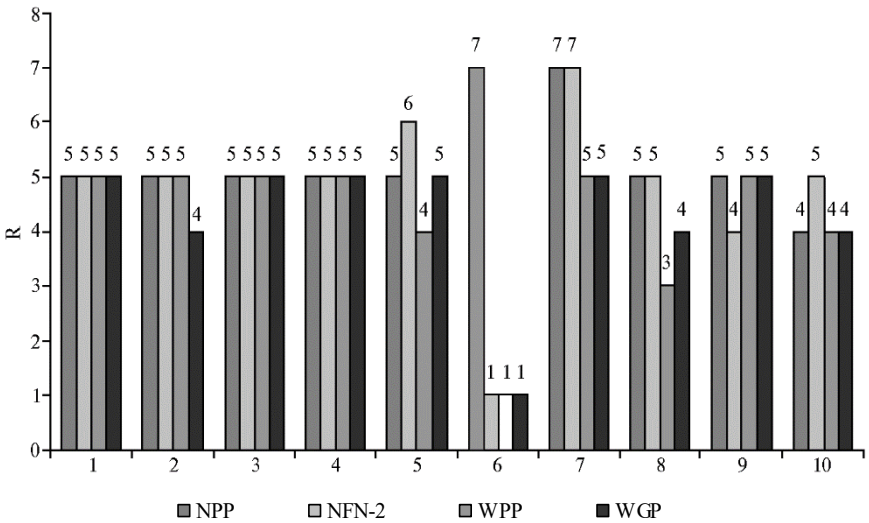

Fig. 2. Nonparametric rank analysis of NPP (the number of pods per plant)**, NFN-2** (the number of fertile nodes with 2 pods per plant)**, WPP** (total weight of pods per plant)**, and WGP** (grain weight per plant) **by M. Nascimento et al.** [19] **in the studied pea (*Pisum sativum* L.) samples:** 1 — 22/16-ob, 2 — 22/16-af, 3 — Kazino-af, 4 — Plovdiv-n, 5 — Echo-af, 6 — Marsy-n, 7 — Shugar dwarf-n, 8 —

B4-34-n, 9 — 1/17-ob, 10 — Vechernitza-n. For R ranks: 1 — high general adaptability; 2 — adaptability under favorable conditions; 3 — adaptability under unfavorable conditions; 4 — low adaptability; 5 — average general adaptability; 6 — adaptability under average favorable conditions; 7 — adaptability under average unfavorable conditions (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020).

According to the centroid classification method of M. Nascimento et al. [19] and the information obtained from Figure 2, a significant proportion of the pea samples showed average overall adaptability (R = 5) with respect to the total number of pods trait. Variety Vechernitza-n (R = 4) does not adapt well not only to this trait, but also to the weight of the pods and the weight of the grains of the plant. Marsy-n and Shugar dwarf-n perform relatively well in the adverse conditions. The Marsy-n variety is characterized by high overall adaptability (R = 1) on the other three grounds by the number of pods, number of fertile nodes with 2 pods per plant, the weight of the pods and the weight of the grains of a plant. Line B4-34 can be relied on to form sufficiently heavy pods (R = 3) when conditions are unfavorable, but not to feed heavy grains from a single plant. Some of the samples during the study period show average overall adaptability to the analyzed traits.

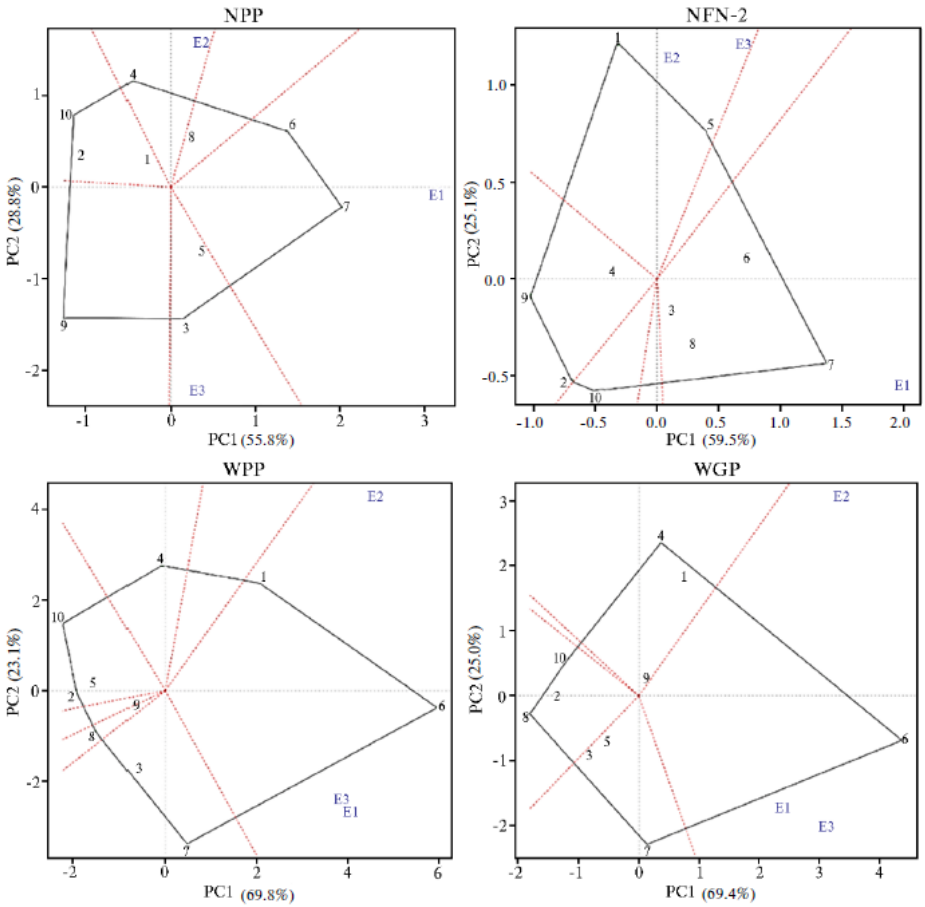

Рис. 3. Graphic of GGE biplot analysis of NPP (the number of pods per plant)**, NFN-2** (the number of fertile nodes with 2 pods per plant)**, WPP** (total weight of pods per plant)**, and WGP** (grain weight per plant) **traints in the studied pea (*Pisum sativum* L.) samples::** 1 — 22/16-ob, 2 — 22/16-af, 3 — Kazino-af, 4 — Plovdiv-n, 5 — Echo-af, 6 — Marsy-n, 7 — Shugar dwarf-n, 8 — B4-34-n, 9 — 1/17-ob, 10 — Vechernitza-n; E1, E2 and E3 — environmental conditions in 2018, 2019 and 2020, respectively (Maritsa Vegetable Crop Research Institute, Plovdiv, Bulgaria, 2018-2020).

GGE biplot analysis. GGE biplot is a complex analysis system designed to show most aspects of the genotype-environment interaction graphically.

The result of the experiment is presented in such a way that the visual evaluation of the samples and the identification of the "mega-environment" are significantly simplified. Only two principal components (PC1 and PC2) are preserved in the graphical model (Fig. 3), as this is the most appropriate way to establish the main patterns and to eliminate unnecessary data. The first two principal components can be plotted on 2D graphics so that the interaction between each genotype and the specific breeding environment can be easily interpreted on the biplot.

GGE biplot analysis for total pod number showed that the first twoprincipal components explained 84.6% of the total variability caused by the genotype-environment interaction. The Marsy-n and Shugar dwarf-n varieties can feed the largest number of pods in the E1 environment (2018), and the Plovdiv-n variety would give the best result in the E2 environment (2019). For Kazino-af and line 1/17-ob, the most suitable environment for the realization of a larger number of pods is E3 (2020), which is the most favorable for plant development compared to the rest of the study period (see Fig. 3).

On the basis of the number of fertile nodes with 2 pods per plant, a polygon is again formed, at the tops of which are the projections of the samples, which have an advantage in a certain environment (or group of environments). Line 22/16-ob and variety Echo-af thrive best in environments E2 (2019) and E3 (2020). Shugar dwarf-n, which as the previous genotype has a level of trait above the average for the studied sample, is positively affected by the climatic conditions of the environment E1 (2019). Genotypes Plovdiv-n, 1/17-ob, Vechernitza-n and 22/16-af are in the sectors without a specific environment and therefore in terms of adaptability of this trait they are inferior to the others (see Fig. 3).

The vertices of the polygon, graphically representing the behavior of the samples in the environment by the weight of the pods per plant, consist of the genotypes Plovdiv-n, occupying the top of the polygon, Vechernitza-n, 22/16-af. and 8-B4-34 located on the left, Shugar dwarf-n with a projection at the top, located at the bottom position and 22/16-ob and Marsy-n, which are on the right side of the polygon. The last two genotypes, especially Marsy-n, manage to form and feed more pods than the others. The Marsy-n variety is able to more fully realize this quality in the nearby first and third environments (E1, E3), while 22/16-ob develops better in E2 environments.

From the GGE biplot analysis of grain weight presented in Figure 3, some similarity was observed, both in the location of the samples and in the environment. It can be seen that in the polygon thus formed, the genotypes of peas are divided into five sectors. The Plovdiv-n, B4-34, Shugar dwarf-n and Marsy-n specimens are located at the tops of the landfill. The Marsy-n variety occupies an extreme right position, defined by a quadrant with a positive value of PC1 and a negative value of PC1, but not very far from the abscissa. This situation is due to the strong superiority of this variety over the others in the total weight of the grains. Among E1 and E3 are located very close to each other and form a mega environment. Environment E2 is in the same sector, but is located at its opposite end.

Threrof, in the study, the three sources of variation, i.e. genotype, environment and genotype-environment interaction, were found statistically significant for the total number of pods, number of fertile nodes with 2 pods per plant, pods weight and grain weight. Similar results in the same crop were previously reported [24, 25], especially with regard to the significance for the seed productivity indicator per plant.

Similar results were obtained in the study of chickpea genotypes [26]. The analysis of variance for pod length showed that more than 60% of the total trait

variation was due to the influence of the growing environment, followed by the genotype-environment interaction. The genotype factor had the least influence on the manifestation of the trait. The findings of A.K. Mukherjee et al. [27] when testing rice varieties are in agreement with those from the study. The authors found that for a small part of the traits the greater sum of the squares for the genotype factor was obtained, from which it can be concluded that the studied samples of them differ significantly in their genetic talents. The influence of other factors of variation was weaker, especially for the genotype-environment interaction. Applying the method of S.A. Eberhart and W.A. Russel [10] to assess the phenotypic stability of quantitative traits in peas, C. Rana et al. [28] obtained similar results and reported statistical significance of the genotype-environment factor for the pods weight and plant seeds. When evaluating genotypes of Pannonian vetch [29] and garden peas [30] the authors report that samples with low seed productivity usually have high trait stability and specific adaptation to different growing conditions. The results in the present study support these findings. Based on the results of their practical experience with peas, Y. Goa and H. Mohammed [31] believe that the most appropriate and desirable genotype is one that combines high productivity and relatively good stability among the tested samples. According to the authors, the most highly productive genotypes are ecologically unstable with negative changes in environmental limits, but responsive under favorable environmental conditions [31]. Their statement is in line with the results of our study.

Our results on the stability of pea varieties with different leaf morphology are in agreement with previous similar studies, such as those of E. Acikgoz et al. [32]. The authors report that genotypes with afila leaf type type of leaves on the basis of plant seed weight are more stable than leaf forms, but the latter are more productive and are therefore preferred for cultivation in different environments.

In our study, a small number of pea samples combined high value and adequate stability of the respective trait. In other crops, such a pattern has also been established. In cowpea, T. Simion et al. [33] reported that a small number of genotypes analyzed by the main quantitative traits showed stability and high expression of the trait. The authors suggested that these genotypes would respond proportionally to changes in the rearing environment.

Y. Rezene et al. [34] in their work with peas reported that the GGE biplot analysis provided additional information about the studied varieties and their future practical use, which is confirmed in our study. In recent years, the GGE biplot technique has been widely used to study the genotype-environment interaction and stability in other crops such as soybeans [35], cowpea [36], chickpeas [37] and barley [38]. O. Sozena et al. [39] recommended that different methods and analyzes should be used to assess the phenotypic stability of the traits in order to obtain a more complete and accurate information about the studied plants. The results of their study show that parametric stability tests are appropriate and reliable.

Thus, we found the statistically significant influence of all factors of variation on such traits as total number of pods, number of fertile nodes with 2 pods per plant, pod weight and grain weight. The strongest effect of the environmental factor was observed for the total number of pods (52.20%) and the number of fertile nodes with 2 pods per plant (59.00%). The genotype factor has the largest part of the total variability of the weight of pods per plant (64.10%) and grain weight per plant (67.40%) traits. Therefore, an effective breeding can be done for improvement of these traits regardless of the environmental conditions. The number of pods per plant and pod length requires longer trials to obtain a more realistic estimate due to the superiority of the genotype×environment interaction variance over the genotype variance. Several stability parameters were calculated for each

trait. The varieties Marsy-n and Echo-af have been identified as the most valuable genotypes for the number of pods per plant. Kazino-af, 1/17-ob and Plovdiv-n are highly variable and with a smaller number of pods. For pod weight, genotypes showed good responsiveness, especially Plovdiv-n (b_i = 2.68), 1/17-ob (b_i = 2.63) and Marsy-n (b_i = 2.18), which have a higher pod weight, while the Echo-af variety has better stability (b_i = 1.39; S_i^2 = 1.91). For the grain weight per plant, the highly productive samples were the Marsy-n (b_i = 3.08), 1/17-ob (b_i = 2.62), Plovdiv-n (b_i = 4.02), but these appeared most variable as well. Vechernitza-n and 22/16-af are close to the ideal genotype with regard to the stability, but are low-yielding.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 981-991
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 634.13:631.527

doi: 10.15389/agrobiology.2022.5.981eng
doi: 10.15389/agrobiology.2022.5.981rus

CREATION OF HIGH-QUALITY VARIETIES OF COMMON PEAR (*Pyrus communis* L.) IN THE LOWER VOLGA REGION

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The authors declare no conflict of interests

Acknowledgements:
The work was carried out within the framework of state task No. 122020100448-6 "Creation of new competitive forms, varieties and hybrids of cultivated, woody and shrubby plants with high productivity, quality and increased resistance to adverse environmental factors, new innovative technologies in seed production and nursery with taking into account varietal characteristics and soil-climatic conditions of arid territories of the Russian Federation".
Received June 22, 2022

Abstract

Nowadays, the creation of high-quality pear varieties of different ripening behavior with early-, mid-, and late-season maturing is still relevant goal for breeders of the Lower Volga region. The long-term pome fruit breeding program, including pear breeding, is carried out in the Laboratory of breeding, seed production and nursery of the Federal Scientific Center for Agroecology, Land Reclamation and Protective Afforestation RAS. The parent forms involved in intervarietal and interspecific hybridization and targeted selection are mainly European varieties of high quality but low resistance to adverse climatic factors and local varieties of high resistance but poor fruit quality. As a result, new pears varieties were released, e.g., Dubovskaya rannyayya, Zimnyaya kubarevidnaya, Yubiley Korneeva, Nadazhda, Andreevskaya, Doktosxaya, Ordinata, Lavanda, Omega, Pamyat' Korneeva, Kapella, Raketa, Positivnaya, Fermata, Banketnaya, Versiya, Nectarnaya, etc. Fully ripened fruits were collected in certain parts of the tree (early-ripening varieties) or at storage sites (late-ripening varieties) to determine quality parameters. Fruits were weighed on a laboratory scale and measured with a ruler to determine the average and maximum fruit weigh and size. Fruit appearance was assessed visually on a five-point scor, taste — by tastings carried out as the fruit ripened, on a five-point scor. The juice concentration of soluble solids was determined refractometrically. The total acidity was assessed by titration of an aqueous extract with 0.1 N alkali solution, sugar content (sum of sugars, mono-, disaccharides) was estimated by Bertrand's method, pectin by carbazole method based on the interaction of carbazole with D-galacturonic acid, the vitamin C content by titration of oxalate extracts with Tillman's reagent (2,6-dichlorophenolindophenol). It was established that the largest fruits (250 g and more) were formed by the varieties Pamyat' Korneeva, Pozitivnaya, Kapella and Banketnaya. Since this trait is inherited from the parental large-fruited Olivier de Serre and Bakhmal cultivars, the new varieties, in addition to high commercial attractiveness, can be successfully used as sources in the creation of new large-fruited pear varieties of different ripening terms. The varieties Pamyat' Korneeva, Nektar, and Fermata with excellent dessert balanced taste (4.7-5 points) and attractive fruit appearance surpass standard varieties in these traits. The genetic sources of the high taste quality of these varieties were the ancestral cultivars Alexandrine Dulyar and Bere Gardi with an exquisite taste and pleasant aroma. These genotypes can also be involved in future breeding for exquisite taste and commercial quality of fruits. The content of sugars in the fruits varied from 8.9 to 15.5 %, with the highest amount for the varieties Doktorskaya (15.48 %), Fermata (14.03 %), Versiya (16.3 %), and Kapella (15.5 %). These varieties also had the most balanced taste due to the low acid content. Biochemical composition, in addition to taste and quality, determines fruit suitability for processing and as an improver of finished (processed) products. For these purposes, the varieties Ordinate, Raketa, Kapella, Versiya, and Fermata and the most suitable. Therefore, of the released pear varieties, the varieties Pamyat' Korneeva and Kapella outstand due to a complex of economically valuable features and are suitable not only for commercial production but also for further breeding program.

Keywords: *Pyrus communis* L., pear, fruits quality, biochemical composition, breeding, new

varieties

In fruit production, special requirements are imposed on quality [1, 2]. Most fruit crops which include pomes (apple, pear, etc.) are valued for the taste and attractive appearance of the fruit [3-5]. However, high-quality varieties should combine good or excellent taste and appearance of fruits with sufficient keeping quality (for varieties of late ripening), transportability, versatility of use, adaptability, and, besides, provide a high economic effect [6, 7].

Pear fruits have always been valued for their delicate balanced taste and harmonious combination of sugars and acids. However, most varieties with high taste scores of fruits have poor winter hardiness which limits their cultivation. Modern fruit varieties, including the common pear, are characterized by many qualitative traits which are determined by genotype and its realization under certain growing conditions and cultivation technology [8-10]. In different natural and climatic conditions, fruits have not only a definite color, the period of harvesting and consumer maturity, but also a specific biochemical composition that determines their specific taste and the potential for universal use [11, 12]. New varieties created under conditions different from growing conditions will almost always have qualitative characteristics other than those declared [13-15]. Therefore, it is extremely important to carry out assortment selection for certain cultivation conditions [16, 17].

For common pear (*Pyrus communis* L.), breeding programs are mainly aimed at improving fruit quality and resistance to adverse environmental factors. [18-20].

In pear breeding for fruit quality, various approaches and methods of hybridization are used, such as simple and complex crosses, intraspecific, distant hybridization, artificial mutagenesis, etc. [19, 21]. One of the modern approaches to the creation of new genotypes is the use of sources and donors of selectively valuable traits in programs in such a way as to achieve a combination of the main (donor) trait with other positive properties [19, 22, 23].

Pear fruits, depending on the variety and growing conditions, are up to 85% water, from 10 to 25% solids, from 8 to 15% sugar (mainly fructose, glucose and sucrose; quantitatively, depending on the variety, either glucose and fructose, or the disaccharide sucrose may predominate), from 0.05 to 0.5% acids (mainly citric and malic). Pear fruits also contain pectins and tannins [24, 25].

For the southern fruit growing zone of the Russian Federation, the model of a new variety of common pear must meet or exceed at least one of the following parameters: average fruit weight form 150-250 g (vs. 130-180 g for the best zoned varieties); fruit quality at 4.7-4.8 points (vs. 4.6 points), yield of 25-30 t/ha (vs. 20-25 t/ha) [4, 37, 43]. Recently, breeders have been faced with the task of creating varieties with the following fruit biochemical composition: 11-14% sugars, 0.2-0.5% acid, 8-12 mg/100 g ascorbic acid, 250-300 mg/100 g, P-active substances [20, 26].

For the first time in the conditions of the Lower Volga region, based on an assessment of the quality characteristics of the fruits of new varieties of common pear, we have identified the most valuable varieties for industrial cultivation and use in breeding.

The purpose of the work is to evaluate the quality indicators of new and promising varieties and hybrids of the common pear in the conditions of the Lower Volga region, to identify the sources of fruit quality traits.

Materials and methods. The studies were carried out in 1999-2020 at the Federal Scientific Center for Agroecology, Integrated Land Reclamation and Protective Afforestation RAS (FSC Agroecology RAS) in collection and breeding pear

plantations. The objects were new and promising varieties and hybrids of common pear bred at the FSC of Agroecology Dubovskaya rannyaya, Zimnyaya cubarevidnaya, Yubileinaya Korneeva, Nadezhda, Andreevskaya, Doctorskaya, Ordinata, Lavanda, Omega, Pamyat' Korneeva, Kapella, Raketa, Positivnaya, Fermata, Banketnaya, Versiya, Nectarnaya. The basis of breeding work to improve the assortment of pears in the Lower Volga region was the method of targeted intervarietal hybridization and subsequent selection using local adaptive and Western European varieties [8, 9].

To determine the quality indicators, fully ripened fruits were selected in certain parts of the tree (early-ripening varieties) or in storage places (late-ripening varieties) in four repetitions [26, 27]. The average and maximum weight of one fruit was determined by weighing on a general purpose laboratory scale Radwag PS 1200.R2 (Radwag, Poland). Fruit size was determined using a measuring ruler. Appearance was assessed visually on a five-point scale, where 5 points mean that fruits are large, with beautiful color and regular shape, 1 point means that fruits are very poor, from 4 to 2 points are intermediate [27]. The taste of the fruits was evaluated according to the results of tastings carried out as the fruits ripened, on a five-point scale, where 5 is an excellent dessert taste, 1 is a very bad taste, the fruits are inedible.

The amount of soluble solids was determined in the juice refractometrically (IRF-454 B2M refractometer, OJSC Kazan Optical and Mechanical Plant, Russia) (GOST 28562-90. Moscow, 2010). The total acidity was assessed by titration of an aqueous extract of 0.1 N alkali solution (conversion factor for malic acid 0.0067, for citric acid 0.0064; an OHAUS ST3100-F benchtop pH meter with a separate electrode holder, a ST310 3-in-1 plastic serviceable pH electrode, OHAUS, China). A VLTE-310 laboratory balance (NPP Gosmetr, Russia), an LT-6 water bath (LabTex, China) (GOST 25555.0-82, paragraph 4. Moscow, 2010) were used.

The content of sugars (sum of sugars, monosugars, disaccharides) was determined by the standard method according to Bertrand, based on the reduction of the oxide form of copper to the ferrous form with invert sugar in Fehling's solution. The ferrous form of copper was converted into the oxide form with the help of ferric sulfate. The formed ferrous oxide was quantitatively determined permanganatometrically. Laboratory scales VLTE-210 (NPP Gosmetr, Russia), general-purpose laboratory scales Radwag PS 1200.R2 (Radwag, Poland), water bath LT-6 (LabTex, China) (GOST 13192-73) were used. Moscow, 2011).

Pectins were quantified by the carbazole method based on the interaction of carbazole with D-galacturonic acid. The pectin solution was acidified with sulfuric acid to pH 1.0-1.5. Then the pectins were precipitated with acidified rectified ethyl alcohol with a pH of 4.7-4.8. The formed precipitate was separated by centrifugation in a DSC-200D laboratory centrifuge (DIGI System, Taiwan) at a speed of 3000 rpm for 10 min and washed with acidified alcohol. The washed precipitate was hydrolyzed with concentrated sulfuric acid, a 0.2% alcohol solution of the carbazole reagent was added, and the optical density was measured at $\lambda = 535$ nm with a green filter (a spectrophotometer PE-5300VI, OOO Izmeritelnaya Tekhnika, Russia).

The vitamin C (ascorbic acid) concentration was determined by titration of oxalic acid extracts with Tillmans dye (2,6-dichlorophenolindophenol) using laboratory balances VLA-220C (NPP Gosmetr, Russia) and Radwag PS 1200.R2 (Radwag, Poland), a Precellys®24 homogenizer (Bertin Technologies, France), ST3100-F pH meter (OHAUS, China), magnetic stirrer (up to 2 L, 380 °C, 1600 rpm) (STEGLER, China; GOST 24556-89. Moscow, 2003).

Statistical processing of experimental data was performed according to recommendations [26] using Microsoft Excel and the STATISTICA 7.0 package (StatSoft, Inc., USA). Means (*M*) and standard deviations (±SD) were calculated.

Results. Work on the selection improvement of the common pear in the Lower Volga region began in the 1950s by breeders V.A. Korneev, R.V. Korneev and L.K. Zhukova. At the initial stages, various varieties of pear were studied and forms valuable for further breeding were identified [8, 9]. From the beginning of the 1980s to the present, the Federal Scientific Center for Agroecology of the Russian Academy of Sciences (until 2016 at the Nizhne-Volzhsky Research Institute of Agriculture) continues to work on the selection improvement and variety study of fruit crops (including the common pear) of various origins [8, 9, 12].

For an objective characterization of a promising variety, a number of qualitative features are evaluated: fruit marketability (size, taste, one-dimensionality), chemical and technological characteristics (biochemical composition), as well as suitability for processing, storage and transportation. It is known that the qualitative characteristics of fruits are a varietal trait that is controlled polygenically [23, 27, 28]. The agro-climatic characteristics of the year and growing conditions can have a significant impact on the variation of quality indicators. In this regard, for an objective assessment of quality, long-term (four or more years) studies and at least five years of fruiting are required.

1. Fruit parameters in promising varieties and hybrids of common pear (*Pyrus communis* L.) bred by the Federal Scientific Center of Agroecology RAS (Dubovka, Volgograd Province, 1999-2020)

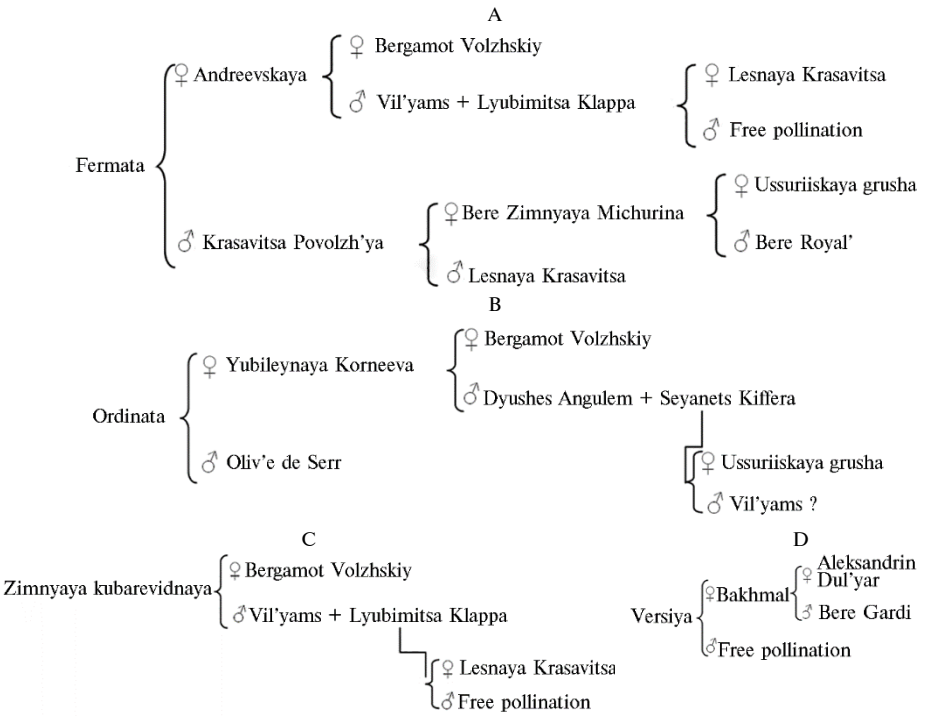
Variety	Parameter				
	appearance, score	taste, score	colour	shape	ripening period
Dubovskaya rannyaya, st	4.5	4.5	Yellow red	Broad pear-shaped	Summer
Nadezhda	4.4	4.4	Gree yellow	Conical	Late summer
Zonal'naya	4.5	4.5	Yellow green	Broad pear-shaped	Early autumn
Doctorskaya	4.5	4.5	Brown yellow	Conical	Early autumn
Andreevskaya	4.5	4.5	Yellow green	Broad pear-shaped	Autumn
Lavanda	4.6	4.5	Yellow	Elongated pear-shaped	Autumn
Fermata	4.8	5.0	Green yellow	Elongated pear-shaped	Autumn
Yubileinaya Korneeva, st	4.3	4.3	Yellow green	Broad pear-shaped	Autumn
Kapella	4,5	4,5	Red yellow	Broad pear-shaped	Autumn
Pamyat' Korneev	4,6	4,6	Green yellow	Broad pear-shaped	Autumn
Raketa	4,5	4,5	Yellow green	Conical	Autumn
Banketnaya	4,5	4,5	Yellow green	Rounded	Autumn
Positivnaya	4,5	4,5	Yellow green	Elongated pear-shaped	Late autumn
Nectarnaya	4,6	5,0	Yellow green	Conical	Late autumn
Omega	4,4	4,4	Yellow green	rounded	Late autumn
Zimnyaya kubarevidnaya, st	4,4	4,4	Red-yellow	Kubariformnaya	Winter
Ordinate	4,4	4,4	Yellow green	Flat-rounded	Winter
Versiya	4,5	4,5	Brown yellow	Broad pear-shaped	Winter

N o t e. Dbovskaya rannyaya, Yubileinaya Korneeva, and Zimnyaya kubarevidnaya are standards for early-ripening, mid-ripening, and late-ripening varieties, respectively.

The study of the qualitative characteristics of pear fruits (Table 1) allowed us to identify the most popular large-fruited varieties with fruits weighing more than 180 g, which turned out to be mostly late-ripening, i.e., Zonalnaya, Doktorskaya, Kapella, Pamyat' Korneeva, Banketnaya, Positivnaya, Nectarnaya, Zimnyaya kubarevidnaya, Versiya. Additionally, these forms exceeded the standard varieties in fruit size. Of the early-ripening varieties, not a single variety was included in the large-fruited group.

Among the large-fruited varieties, forms with very large fruits were distinguished, namely, Pamyat' Korneeva, Pozitivnaya, Kapella, Banketnaya (see Table 1). These varieties inherited the trait of large fruit from parental forms, the varieties Bakhmal and Olivier de Serre with very large fruits. The trait is controlled

polygenically and almost always manifests itself in a part of the offspring, and therefore new varieties, along with their parental forms, can also serve as a source of the large-fruited trait when creating new varieties [29-31].



Pedigree of varieties Fermata (A)**, Ordinata** (B)**, Zimnyaya kubarevidnaya** (C)**, Versiya** (D) **based on genealogical analysis** (FSC Agroecology RAS, Dubovka, Volgograd Province, 1999-2020).

For the new varieties Fermata, Pamyat' Korneeva, and Nektarnaya, the most attractive appearance and a harmonious, dessert taste of fruits was characteristic. Their assessment of fruit appearance varied from 4.6 to 4.8 points, taste characteristics from 4.6 to 5.0 points (see Table 1). In the varieties Pamyat' Korneeva and Nectarnaya, the source of high palatability of the fruit was the ancestral forms, the varieties of European origin Alexandrin Dulyar and Bere Gardi which conveyed good taste and exquisite aroma. The Fermata variety inherited the attribute of dessert taste and rich aroma from its parent forms Williams, Clapp's Favorite and Forest Beauty (Fig., A) [19]. These varieties also significantly exceeded the standard varieties in taste and external characteristics.

Statistical analysis showed (Table 2) that among the varieties of early fruit ripening, the Doktorskaya variety had the highest average fruit weight (182.6 g), and the minimum values were noted in the Nadezhda variety (121.3 g) (see Table 2). In varieties with fruits of medium ripening, the leader was Banketnaya (222.1 g; this value was the highest among all the varieties studied). The minimum indicators of fruit size among the entire studied assortment were observed in the Fermata variety (102.7 g). In varieties with fruits of late ripening, the best indicator was recorded in the variety Pozitivnaya (194.1 g), the minimum in the variety Ordinata (149.1 g). The average fruit weight varied from 102.7 to 222.1 g depending on the variety. The standard deviation of the coefficient of variation did not exceed 10%, which indicates a slight variability of the values. The accuracy of the experiment was kept within acceptable limits.

Fruit color is an important commodity and aesthetic characteristic of the variety. The fruits of most of the varieties studied had a greenish-yellow color as

the main color. Integumentary coloration was either completely absent or present in the form of a small red blush. When studying new and promising varieties, the brightest color was formed on the fruits of the varieties Dubovskaya rannyaya (yellow with a red blush on most of the fruit), Kapella (yellow with a bright carmine blush on half of the fruit), Doktorskaya (bright yellow with a brown-rusty color on most of the fruit). parts of the fruit), Versiya (golden-orzhavlennaya).

2. Statistical parameters of the fruit weight of promising pear (*Pyrus communis* L.) varieties of the FSC Agroecology RAS (Dubovka, Volgograd Province, 1999-2020)

Variety	min	max	*M*	Q_1	*Me*	Q_3	±SD	Accuracy, %
Varieties of early fruit ripening								
Dubovskaya rannyaya, st	109.0	150.0	128.5	115.0	130.0	140.0	1.13	0.38±0.08
Nadezhda	85.0	151.0	121.3	108.0	125.0	141.0	1.23	0.40±0.25
Zonalnaya	106.0	215.0	176.5	164.0	180.0	191.0	1.21	0.28±0.06
Doctorskaya	95.0	215.0	182.6	177.0	192.0	200.0	1.07	0.27±0.06
Varieties of medium ripening fruit								
Andreevskaya	109.0	181.0	139.5	115.0	130.0	153.0	1.20	0.35±0.07
Lavanda	75.0	180.0	110.0	81.0	109.0	120.0	1.32	0.44±0.09
Fermata	80.0	150.0	102.7	87.0	92.0	123.0	1.24	0.48±0.01
Yubileinaya Korneeva, st	89.0	160.0	118.4	98.0	109.0	145.0	1.24	0.42±0.09
Kapella	125.0	320.0	217.3	148.0	208.0	260.0	1.40	0.22±0.05
Pamyat' Korneev	175.0	350.0	221.8	180.0	200.0	250.0	1.28	0.22±0.05
Raketa	122.0	180.0	152.2	128.0	148.0	176.0	1.18	0.32±0.07
Banketnaya	115.0	313.0	222.1	166.0	233.0	261.0	1.38	0.22±0.05
Varieties of late fruit ripening								
Positivnaya	115.0	285.0	194.1	135.0	185.0	285.0	1.43	0.25±0.05
Nectarnaya	132.0	217.0	175.5	140.0	180.0	207.0	1.22	0.28±0.06
Omega	115.0	195.0	164.5	150.0	161.0	185.0	1.18	0.30±0.06
Zimnyaya kubarevidnaya, st	108.0	214.0	167.1	134.0	172.0	198.0	1.29	0.29±0.06
Ordinate	105.0	195.0	149.1	112.0	143.0	187.0	1.27	0.33±0.07
Versiya	126.0	195.0	161.0	142.0	166.0	190.0	1.18	0.30±0.06

Note. min, max — minimum, maximum values of indicators, *M* — mean value, Q1, Q3 — quartiles, *Me* — median, SD — standard deviation.

One of the characteristic features of the variety is the ripening period of the fruit. The varieties with late ripening fruits that can be stored for a long time are of the greatest value. Among the studied varieties with fruits of late-autumn and winter terms of consumption and a long storage period, three were distinguished: Winter kubariform, Ordinata and Versiya. This trait is controlled polygenically and is inherited from parental or grandparental forms [27]. Genealogical analysis showed the probable heredity of the trait in the Ordinata variety from parental and grandparental varieties Olivier de Serre and Kieffer Seedling, which have fruits of late ripening (see Fig., B) [19, 21, 32]. Varieties Versiya and Zimnyaya kubarevidnaya did not have direct parental forms with late ripening fruits, but there was an unknown paternal variety (or group of varieties) potentially capable of transmitting the trait of late fruit ripening (see Fig., C, D) [19].

The balanced taste of fresh fruits is determined by the biochemical composition, which is also related to varietal characteristics and is inherited [27, 33]. However, depending on the climatic conditions of the year of cultivation, the content of sugar, solids and acid in the same varieties may vary.

For technical processing, pear varieties with high acidity (more than 0.35%) and tart taste are better suited. From sweet-fruited varieties with a dessert taste, practically no high-quality canned food is obtained [23, 27]. Among the studied pear varieties, the Andreevskaya (0.37%), Fermata (0.47%) and Omega (0.39%) varieties are most suitable for the manufacture of canned compotes (Table 3).

Pear juice with a high content of mono- and polysaccharides glucose, fructose and sucrose in Western countries serves as a raw material for the production of flavored juices and wines [21, 34, 35)]. The most suitable for these purposes are varieties with a total sugar content of more than 12% and an acid content of

less than 0.3%. In new pear varieties Doktorskaya, Kapella, Raketa, Ordinata and Versiya, which have a high content of sugars in the juice and a low amount of acid, the fruits can also be suitable for adding flavorings and taste improvers to juices of other fruits and berries (see Table 3).).

3. Fruit biochemical composition of promising pear (*Pyrus communis* L.) varieties of of the FSC Agroecology RAS (M±SD, n = 10; Dubovka, Volgograd Province, 1999-2020)

Variety	Dry matter, %	Sugar, %	Titratable acids, %	Sugar acid index	Vitamin C, mg/100 g wet weight	Pectin, % dry weight
Varieties of early fruit ripening						
Dubovskaya rannyaya, st	14.1±0.4	11.1±0.1	0.2±1.70	50.5±12.0	3.2±0.9	5.6±0.48
Nadezhda	13.8±0.4	11.2±0.1	0.2±1.60	46.7±11.9	4.1±0.9	6.2±0.5
Zonalnaya	15.2±1.1	11.7±0.01	0.1±0.40	83.6±2.1	3.7±0.4	6.5±0.5
Doctorskaya	21.3±1.0	15.5±0.02	0.3±0.90	53.4±2.8	3.8±0.4	6.8±1.1
Varieties of medium ripening fruit						
Andreevskaya	16.7±0.5	9.1±0.5	0.4±0.10	24.5±5.2	7.9±3.0	7.4±1.2
Lavanda	16.9±0.5	9.2±0.6	0.1±0.02	82.1±13.3	7.4±0.4	8.1±1.3
Fermata	17.9±0.3	14.0±1.4	0.5±0.04	29.8±1.8	4.1±0.3	4.5±0.4
Yubileinaya Korneeva, st	12.6±0.6	8.9±1.7	0.1±0.02	127.1±21.2	3.8±0.8	7.4±1.2
Kapella	18.7±0.4	15.5±1.0	0.3±0.01	55.5±3.1	2.6±0.3	8.1±1.3
Pamyat' Korneev	17.5±1.8	10.9±0.9	0.3±0.10	35.0±10.4	3.1±6.7	5.9±0.8
Raketa	18.8±1.2	13.0±1.4	0.3±0.10	48.3±14.6	3.8±0.5	8.1±1.3
Banketnaya	18.2±0.2	11.9±1.4	0.3±0.01	44.1±13.9	4.0±0.1	6.5±1.0
Varieties of late fruit ripening						
Positivnaya	18.4±0.8	13.9±1.4	0.4±0.02	33.0±2.9	3.6±0.4	6.5±1.1
Nectarnaya	17.6±0.3	11.8±1.8	0.4±0.10	33.6±1.3	3.8±0.5	6.8±1.2
Omega	15.6±0.5	9.2±0.1	0.4±0.10	26.0±5.8	3.2±0.2	6.9±1.2
Zimnyaya kubarevidnaya, st	14.4±1.3	10.8±1.5	0.2±0.10	63.5±22.7	4.6±2.1	6.9±1.2
Ordinate	18.1±0.8	12.6±1.4	0.3±0.10	50.4±11.0	3.8±0.7	7.3±1.1
Versiya	19.6±0.3	16.3±0.6	0.2±0.02	67.9±2.8	3.0±0.2	6.2±1.0
LSD_{05}	0.85	0.6	0.013		0.21	0.34

The content of ascorbic acid, the fruits of almost all varieties of common pear are not rich. The varieties bred by the FSC Agroecology RAS were no exception. On average, the content of ascorbic acid in their fruits ranged from 2.6 mg/100 g (Kapella) to 4.6 mg/100 g (Zimnyaya kubarevidnaya). However, the fruits of Andreevskaya and Lavanda cultivars had a higher content of ascorbic acid, 7.9 and 7.4 mg/100 g, respectively (see Table 3).

By the amount of pectin substances, pear fruits are inferior to apple fruits. In the fruits of the studied varieties, the content of pectin substances varied in the range of 4.5-8.1% per dry weight. The highest content was recorded in the fruits of the Lavanda, Kapella, and Raketa varieties (see Table 3).

An analysis of the matrix of paired correlation coefficients showed that the resulting value is most closely related to varieties of early ripening, for Nadezhda, it was a close correlation between acidity and sugar (r = 0.71, p = 0.021), Doctorskaya a close relationship between the sugar-acid index and sugar (r = 0.80, p = 0.005), dry matter and vitamin C (r = 0.79, p = 0.006), for Dubovskaya rannyaya (st) a close relationship between acidity and sugar acid index (r = 0.73, p = 0.016). Among the mid-ripening varieties, the Fermata variety stood out which has a moderate relationship between the acid and sugar amount (r = 0.67, p = 0.034), sugar-acid index and acid (r = 0.64, p = 0.044), vitamin C and sugar-acid index (r = 0.69, p = 0.028) and a close correlation between the sugar-acid index and sugar content (r = 0.76, p = 0.012). Among the late-ripeningvarieties, the leader was the Nectarnaya variety with a close correlation between the sugar-acid index and dry matter; sugar-acid index and sugar; vitamin C and acidity (r = 0.74, p = 0.014), and the Ordinata variety with a close relationship between the sugar-acid index and dry matter (r = 0.85, p = 0.001); sugar-acid index and acidity (r = 0.78, p = 0.008). The rest of the studied varieties had either a weak correlation, or it was absent.

Thus, at the FSC Agroecology RAS, the breeding of common pear resulted in the creation of new forms with high fruit quality parametersm including both taste and consumer qualities, due to the biochemical composition and technological characteristics of the fruits. The study of new pear varieties revealed forms that most fully meet modern requirements for the quality characteristics and biochemical composition. In terms of fruit size, varieties Pamyat' Korneeva, Pozitivnaya, Kapella and Banketnaya with fruits weighing more than 200 g stood out. These varieties serve as sources of large-fruited traits combined with high taste, and a balanced biochemical composition. In addition to these varieties, Fermata and Nektarnaya stood out in terms of taste characteristics and attractive appearance. For processing, based on the data on the biochemical composition of fruits, the most suitable varieties are Andreevskaya, Fermata and Omega, and the varieties Doktorskaya, Kapella, Raketa, Ordinata and Versiya as improvers of juice products. The best new pear cultivars with high fruit quality are obtained from the parental forms Lyubimitsa Clapp, Forest Beauty, Olivier de Serre and Bakhmal. According to the complex of economically valuable traits, the varieties Pamyat' Korneeva and Kapella stood out.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 992-1000
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Mycotoxins

UDC 633.85:581.192.6:579.222:543.9

doi: 10.15389/agrobiology.2022.5.992eng
doi: 10.15389/agrobiology.2022.5.992rus

THE COMPLEX OF MYCOTOXINS IN OILSEED RAPE AND TURNIP RAPE DURING SPRING AND SUMMER SEASONS

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The authors declare no conflict of interests
Acknowledgements:
The work was carried out within the framework of the cooperation agreement between the Federal Scientific Center Williams VIK and VNIIVSGE — Branch of the Federal Scientific Center VIEV RAS (dated March 17, 2021).
Received April 11, 2022

Abstract

Oilseed rape and turnip rape crops are widely used to produce seeds and green mass (T.A. Egorova et al., 2015; A.V. Valitov et al., 2018; V.T. Volovik, 2020). The plants are also considered promising siderates that saturate the soil with potassium, phosphorus and nitrogen, and their introduction into crop rotation has a positive effect on grain yields. Recently, the composition and content of mycotoxins were studied in vegetating white mustard and meadow grasses of the *Cruciferous* family with an assessment of seasonal variability and organotropy (A.A. Burkin et al., 2019; A.A. Burkin, G.P. Kononenko, 2022). In this study, it was established for the first time that cyclopiazonic acid. ergot alkaloids, alternariol and emodin are included in the group of the main contaminants of oilseed rape and turnip rape before flowering, as well as data on the expansion of the composition of the mycotoxin complex during budding and the heterogeneous distribution of these substances by plant organs has been received. The aim of this work was mycotoxicological examination of winter turnip rape *Brassica campestris* fr. *biennis* and winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk in the spring-summer growth period — from the rosette phase to the completion of budding, as well as in vegetative and generative organs of plants during flowering and formation of siliques. Vegetating plants were collected from the experimental plots of the Williams Federal Research Center VIC. Winter oilseed rape and turnip rape (sown on September 8, 2020) were collected starting from April 23, 2021, spring rapeseed (sown on May 21, 2021) — from June 25, 2021 weekly. The aboveground parts of whole plants were cut at a height of 3-5 cm from the soil surface, in the phases of flowering and silique formation, the plants were divided into leaves, stems, flowers and siliques. After drying and grinding in a laboratory mill, 349 samples were analyzed. The content of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins of group B (FUM), ergot alkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR-toxin (PR) were determined according to a unified methodology (GOST 31653-2012. Feed. Method of enzyme immunoassay of mycotoxins. Moscow, 2012) using a panel of 15 certified commercial and research enzyme immunoassay systems. The ground samples were extracted with a mixture of acetonitrile and water, 84:16 v/v, at 10 ml per 1 g sample. Indirect competitive enzyme-linked immunosorbent assay (ELISA) was performed after tenfold dilution of extracts with phosphate-salt buffer solution (pH 7.4) with Tween 20. In the entire sample of samples, 14 analytes out of 15 were detected (no POA was found). In winter crops in the rosette-stemming phases, EA, AOL, CPA and EMO were detected in part of the samples with the values located near the limits of the method definition, in the budding phase, an increase in the accumulation of EA, AOL, CPA was observed with cases of detection of EMO, AB_1, STE, OA, MPA and the appearance of fusariotoxins ZEN, FUM. Spring oilseed rape was less contaminated than winter form. During flowering and maturation of siliques, plants showed common patterns of the distribution of mycotoxins by organs, i,e., a greater accumulation in leaves compared to stems and a decrease in the content in ripening siliques. In the flowers of all crops, frequent contamination of MPA was detected, and, as a rule, in combination with EMO, and mycotoxins were

found in winter crops that were absent during the initial growth period (CIT, PR, T-2, and DON). The possibility is discussed of participation of potentially toxigenic micromycetes of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Mucor* in plant contamination.

Keywords: winter turnip rape, winter oilseed rape, spring oilseed rape, mycotoxins, enzyme immunoassay

Oilseeds turnip rape *Brassica campestris* fr. *biennis* and winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk are universal forage crops. In addition to waste from seed processing (cake and meal), their herbage and silage are widely used [1]. The intensive rate of crop formation, good regrowth after mowing in the early stages and the possibility of sowing every 10-15 days, can provide a continuous green conveyor [2]. Both species are also important for agrotechnical practice. As green manure, they enrich the soil with potassium, phosphorus and nitrogen and have a positive effect on grain yields in crop rotation. Almost all types of soils and zones are suitable for these crops; green mass can be harvested from early spring to late autumn, until the snow cover is formed [3, 4]. Of the variety of economically valuable cruciferous crops, the nature of mycotoxin contamination has been studied only for white mustard [5]. For vegetative plants of turnip rape and oilseed rape, such an assessment was not carried out.

In recent decades, significant progress has been made in the study of biodiversity and the functional role of microscopic fungi living inside plants [6, 7]. Information about the phylogenetic position, genetic potential, and metabolic capabilities of these organisms is important for accumulating information about their participation in the processes of plant development and adaptation [8, 9]. The study of low molecular weight metabolites the appearance of which is associated with associated toxigenic fungi, in representatives of the *Cruciferous* family began quite recently. For meadow grasses of 13 genera, these investigations revealed composition of mycotoxins and the features of seasonal dynamics and distribution over vegetative and generative organs [10]. The continuation of these works on cultivated plants is of particular interest because of their stable adaptation to the climatic conditions of areas of long-term cultivation and the formation of winter or spring forms.

This paper is the first to report that cyclopiazonic acid, ergoalkaloids, alternariol, and emodin are among the main contaminants of turnip rape and oilseed rape before flowering, and there is an expansion of the mycotoxin complex during budding and the heterogeneous distribution of these substances over plant organs.

The aim of this work was mycotoxicological examination of winter turnip rape, winter oilseed rape, and spring oilseed rape in the spring-summer period of growth, from the rosette stage to the completion of budding, and the vegetative and generative organs of these plants at flowering and pod formation.

Materials and methods. The vegetative plants of the winter rape *Brassica campestris* fr. *biennis*, winter and spring rapeseed *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk cultivars were grown at the experimental plots of the Williams FSC VIK (Moscow Province).

Winter turnip rape and oilseed rape plants sown on September 8, 2020 were cut weekly starting from April 23, 2021, spring oilseed rape sown on May 21, 2021, from June 25, 2021. Plants were cut at 3-5 cm above soil surface; the plants cut at flowering and fruit formation were divided into leaves, stems, flowers, and pods. After drying and grinding in a laboratory mill, 349 samples were analyzed.

The content of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), group B fumonisins (FUM), ergoalkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR-toxin (PR) were determined according to the unified method (GOST

31653-2012). Method for enzyme immunoassay for the determination of mycotoxins. Moscow, 2012) using a panel of 15 certified commercial and research enzyme immunoassay test systems (STO 00494143.01-2015 Test systems for indirect competitive enzyme immunoassay. General specifications, VNIIVSGE). The lower limits of quantitative measurements corresponded to 85% antibody binding and amounted to 1 (AB_1, EA), 2 (T-2, OA, STE), 5 (ROA), 10 (AOL, MPA, ZEN, EMO, CIT, CPA), 40 (DON, FUM), 100 rg/kg (PR).

For the extraction, a mixture of acetonitrile:water (84:16, 10 ml per 1 g of milled samples) was used. Indirect competitive enzyme immunoassay was performed using 10-fold dilution of the extracts with phosphate-buffered saline pH 7.4 with Tween 20.

Results. In 349 aerial parts of turnip rape and oilseed rape plants, and in vegetative and generative organs of these plants, 14 out of 15 mycotoxins were detected at flowering and maturation, although with various frequencies and often sporadically at a basal concentrations). ROA was not detected.

1. Mycotoxin occurance ($n+$) **and accumulation in winter turnip rape *Brassica campestris* fr. *biennis*, winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk of different varieties at the rosette—stem extention** (1) **and flowering** (2) **stages** (Moscow Province, 2021)

Mycotoxin	Winter turnip rape *B. campestris* fr. *biennis*		Winter oilseed rape *Brassica napus* L. ssp. *oleifera*		Spring oilseed rape *Brassica napus* L. ssp. *oleifera*
	1 (n = 15)	2 (n = 6)	1 (n = 30)	2 (n = 24)	2 (n = 8)
ZEN	–	5	–	2	–
		10-22-26		19, 24	
FUM	–	4	–	2	–
		305-325-340		315, 390	
EA	4	6	9	24	7
	4-7-10	15-21-30	2-3-6	12-32-84	5-19-33
AOL	1	6	3	8	4
	30	30-25-46	16-20-25	26-32-37	(16-19-21)
AB_1	–	2	1	1	–
		1, 1	3	1	
STE	–	2	–	1	–
		10, 20		9	
CPA	3	6	15	24	8
	135-150-170	190-270-400	89-120-160	160-360-980	83-135-200
EMO	3	2	–	4	1
	28-31-33	30, 40		32-34-37	38
OA	–	1	–	1	–
		9		9	
MPA	–	–	–	1	–
				33	

N o t e. ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB_1 — aflatoxin B_1, STE —sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, MPA — mycophenolic acid; n is the number of studied samples. The top figure in the rows is the number of positive samples ($n+$) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

At the beginning of spring regrowth, in the rosette—stem extension stages, EA, AOL, and CPA were detected in both winter crops, and EMO in oilseed rape but only in some samples and in an amount within the detection limits of the method. AB_1 was detected in one oilseed rape sample (Table 1). The onset of the budding stage led to the appearance of fusariotoxins ZEN, FUM and an increase in the EA and CPA accumulation, these toxins were detected in all samples. The average levels of mycotoxin accumulation in turnip rape and oilseed rape were comparable, 21 and 32 μg/kg for EA, 270 and 360 μg/kg for CPA, EMO, AB_1, STE, OA were found rarely, MPA in one of the samples of winter oilseed rape.

AOL was produce in all turnip rapeseed samples (average content of 25 μg/kg) and only in 8 out of 24 oilseed rape samples in comparable amounts. The unequal increase in AOL contamnation in winter turnip rape and oilseed rape

rapeseed during stage changes could be due to differences in the species composition of *Alternaria* fungi. Unfortunately, the available information on this issue is still limited. Isolates of the potentially toxigenic endophytic species *A. alternata* were detected in the stems and leaves of oilseed rape [11]. M.J. Kelman et al. [12] found that in the Canadian population of *Alternaria* colonized oilseed rape plants, the proportion of AOL producing species is small. Representatives of the *A. infectoria* group that do not form this toxin are much more common.

Spring oilseed rape at budding stage was less contaminated than winter turnip rape and oilseed rape (see Table 1). Thus, the accumulation of EA, AOL, and EMO here did not exceed 50 μg/kg, CPA 200 μg/kg, and other toxins were absent. In contrast, 10 out of 15 mycotoxins were found in both winter crops. It is possible that the number of detected mycotoxins increased relative to spring rapeseed was the result of a long spring-spring period of growth, during which a larger part of toxigenic fungi could accumulate mass.

In all examined plants, along with AOL, CPA and EA were typical contaminants. The same mycotoxins dominated in vegetative white mustard and meadow grasses [5, 10]. Apparently, cruciferous communities of associated fungi always contain micromycetes capable of their biosynthesis and begin to function actively from the very beginning of growth.

Any participation of endophytic fungi in the biosynthesis of mycotoxins should be confirmed by the identification of potentially toxigenic species in the deep mycobiota after surface disinfection of tissues. Data which would allow us to make reasonable assumptions about the sources of the appearance of mycotoxins that we found in cruciferous plants are very little. Thus, *Aspergillus flavipes* was found in the stems of vegetative oilseed rape plants, and *Fusarium proliferatum* was found in the leaves [11]. For *A. flavipes* and related species, the ability to biosynthesize STE has been described [13], *F. proliferatum* produces FUM [14]. Unfortunately, so far the interest of researchers in cruciferous endophytes is mainly associated with the search for biological means of protecting these plants from pathogens of fungal diseases [11, 15-17].

The production of CPA and EA is known for micromycetes of many taxa [18]. Some species belong to the endophyte community, for example, *Aspergillus fumigatus* [19], *Penicillium chrysogenum*, *P. commune*, *Mucor hiemalis* [20]. Among the endophyte-dwelling fungi, *A. versicolor* [21], *P. chrysogenum* [20], and *P. brevicompactum* [7] synthesized STE [22], EMO and MPA derivatives [23] were also identified. Detection of OA in turnip rape and oilseed rape during budding may be associated with fungi *P. verrucosum* var. *cyclopium* and *P. chrysogenum* which were previously found in oilseed rape seeds and produced this toxin [24]. In addition to the potentially toxigenic species already known, one should consider the role of other micromycetes, which, as recently found out, possess the corresponding gene clusters [25], and the extensive associations of non-cultured fungi, the presence of which in plant microbiomes has been confirmed by molecular methods.

From the beginning of flowering to the maturation of rapeseed and rapeseed plants, we analyzed the content of mycotoxins in various organs. i.e., stems, leaves, flowers and pods (Tables 2-4). During this period, all plants showed a greater accumulation of mycotoxins in leaves compared to stems and a decrease in the content in ripening pods.

The same patterns were previously noted in white mustard seed and wild herbs [5, 10], which obviously indicates the general directions of the reorganization of their internal microbiome, which are still unknown. Extremely low contamination of ripening pods, especially in spring oilseed rape, is consistent with the absence of mycotoxins in the seeds of this plant (unpublished data of the authors).

2. Mycotoxin occurrence (n+) and accumulation in various organs of winter turnip rape *Brassica campestris* fr. *biennis* plants at flowering, fruit formation and ripening stages (Moscow Province, 2021)

Mycotoxin	Stems (n = 21)	Leaves (n = 11)	Flowers (n = 5)	Green pods (n = 15)	Yellow pods (n = 10)
T-2	–	–	–	–	–
DON	–	–	4 79-82-89	–	–
ZEN	–	–	4 12-13-17	–	–
FUM	–	–	2 105, 125	–	–
EA	18 6-19-40	11 10-15-26	4 10-13-20	15 5-19-42	3 6-13-25
AOL	4 26-32-48	4 18-28-33	5 30-4-49	–	1 54
AB1	–	–	3 1-1-2	–	–
STE	–	–	–	–	–
CPA	18 63-145-350	11 100-195-295	5 155-185-240	13 79-115-235	1 50
EMO	–	4 38-47-58	3 32-34-39	1 31	5 30-42-59
OA	–	–	1 8	–	–
CIT	–	–	2 16, 18	–	–
MPA	–	–	3 40-41-42	1 40	3 13-16-21
PR	–	–	–	–	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin , OA — ochratoxin A, MPA — mycophenolic acid, PR — PR-toxin; n is the number of samples examined, the top figure in the rows is the number of positive samples (n+) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

3. Mycotoxin occurrence (n+) and accumulation in various organs of winter oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk plants at flowering, fruit formation and ripening stages (Moscow Province, 2021)

Mycotoxin	Stems (n = 36)	Leaves (n = 25)	Flowers (n = 16)	Green pods (n = 27)	Yellow pods (n = 28)
T-2	–	–	1 6	–	–
DON	–	–	8 83-105-130	–	–
ZEN	–	–	13 9-16-24	–	–
FUM	–	–	1 100	–	–
EA	31 4-13-30	25 8-83-710	16 6-10-16	27 3-21-63	10 4-10-20
AOL	3 21-23-26	19 17-36-50	16 30-42-56	6 12-24-30	1 24
AB1	–	–	10 1-1-2	–	–
STE	–	1 18	–	–	–
CPA	25 54-130-245	22 105-205-415	15 100-145-200	22 100-150-245	3 79-87-91
EMO	3 30-31-32	4 31-33-39	10 31-37-48	2 (30, 30)	–
OA	–	3 8	3 9-10-10	–	–
CIT	–	1 16	1 16	–	–
MPA	–	1 62	12 30-37-52	3 32-37-40	–
PR	–	–	4 320-380-400	–	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin,

OA — ochratoxin A, MPA — mycophenolic acid, PR — PR-toxin; *n* is the number of samples examined, the top figure in the rows is the number of positive samples (*n*+) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

4. Mycotoxin occurrence (*n*+) and accumulation in various organs of spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk plants at flowering, fruit formation and ripening stages (Moscow Province, 2021)

Mycotoxin	Stems (*n* = 19)	Leaves (*n* = 12)	Flowers (*n* = 5)	Green pods (*n* = 19)	Yellow pods (*n* = 17)
T-2	–	–	–	–	–
DON	–	–	–	–	–
ZEN	–	–	–	–	–
FUM	–	–	–	–	–
EA	4	10	5	3	1
	3-5-6	4-8-20	4-5-6	4-5-6	5
AOL	–	2	3	–	–
		21, 26	20-24-26		
AB1	–	–	2	–	–
			2, 3		
STE	–	–	1	–	–
			7		
CPA	4	10	4	4	–
	84-160-250	94-255-300	105-185-290	79-195-315	
EMO	2	3	3	1	–
	43, 50	36-45-50	41-44-51	48	
OA	–	–	2	–	–
			6, 7		
CIT	–	–	–	–	–
MPA	–	3	5	5	-
		16-18-20	15-25-38	15-20-29	
PR	–	–	–	–	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin , OA — ochratoxin A, MPA — mycophenolic acid, PR — PR-toxin; *n* is the number of samples examined; *n* is the number of studied samples. The top figure in the rows is the number of positive samples (*n*+) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

In flowers of winter plants (see Tables 2, 3), the number of detected metabolites increased. The flowers of winter turnip rapeseed and winter oilseed rape contained fusariotoxins which were not present at the generative phase either in the vegetative organs (leaves, stems) or in the pods. This has been described in sowing and wild-growing annuals, the white mustard and field cabbage [5, 10]. Formally, winter plants are classified as annuals, since the entire development period does not exceed one year, although it begins in autumn, is interrupted by a period of winter dormancy, and resumes in spring. No fusariotoxins were found in the flowers of spring rapeseed (see Table 4). Perhaps this is due to a shortened initial rosette-stem phase and a rapid transition to budding and flowering.

A clear difference in the accumulation of fusariotoxins in flowers in winter and spring forms definitely indicates the unequal involvement of *Fusarium* toxin-forming fungi in ontogeny. In this case, either a directed movement of the pathogen through the tissues or the transfer of the resulting metabolites from remote points of localization of producers can occur. Given the available information, the second way seems to be more realistic. Thus, it has been shown that phytotoxins spread throughout the plant, while pathogens of the genus *Fusarium* ascend the stem only a few centimeters above the soil level or remain in the root collar.

In the flowers of all crops (see Tables 2-4), frequent MPA contamination was detected, as a rule, in combination with EMO, and in winter crops, mycotoxins were found that were absent in the initial period of growth, the CIT, PR, fusariotoxins T-2 and DON. There are indications in the literature that some species of *Penicillium* and *Fusarium* capable of biosynthesis of MPA (*P. brevicompactum*), PR

(*P. chrysogenum*) [23], T-2 (*F. sporotrichioides*), DON (a complex of species related to *F .graminearum*) [26] are present in the internal mycobiota of plants [20, 27]. Information on belonging to the endophytes of the fungus *Aspergillus pseudoglaucus* which is characterized by co-formation of MPA and EMO (unpublished data of the authors), could not be found. The fact that almost all analyzed mycotoxins, except for ROA, were found in the tissues of the plants examined by us indicates the diversity of toxigenic fungi present in the microbiome.

Thus, for winter turnip rape, winter and spring oilseed rape, a weak contamination with mycotoxins was established with the most frequently detected cyclopiazonic acid in amounts of no more than 360 μg/kg, alternariol and emodin at 32 and 34 μg/kg, respectively, ergoalkaloids at 3-32 μg/kg. A greater accumulation of the components of the main complex and the appearance of zearalenone and fumonisins, as well as aflatoxin B_1, sterigmatocystin, ochratoxin A and mycophenolic acid detected in winter crops at budding stage, after the end of the initial period of growth, was also weakly expressed. Therefore, both economically valuable crops belong to the group with a reduced risk of negative effects on animals. At the generative phase, plants of both winter and spring forms showed an increased content of mycotoxins in leaves compared to stems, a weakening of contamination of ripening pods, and an accumulation of mycophenolic acid and emodin in flowers. The appearance of fusariogenic toxins and citrinin was noted only in flowers of winter plants. Signs of similar shifts and differences in the complex of mycotoxins in cruciferous crops of winter and spring types, established for the first time, indicate complex multidirectional processes of involvement of toxin-forming micromycetes in the development of these organisms. The obtained data may be a basis for further study of the mechanisms regulating the cohabitation of plants and associated fungi.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 1001-1009
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 633.85:581.192.6:579.222:543.9

doi: 10.15389/agrobiology.2022.5.1001eng
doi: 10.15389/agrobiology.2022.5.1001rus

A TYPICAL MYCOTOXIN PROFILE FOR ORIGINAL (REPRODUCTIVE) OILSEED RAPE SEEDS

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Acknowledgements:
The authors are grateful to E.A. Piryazeva (the Laboratory of Mycotoxicology and Feed Sanitation, All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology) for performing mycological analysis and assistance in searching scientific literature.
The work was carried out within the framework of the cooperation agreement between the Federal Scientific Center Williams VIK and VNIIVSGE — Branch of the Federal Scientific Center VIEV RAS (dated March 17, 2021).
Received May 7, 2022

Abstract

For rapeseed, the world's third largest source of vegetable oil, there are special requirements for post-harvest processing, which are rapid drying to the required moisture content due to the threat of mass mold during storage (J.T. Mills, 1987; J.T. Mills, R.N. Sinha, 1980). The authors of publications explained all known cases of mycotoxin detection in seeds of this crop by either infection of plants in the field or by the impact of unfavorable factors during harvesting (I. Brazauskiene et al., 2006; A. Mankeviciene et al., 2011, L. Wu et al., 2017). In this study, for the first time, we have confirmed that the presence of toxic metabolites of fungi of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Myrothecium* and a number of others is not typical for seeds of this crop. The purpose of this work was mycotoxicological examination of oilseed rape *Brassica napus* L. ssp. oleifera (Metzg.) Sinsk seeds under proper phytosanitary and technological conditions during cultivation, harvesting and storage. Original (reproduction) seeds were harvested in 2009-2021 from experimental plots (the Laboratory of fodder crops and field feed production systems, the Williams Federal Research Center, Moscow Province). After grinding in a laboratory mill, 158 samples were analyzed by a unified methodology using certified commercial and research enzyme immunoassay test systems (GOST 31653–2012). For extraction, a mixture of acetonitrile and water was used (84:16 v/v), 5 ml per 1 g sample. Mycotoxins (T-2 toxin, deoxynivalenol, zearalenone, fumonisins of group B, ergot alkaloids, alternariol, roridin A, aflatoxin B_1, sterigmatocystin, cyclopiazonic acid, emodin, ochratoxin A, citrinin, mycophenolic acid, PR-toxin) were determined in extracts after 10-fold dilution with phosphate-salt buffer solution (pH 7.4) with Tween 20. The analyzed mycotoxins were not found in the seeds of the 2009-2020 harvests collected under normal weather conditions and without violations of the drying and storage regimes. For only one sample of spring rapeseed (Bison variety, 2019), weak contamination with mycophenolic acid was detected and the producer *Aspergillus pseudoglaucus* Blochwitz was identified in the mycobiota. In the seeds of the 2021 harvested under elevated temperature and humidity, contamination with ergot alkaloids (from 2 to 12 μg/kg = ppb) was found in 67.5 % of spring and 25.6 % of winter crops. In addition, 28.6 % of spring rape samples contained alternariol, mainly in low concentrations (from 8 to 32 ppb) and rarely with a more pronounced accumulation (from 46 to 775 ppb). In one spring rapeseed sample which was stored in an under-dried state for the longest time, the greatest contamination with alternariol occurred and intensive infection with the fungus *Alternaria tenuissima* (Nees et T. Nees:Fries) Wiltshire were determined with an experimentally confirmed ability to toxin formation.

Keywords: oilseed rape, seeds, mycotoxins, enzyme immunoassay

For seeds of oilseed rape, the world's third-largest source of vegetable oil, accounting for 14.1% of global production in 2020-2021 [1], there is a special post-harvest requirement for drying as quickly as possible to a standard moisture

content of 5-8%. This is associated with a real threat of mass molding of seeds, which is especially relevant in zones with a warm and humid harvesting period, as well as in storage areas where deviations from optimal parameters are observed [2, 3]. Studies performed in Romania [4, 5], Lithuania [6, 7], Poland [8, 9], Serbia [10, 11], India [12, 13], Ethiopia [14] showed that at harvesting and during storage of the rape seeds there is an extensive community of associated fungi, including both pathogens of these plants and saprotrophic fungi. Information on the contamination of rape seeds with mycotoxins is scarce. Researchers explain their appearance either by infection of plants in the field, or by the impact of unfavorable factors during harvesting [15, 16].

In this study, for the first time, confirmation is presented that the presence of toxic metabolites of fungi of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Myrothecium* and a number of others is not typical for rapeseed seeds.

The purpose of the work is mycotoxicological examination of oilseed rape seeds obtained in compliance with the appropriate phytosanitary and technological conditions during cultivation, harvesting and storage.

Materials and methods. The objects of the study were 158 samples of original (reproductive) seeds of winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk from the experimental plots of the Williams Federal Scientific Center for Forage Production and Agroecology (VIK) in 2009-2021.

Comparison of data on air temperature and humidity during seed harvesting was carried out based on data of the Lugovaya meteorological station (Moscow Province).

Mycotoxicological analysis of seeds of the 2021 harvest (120 samples) was carried out 2 months after harvest. Seeds of the 2020 harvest and samples of previous collections taken from the seed warehouse were analyzed in March 2021, that is, the shelf life was about 9 months (harvest 2020) and from 2 to 10 years.

The content of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), group B fumonisins (FUM), ergoalkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR-toxin (PR) were determined according to the unified method (GOST 31653-2012). Feeds. Method for enzyme immunoassay for the determination of mycotoxins. Moscow, 2012) using a panel of 15 certified commercial and research enzyme immunoassay test systems (STO 00494143.01-2015 Test systems for indirect competitive enzyme immunoassay. General specifications, VNIIVSGE). The lower limits of measurements corresponded to 85% antibody binding and amounted to 1 (AB_1, EA), 2 (T-2, OA, STE), 5 (ROA), 10 (AOL, MPA, ZEN, EMO, CIT, CPA), 40 (DON, FUM), 100 μg/kg (PR).

For the extraction of ground seed samples, a mixture of acetonitrile and water was used in a ratio of 84:16, 5 ml per 1 g sample. Indirect competitive enzyme immunoassay was performed after 10-fold dilution of the extracts with phosphate-buffered saline (pH 7.4) with Tween 20.

Results. The data on the rape seed samples used in the work are shown in Table 1. When using a unified methodology and a panel of 15 enzyme immunoassay test systems, no mycotoxins were detected in 38 rapeseed seed samples from the 2009-2020 crops.

Their complete absence indicated that this state remained stable and did not depend on the seed storage time. During harvesting in these years, weather conditions corresponded to long-term climatic parameters, and no deviations from the standards for drying seeds were recorded. It should be noted that earlier, in five samples of oilseed rape seeds harvested in 2015-2018 from eastern Poland, none of the 13 analytes were fund, including fusariotoxins diacetoxyscirpenol, T-

2 toxin, HT-2, nivalenol, deoxynivalenol, 3-acetyl- deoxynivalenol, fusarenone X, zearalenone, as well as aflatoxins B1, B2, G1, G2 and ochratoxin A [17].

1. Characterization of spring and winter oilseed rape *Brassica napus* L. ssp. oleifera (Metzg.) Sinsk seeds used in mycotoxicological examination (Moscow Province, 2009-2021)

Crop	Year	Varieties, hybrids, breeding samples
Winter rapeseed	2012-2018	Varieties: Horizon (2018), Severyanin (2012, 2013, 2017, 2018), Capital (2014, 2017, 2018)
	2019	Varieties: Garant, Severyanin
	2020	Variety: Severyanin
	2021	Varieties: Garant (two sowing dates), Horizont (two sowing dates), Dobrodey, Kazimir, Laureate, Loris, Nord (two sowing dates), Olivine, Progress, Sarmat, Severyanin (two sowing dates), Severyanin (another reproduction), Seligor, Capital (two sowing terms), Elvis, Asparagus, Brauen Schnitthole, Dunne, Grunder Sch., Hangry Gap, Imperial, Jot Neuf, Jrig Scedrap, Lider, Symmons, Taisetze note, Victor, Zenit, Zorni Samples: No. 4, No. 2 NPTs, No. 4 NPTs, No. 6 NPTs, VN-360-14L, VN-364-14R, VN-469-15L, VN 726-17R, VN 912-15
Spring rapeseed	2009-2018	Varieties: Bizon (2016), Vikros (2011, 2017), Grant (2009), Lugovskoy (2017), Novosel (2015), Podmoskovny (2012, 2015, 2018)
	2019	Varieties: Bison, Veles, Grant, Novosel, Podmoskovny
	2020	Varieties: Vikros, Novosel, Podmoskovny, Svetozar
	2021	Varieties: Bison, Veles, Vikros, Grant, Lugovskoy, Novik, Novosel, Podmoskovny Hybrids: Astra 4, Astra 5, Astra 6, VIK 1, VIK 2, VIK 3, VIK 4, VIK 5, Vikros, Drago, Karalino, Lagonda, Casket, Lumen, Miracle, Macro, Salsa M 45, Smilla, Chevy KL Samples: Vikros M 48.2, Vikros MD 38, Vikros MD 69.1, Vikros MD 70, Vikros ML 35, Vikros ML 38, Vikros ML 49, Vikros ML 69, Vikros ML 102, Grant MD 4.1, Novik MA 81, 25-3, 379-13, 557-15, 580-15, 902, 951-3, 948-3, 948-4, 948-6, 949-2; 951-1, 951-18, 7, 10, 15, 17, 19, 23, 26, 27, 38, 39, 40, 1/20, F 1/21, 2-7/15 M 74, 8/20 , 18/20, 29/20, 32/20, 33/20, 75/20, 88/20, 359/20, 369/20, 372/20, 388/20

The only exception for the period from 2009 to 2019 was a sample of spring oilseed rape (variety Bizon, 2019) which contained MPA at a low concentration close to the limit of detection (20 μg/kg). One of the strains of *Aspergillus pseudoglaucus* Blochwitz (No. 448/2) detected by mycological analysis of the sample according to common procedure including isolation and identification of pure cultures, produced MPA (2000 ng/g) and an anthraquinone toxin EMO (70 ng/g) during express testing (7 days, 25 °C, wort agar). The appearance of MPA in this sample was probably the result of a short-term violation of its storage conditions, and a longer exposure to negative factors could lead to combined contamination. The production of two toxins with a quantitative predominance of MPA is typical for strains of this fungus found in grain products (unpublished data of the authors).

The seeds of the 2021 harvest also lacked T-2, DON, ZEN, FUM, OA, CIT, AB_1, STE, CPA, PR, EMO, and ROA. However, EA and AOL were found in some samples (Table 2). EA, the biosynthesis of which is known for fungi of many taxa, including the genera *Penicillim* and *Aspergillus* [18], were contained at a basal amounts of 2 to 12 μg/kg, and the frequency of their detection in spring varieties was higher than in winter varieties (67.5 % vs. 25.6%). AOL, a toxic metabolite of small-spore *Alternaria* species, was detected in 28.6% of spring oilseed rape seed samples. The content of this toxin mostly was low, from 8 to 32 μg/kg, only in 6 samples, it varied from 46 to 775 μg/g. Most of the positive samples contained only EA (32 samples) or were co-contaminated (20 samples), two had only AOL.

The 2021 growing season was generally warmer than usual. In 34 decades of the year out of 37, the average daily air temperature was above the norm. The average daily temperature exceeded the long-term average in summer by 4.6 °C, in autumn by 3.1 °C, the growing season lasted until the end of the first decade

of November. Precipitation was 1.36 times more than the annual averages, and the excess was unevenly distributed from April to September.

2. Mycotoxin occurrence ($n+$) and accumulation in original (reproduction) seeds of spring and winter rapeseed *Brassica napus* L. ssp. oleifera (Metzg.) Sinsk (Moscow Province, 2021)

Crop	n	n^+ (mycotoxin concentration min-max, μg/kg)	
		ergoalkaloids	alternariol
Winter rapeseed	43	11 (2-12)	—
Spring rapeseed	77	52 (2-5)	22 (8-775)

N o t e. n is the number of samples examined, $n+$ is the number of positive samples containing mycotoxins in an amount exceeding the lower limit of measurements. A dash means that the mycotoxin was not detected.

The harvesting of winter seeds at the end of July 2021 took place under more favorable conditions than spring ones: the increase in precipitation in July was the least and amounted to 13.6% of the monthly norm. Spring seeds were collected at the end of the growing season as they matured, when the humidity exceeded the norm by 70.9% (in August) and 52.8% (in September). The samples contained many weed seeds, the moisture content of which turned out to be especially high, and it was not always possible to rapidly dry seeds to the standard moisture content. The combination of these circumstances, apparently, caused the active growth of toxigenic micromycetes and the accumulation of mycotoxins in seeds, which is more pronounced in spring crops. One of the samples of spring rapeseed, designated as F 1/21 (see Table 1), which was not dried to the normative humidity, along with EA (3 μg/kg) contained AOL in the largest amount, 775 μg/kg. Mycological analysis revealed intense (more than 50%) infection with *Alternaria tenuissima* (Nees et T. Nees: Fries) Wiltshire. Three isolates (Nos. 456/1, 456/2, and 456/4) during cultivation (7 days, 25 °C, malt agar) confirmed the ability to produce AOL in amounts of 980, 1520, and 2500 ng/g of medium.

Previously, for fungi found in oilseed rape seeds, the toxic effects of metabolites of *Alternaria* spp. were revealed [7] and aflatoxigenicity was found in three isolates of *Aspergillus flavus* [19]. The species *Aspergillus flavipes* found in the composition of endophytes of oilseed rape stems is also potentially toxigenic [20]. The search for micromycetes involved in the contamination of the seeds of this crop with toxic metabolites should be continued within the framework of more detailed mycological examinations.

The generalization of the obtained results showed that, subject to the phytosanitary and technological rules, regardless of the type of cultivation of oilseed rape, there were no mycotoxins in its seeds. This new fact allows us to assume their extremely low content in vegetative plants. Earlier, it was shown that in sunflower, the contamination of achenes is residual compared to the green mass [21], and in mature fruits (pods) of mustard and meadow herbs of the *Cruciferous* family, the content of mycotoxins is less than in the vegetative part [22-24]. The source of the appearance of mycotoxins in vegetative plants is probably fungi that live in the form of stable associations with the main organism and retain the composition and ratio of components according to species ranks. For extensive communities of endophytic fungi [25], the transmission is known [26] of their metabolites into seeds (through the migration of producers from vegetative parts or vertical transfer in the form of conjugated forms along conducting pathways)

Potentially toxigenic *Alternaria* species are not only among the causative agents of *Alternaria*, which annually leads to significant losses in rape crops [27], but are also capable of semi-parasitic habitation with broad substrate specificity [28] and have recently been identified as dominant among endophytes. in the roots, stems and leaves of rapeseed plants [20]. Given this, it is still premature to conclude about the sources of AOL in the composition of contaminants in spring

oilseed rape seeds under changing external conditions. The possibility of its transmission as one of the metabolites of endophytic fungi under the influence of biotic or abiotic displacements cannot be ruled out. In a single attempt to detect alternariotoxins in the seeds of this crop, none of the six were detected [19].

3. Selective mycotoxicological analysis of seeds from different regions

Region	Mycotoxins, n^+/n, concentration (min-max, μg/kg)	References
Romania	Aflatoxins, 7/7; deoxynivalenol, 7/7; zearalenone, 7/7	[4]
Lithuania	At harvest: aflatoxins, 5/5, (1.0-3.1); ochratoxin A, 5/5 (1.9-7.0); deoxynivalenol, 5/5 (164-183) After 8 months of storage: aflatoxins, 3/5 (2.1-3.3); ochratoxin A, 5/5 (1.3-1.9); deoxynivalenol, 0/5	[15]
Lithuania	Winter barley: deoxynivalenol, 8/8 (153.5-176.5); zearalenone, 12/12 (10.6-25.6); T-2 toxin, 8/8 (8.5-10.2) Spring barley: deoxynivalenol, 6/8 (0-181.0); zearalenone, 12/13 (0-25.10); T-2 toxin, 8/8 (8.2-10.1)	[16]

N o t e. n is the number of samples examined, $n+$ is the number of positive samples containing mycotoxins.

4. A detailed mycotoxicological analysis of rape seeds from different regions

Region	n	Mycotoxins		References
		detected n^+ (concentration, min-max, μg/kg)	not detected	
Spain (Catalonia)	20	Aflatoxin B1 — 1 (0.25)	Aflatoxins B_2, G_1, G_2, alternariol, alternariol monomethyl ether, tenuazonic acid, altertoxins I and II	[19]
China	29	Aaflatoxin B1 — 10 (0.2-0.8); bovericin — 8 (137.6-898.8); fumonisin B_1 — 6 (157.8-474.5); aflatoxin B_2 — 2 (0.6; 1.4)	Aflatoxins G_1, G_2, ochratoxin A, sterigmatocystin, zearalenone, 3- and 15-deoxynivalenol monoacetates, fumonisins B_2, B_3, T-2 toxin, HT-2	[29]

N o t e. n is the number of samples examined, $n+$ is the number of positive samples containing mycotoxins.

According to our data, under difficult harvesting conditions, EA, AOL, MPA, and EMO can be expected among the contaminants of oilseed rape seeds, but such contamination may well be characteristic only of the agrozone in which the observation was carried out. Mycotoxicological evaluation of seeds from other regions, performed on smaller samples, revealed aflatoxins, OA, and fusariotoxins (Tables 3, 4). Thus, from the group of fusariotoxins in Romania in 2002-2004, DON and ZEN were found [4], in Lithuania DON in the crop obtained from experimental crops of winter rapeseed [15]. DON, ZEN and T-2 were identified in 2007-2009 in the seeds of spring and winter rapeseed grown using traditional technology in the Kedai and Panevezys districts of central Lithuania [16]. In China, in seeds obtained from industrial crops in 11 provinces, only fumonisin B_1 and bovericin were found, while ZEN, DON monoacetates, and T-2 were not detected [29]. Apparently, the appearance of fusariotoxins in the seeds was the result of focal damage to plants by complexes of fusarium pathogens that differ in species composition. The capabilities of the analytical approach we used were quite sufficient to confidently determine the degree of contamination of seeds with aflatoxins, OA, and fusariotoxins identified by other researchers (see Tables 3, 4).

In Russia, the oilseed rape seed production and processing had grown rapidly in recent decades, with acreage nearly doubling and seed yields reaching a record 2.4 million tonnes in 2020-2021 [30]. This crop is cultivated in the regions of all federal districts - from the North-Western to the Far East, which differ significantly in soil-climatic and agrotechnical conditions. By-products from the processing of seeds into oil, cake and meal are becoming more and more in demand in feed production, and rapeseed cake biomodification products are considered as promising functional ingredients for the food industry [31]. Under such conditions, the need for a systematic approach to monitoring this raw material in the country is beyond doubt. Unfortunately, regular control of its sanitary quality has not yet been organized.

The results of a point assessment are all the more interesting: a sample of rapeseed cake received from the Kaliningrad region in 2021 did not contain the studied mycotoxins, while that obtained in 2018 from the Krasnodar Territory was contaminated with CPA (50 μg/kg) and EMO (38 μg/kg) (unpublished data of the authors).

The possible accumulation of individual mycotoxins in oilseed rape seeds should not be ignored in agronomic practice, given their negative impact on germination. Effective elimination of seed infection can be ensured by pre-sowing preparation (drageeing, encrustation or disinfection using fungicides), but mycotoxicological control is necessary to reduce the risk of seed contamination.

Thus, on the original (reproductive) seeds of spring and winter crops of oilseed rape, it was shown that, subject to the phytosanitary and technological rules of cultivation and storage, there is no reason to worry about the threat of their contamination with toxins characteristic of fungi of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Myrothecium* and others. All identified cases of seed contamination with mycotoxins were samples collected under a combination of adverse weather conditions or violations of storage rules.

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AGRICULTURAL BIOLOGY,
2022, V. 57, Iss. 5, pp. 1010-1020
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Plant biologicals and biostimulants

UDC 633.511:581.1:631.811.98

doi: 10.15389/agrobiology.2022.5.1010eng
doi: 10.15389/agrobiology.2022.5.1010rus

INFLUENCE OF NANOPREPARATIONS ON LABORATORY SEED GERMINATION, GROWTH, DEVELOPMENT AND YIELD OF COTTON (*Gossypium hirsutum* L.)

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The authors declare no conflict of interests
Received April 26, 2022

Abstract

Currently, an important role is given to technologies based on a wide range of environmentally friendly plant protection products. The nanopolymer bioactive preparations based on chitosan from the silkworm (*Bombyx mori* Linnaeus, 1758) pupae with fungicidal and bactericidal properties are well suited for pre-sowing treatment of crop seeds. Here, for the first time, it was revealed that the seed treatment with nanopolymer preparations containing copper and silver ions accelerates the growth and development of seedlings and increases the yield of cotton cultivar Andijan 36. Our aim was to evaluate the effect of biologically active nanopolymer complexes based on chitosan and its derivatives on the morphophysiological and sowing parameters of seeds, the economically valuable qualities of raw cotton, and the yield of cotton in lab tests and under field conditions (the Research Institute of Selection, Seed Production and Agro-Technology of Cotton Growing, 2018-2020). In the experiments, we used nanopolymeric preparations PMC (polymer-metal complex) Cu^{2+}:Ag 7:3, PMC Cu^{2+}:Ag 8:2, Nanochitosan (NanoChS, 0.5 %, 90 kDa), Nanoascorbachitazan (NanoAChS, 0.5 %, chitosan:ascorbic acid 4:1) and polymer preparations Chitosan initial 0.5 %, Kuprimhit 0.5 %, Ascorbatchitosan (AChS). Preparations UZKHITAN (Institute of Chemistry and Physics of Polymers of the Academy of Sciences of the Republic of Uzbekistan) and Dalbron (Dalston associated SA, Panama) served as standards, seeds without processing served as control. Seeds (n = 36) of cotton (*Gossypium hirsutum* L.) cultivar Andijan 36 were soaked in preparations (at the rate of 20 l/t seeds) at least 3-4 days before sowing. In each variant of the experiment, 6 kg of cotton seeds were treated. In lab tests, the seeds were germinated in sand at 25 °C and a 60-65 % air humidity. Germination energy was determined on day 4, germination on day 12. The length of the aboveground and underground parts of the seedlings was measured on days 3, 5, 7, 9, and 10. Field experiments were carried out at the experimental farm of NIISSAVKh (Tashkent region, Kibray district, Salar settlement). The emergence of seedlings was registered, field germination was assessed, and phenological observations were made over the plant growth and development period from June 1 to September 1. To assess the economically valuable characteristics of raw cotton (fiber yield, weight per box, fiber length), test samples were collected before harvesting. Yields were recorded on September 15, October 1, October 15, and November 1. In lab tests, the seeds treated with PMC Cu^{2+}:Ag 8:2, NanoAChS 0.5 %, 4:1 or PMC Cu^{2+}:Ag 7:3 had the best germination rate, the 96.0, 96.0, and 97.0 %, respectivey, that exceeded the control by 4.0-5.0 % and the Dalbron standard by 2.0-3.0 %. The aboveground part of the seedlings with NanoAChS treatment turned out to be 0.6-4.6 mm longer than that with AChS, the underground part was 0.1-2.1 mm longer. The differences between NanoChS and Chitosan were 0.3-2.1 mm and 0.8-2.3 mm, respectively, between PMC Cu^{2+}:Ag 8:2 and Kuprumhit — 0.3-4.1 mm and 0.1-2.7 mm. On days 3, 5, 7, 9, and 10, plants from seeds treated with nanopolymer preparations outperformed those threated with polymer preparations. PMC Cu^{2+}:Ag 8:2, NanoAChS and NanoChS had a more pronounced effect on seed germination and length of the aboveground and underground parts of the seedlings than their polymer counterparts. In field tests, the best indicators of growth and development were recorded for PMC

$Cu2^{+}$:Ag 8:2, PMC Cu^{2+}:Ag 7:3, and NanoAChS 0.5 %, specifically, the plant height on September 1 exceeded the control by 7.4; 8.0; 7.7 cm, the number of sympodial branches by 1.5; 1.2; 0.6 pcs, the number of boxes by 2.4; 1.8; 2.5 pcs. Also, upon seed treatments with PMC Cu^{2+}:Ag 8:2 and PMC Cu^{2+}:Ag 7:3, the cotton yields exceeded the control by 4.0 and 3.7 c/ha, respectively, and the Dalbron standard by 3.3 and 3.0 c/ha. A trend towards higher yields was observed from the first crop count. Therefore, nanopolymeric preparations PMC Cu^{2+}:Ag 7:3 and PMC Cu^{2+}:Ag 8:2 can be used for cotton seed encapsulation.

Keywords: *Gossypium hirsutum* L., cotton, seeds, variety, nanopreparations, germination, seedling length, phenological observation, growth, development, raw cotton, yield

Nanotechnology is a promising area of interdisciplinary research with great opportunities in medicine, pharmaceuticals, electronics, and agriculture. Advances in nanotechnology can be used to control insect pests using pesticides and insecticides based on nanomaterials, to increase crop productivity through the use of bioconjugated nanoparticles (encapsulation) to slowly release nutrients and water, for nanoparticle-mediated gene or DNA transfer in plants when creating varieties resistant to pests [1, 2].

In many countries (in Russia, Japan, India, China, USA, Vietnam, Germany, Uzbekistan), the effect of nanopolymer preparations on the sowing qualities of seeds, growth, development and productivity of agricultural crops, and metabolic processes in plants is being studied. Methods are being developed to obtain this information. In the Republic of Uzbekistan, work is also being carried out on the use of domestic nanomaterials. Nanopolymer preparations based on chitosan and its derivatives are being investigated. It has been shown that nanoparticles can have both positive and negative effects on plants depending on their size, concentration, chemical composition, stability and shape [3]. In addition, thanks to nanomaterials, it is possible to increase the efficiency of the restoration of soil contaminated with metals and metalloids [4]. Engineering nanomaterials with particle sizes of approximately 100 nm, where biological interactions are inevitable, have contributed to many revolutionary developments in various fields, including agriculture [5-10].

Encapsulation of seeds with preparations of chitosan and its derivatives has a positive effect on the process of photosynthesis in cotton plants even against a wilt background [11]. Chitosan nanoparticles are a natural material with excellent physicochemical, antibacterial and biological properties. Chitosan nanoparticles have a beneficial effect on the environment, have biological activity and do not adversely affect the human body [12-15]. They are widely used in agriculture, especially for crop protection, aided by their size, high surface area to volume ratio, and unique optical properties [16, 17]. The use of chitosan nanoparticles increases the content of chlorophyll and improves the absorption of nutrients by plants, affects germination, seedling growth and wheat yield [18, 19]. Chitosan nanopurges have an effect at lower concentrations than chitosan, in particular, they can enhance the growth of wheat, which reduces the use of fertilizers [20].

Since the end of the 20th century, the Research Institute of Breeding, Seed Production and Agricultural Technology of Cotton Growing (Republic of Uzbekistan) has been studying the physicochemical and biologically active properties of chitin and chitosan isolated from silk-reeling production waste, the silkworm (*Bombyx mori* Linnaeus, 1758) pupae [21]. A technology has been developed and production of *Bombyx mori* chitosan and its various derivatives for agricultural use has been established [21].

In this work, it was revealed for the first time that the treatment of cotton seeds of the Andijan 36 variety with preparations containing copper and silver ions

based on chitosan nanopolymers from silkworm pupae accelerates the growth and development of seedlings, and also increases the yield of cotton.

The purpose of the work is to evaluate the effect of biologically active nanopolymer complexes based on chitosan and its derivatives on the morphophysiological and sowing parameters of seeds, the economically valuable qualities of raw cotton, and the yield of cotton.

Materials and methods. The studies were carried out in laboratory and field conditions in 2018-2020 at the Breeding, Seed Production and Agricultural Technology of Cotton Growing (NIISSAVKh). Environmentally safe biologically active nanopolymer preparations based on chitosan and its derivatives were synthesized at the Institute of Chemistry and Physics of Polymers of the Republic of Uzbekistan Academy of Sciences (ICPPRUAS). In the experiments, we used nanopolymer preparations of PMC (polymer metal complex) $Cu2^{+}$:Ag 7:3, PMC Cu2+:Ag 8:2, Nanochitosan (NanoChZ, 0.5%; 90 kDa), Nanoascorbatchitosan (NanoAChZ, 0.5%, ratio of chitosan to ascorbic acid 4:1) and polymer preparations Chitosan initial 0.5%, Kuprimhit 0.5%, Ascorbatchitosan (AChZ). Preparations UZKHITAN (ICPPRUAS) and Dalbron (Dalston associated SA, Panama) served as standards. Control seedswere not treated with preparations.

We used cotton (*Gossypium hirsutum* L.) seeds of the Andijan 36 variety which is included in the State Register of crops recommended for sowing on the territory of the Republic of Uzbekistan.

Seeds were soaked in preparations (20 l/t of seeds) at least 3-4 days before sowing. In each option, 6 kg of cotton seeds were processed. In a lab tests, the seeds were germinated in a thermostat in sand at 25 °C and the air humidity of 60-65%. Germination energy was determined on day 4, germination rate on day 12. The length of the aboveground and underground parts of the seedlings was measured on days 3, 5, 7, 9, and 10.

Field trials were carried out at the experimental farm of NIISSAVKh (Tashkent region, Kibray district, Salar village) as accepted [22] on 50-hole plots, sowing was carried out manually, 5 seeds per hole.

Seedling emergence was recorded, and field germination was calculated. Phenology of plant growth and development was examined from June 1 to September 1. To assess the economically valuable characteristics of raw cotton (fiber yield, weight of one box, fiber length), test samples were collected before harvesting. Yields were assessed on September 15, October 1, October 15 and November 1 in accordance with the current instructions. Sowing and varietal qualities were determined based on existing standards [23-25].

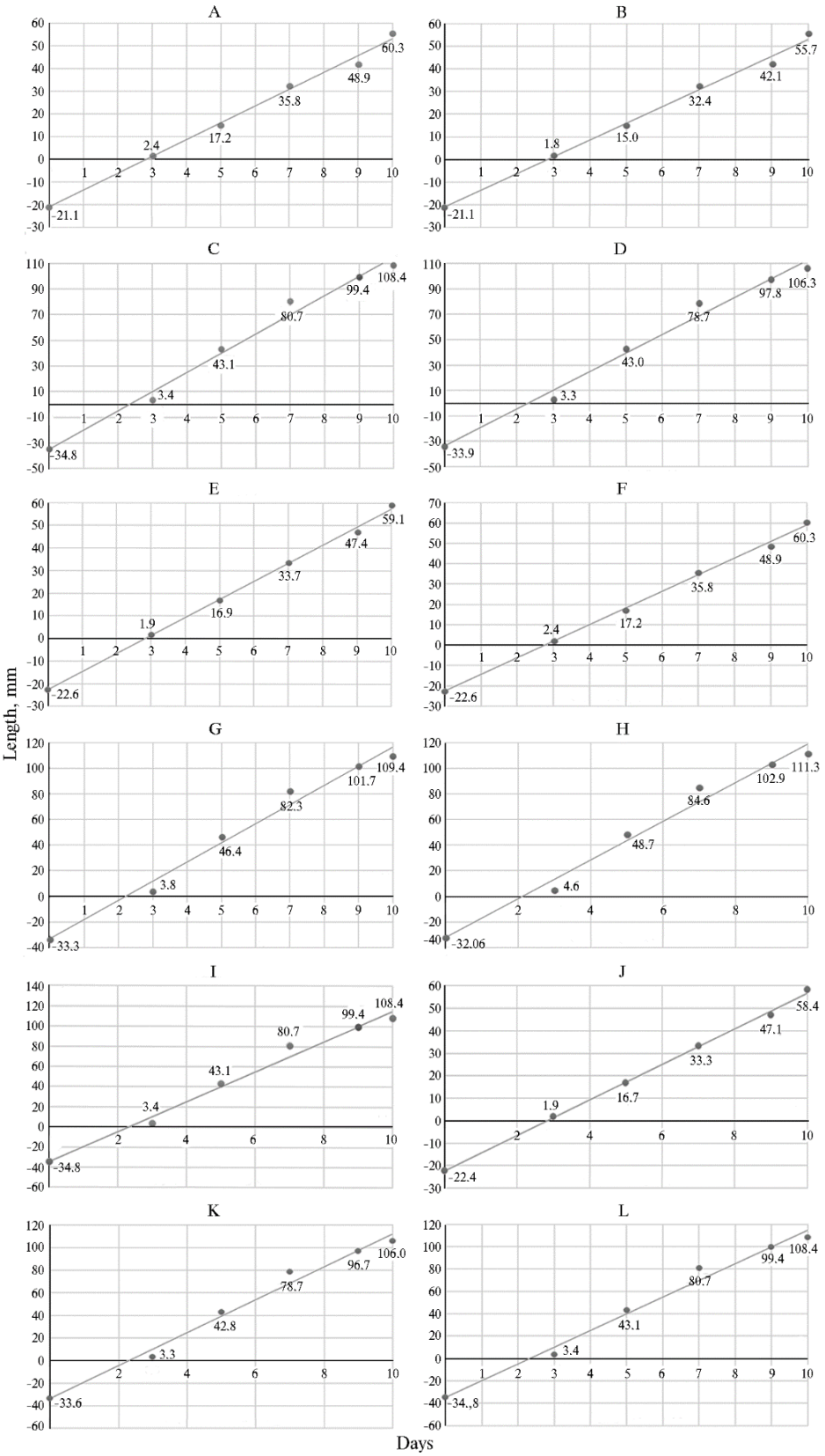
Mathematical processing of the obtained results was carried out according to B.A. Dospekhov [26]. Means (M) and standard errors of means (±SEM) were calculated, and the least significant difference for the 5% significance level (LSD_{05}) was calculated. The least squares method [27] was also used for analysis.

Results. For high and guaranteed raw cotton yields, the use of a wide range of environmentally friendly plant protection products for pre-sowing seed processing are promising. Metal nanopowders and nanoparticles easily penetrate cells and actively influence enzymes and physiological and biochemical reactions, increase laboratory and field germination [28, 29]. Analysis of variance showed that pre-sowing treatment of seeds with nanopreparations has a significant effect on most plant traits: the length of seedlings, roots and shoots, their wet and dry weight, resistance to pathogens and pests, as well as on indicators of crop structure and yield [30].

1. Germination and length of cotton (*Gossypium hirsutum* L.) cv. Andijan 36 seedlings upon seed treatment with nanopolymer and polymer preparations (lab tests, $N = 4$, 2018-2020)

Treatment	Germination energy/germination rate, %			Length, ьmm									
				day 3		day 5		day 7		day 9		day 10	
	day 4	day 12	to control	AG	UG	AG	UG	AG	UG	AG	UG	AG	UG
Control (no treatment)	90.0±0.95	92.0±0.6	0	1.4±0.06	3.0±0.05	14.6±0.11	39.4±0.25	31.7±0.22	76.8±0.21	40.6±0.30	90.2±0.51	49.7±0.32	101.5±0.31
Dalbron (reference)	92.0±0.2	94.0±0.9	+2.0	1.5±0.04	3.1±0.05	15.1±0.09	42.1±0.37	32.1±0.25	77.2±0.29	42.3±0.31	91.9±0.37	50.3±0.31	103.7±0.35
UZKHITAN (standard)	94.3±0.4	95.0±0.2	+3.0	1.7±0.05	3.4±0.04	14.8±0.12	42.8±0.29	31.9±0.33	78.4±0.19	43.4±0.25	94.2±0.39	54.1±0.32	105.2±0.38
Chitosan original 0.5%	94.2±0.7	95.0±0.2	+3.0	1.9±0.04	3.8±0.06	16.9±0.13	46.4±0.41	33.7±0.30	82.3±0.28	47.4±0.26	101.7±0.56	59.1±0.38	109.4±0.39
NanoChZ 0.5%	94.8±0.6	95.0±0.2	+3.0	2.4±0.06	4.6±0.07	17.2±0.14	48.7±0.23	35.8±0.25	84.6±0.31	48.9±0.34	102.9±0.41	60.3±0.47	111.3±0.52
PMC $Cu2^{+}$:Ag 7:3	94.9±0.1	97.0±0.3	+5.0	1.6±0.05	3.5±0.04	16.1±0.09	43.2±0.33	33.1±0.23	79.8±0.28	43.1±0.33	98.8±0.54	56.8±0.41	107.9±0.45
PMC Cu^{2+}:Ag 8:2	95.1±0.2	96.0±0.4	+4.0	1.9±0.03	3.4±0.05	16.7±0.10	43.1±0.27	33.3±0.28	80.7±0.30	47.1±0.18	99.4±0.37	58.4±0.47	108.4±0.42
Kuprumhit	93.6±0.3	95.0±0.2	+3.0	1.6±0.04	3.3±0.03	15.7±0.13	42.8±0.36	32.3±0.19	78.7±0.23	43.0±0.21	96.7±0.38	55.6±0.39	106.0±0.42
AChZ	93.7±0.8	95.0±0.2	+3.0	1.8±0.06	3.3±0.03	15.0±0.08	43.0±0.28	32.4±0.29	78.7±0.33	42.1±0.32	97.8±0.42	55.7±0.37	106.3±0.38
NanoAChZ 0.5%, 4:1	94.5±0.5	96.0±0.4	+4.0	2.4±0.04	3.4±0.04	17.2±0.17	43.1±0.42	35.8±0.31	80.7±0.25	48.9±0.27	99.4±0.45	60.3±0.35	108.4±0.43
$LSD_{05} = 2.21$ %													

N o t e. For a description of the preparations and the experimental design, see the Material and methods section; AG — aboveground part, UG - underground part.



The length of roots and seedlings of cotton (*Gossypium hirsutum* L.) cv. Andijan 36 on days 3-10 upon

seed treatment with nanopolymer and polymer preparations: A — NanoAChZ, seedlings (k = 7.68), B — AChZ, seedlings (k = 7.42), C — NanoAChZ, roots (k = 14.74), D - AChZ, roots (k = 14.66), E — Chitosan original, seedlings (k = 8.00), F — NanoAChS, seedlings (k = 8.16), G — Chitosan original, roots (k = 15.01), H — NanoChZ, roots (k = 15.07), I — Kuprumhit, seedlings (k = 7.46), J — PMC Cu^{2+}:Ag 8:2, seedlings (k = 7.92), L — Kuprumkhit, roots (k = 14.58), M — PMC Cu^{2+}:Ag 8:2, roots (k = 14.98) (lab test, 2018-2020). For a description of the preparations and the experimental design, see the Material and methods section.

In our lab tests, the seeds treated with PMC Cu^{2+}:Ag 8:2 (96.0%), NanoAChZ 0.5%, 4:1 (96.0%) and PMC Cu^{2+}:Ag 7:3 (97.0%) had the best germination which was 4.0-5.0% higher than in the control and 2.0-3.0% higher comared to Dalbron (reference) (Table 1).

Previously, we found that the nanopolymer preparations Nanoascorbatchitazan, PMC Cu^{2+}:Ag 7:3 and PMC Cu^{2+}:Ag 8:2 have a positive effect on the laboratory germination of soybean seeds. In addition, the yield of Selecta 302 and Baraka varieties when treated with these preparations was higher than that for Gaucho WS 70 standard (wetting powder, Bayer Crop Science, Germany) by 6.1-4.5 and 5.1-3 .3 c/ha, respectively [31]. The effectiveness of the action of nanopolymer preparations on the activity of peroxidase and polyphenol oxidase enzymes and protein content in 7-day-old soybean seedlings has also been proven [31].

V. Saharan et al. [32] revealed higher values of germination, length of shoots and roots, number of roots, length of seedlings, wet and dry weight of corn seedlings treated with Cu-chitosan nanoparticles (NP) at concentrations of 0.04-0.12% compared to water, $CuSO_4$ and loose chitosan. Cu-chitosan NPs at the same concentrations induced the activity of α-amylase and protease enzymes. Cu-chitosan NPs at a concentration of 0.16% had an inhibitory effect on the growth of seedlings, which can be explained by the toxicity of copper excess [32].

In this work, we compared the effectiveness of the use of nano-preparations with polymer preparations on cotton. When measurements were carried out at all times, the length of the above-ground part of the seedlings in the variant with NanoAChZ turned out to be 0.6-4.6 mm longer than in the variant with ACP, and the length of the underground part by 0.1-2.1 mm. When comparing NanoChZ with Chitosan, the differences were 0.3-2.1 mm and 0.8-2.3 mm, respectively; when comparing PMC Cu^{2+}:Ag 8:2 and Kuprumkhit, by 0.3-4.1 mm and 0.1-2.7 mm (see Table 1, Fig.). In all measurements on days 3, 5, 7, 9, 10, plants from seeds treated with nanopolymer preparations outperformed the variants where polymer preparations were used. PMC Cu^{2+}:Ag 8:2, NanoAChZ and NanoChZ had a more effective effect on the germination and length of the above-ground and underground parts of seedlings than their polymer counterparts.

An analysis of the results obtained using the least squares method [27] and summing up the influence of various composite compositions is given in the form of the dynamics of biological effects over time (five points, days 3, 5, 7, 9 and 10). It seems that the most realistic comparative indicator of these dependencies can be linear:

$$y = kx + b,$$

where y is an indicator characterizing the growth of the object, x are the observation periods, k and b are the internal parameters of the phenomenon under study. As can be seen from the figure, when using nanopreparations, the value of the coefficient k, which was considered as a collective indicator of the effect of preparations on the efficiency of plant growth, was higher than in the variants with polymer preparations.

In 2018-2020, in the experimental farm of NIISSAVKh, we studied the effect of nanopolymer preparations on the sowing qualities of seeds, the growth and development of plants, as well as the yield of cotton.

2. Yield and economically important traits of cotton (*Gossypium hirsutum* L.) cv. Andijan 36 upon seed treatment with nanopolymer and polymer preparations ($N = 4$, experimental farm NIISSAVKh, Republic of Uzbekistan, 2018-2020)

Вариант	The number of plants per ha	Yield, c/ha					Economically important traits					
		10/01	10/15	11/01	total	± to control	fiber output, %	± to control	pod weight, g	± to control	fiber length, mm	± to control
Control (no treatment)	66.7±0.2	27.3±0.4	6.4±0.2	3.4±0.2	37.1±0.4	0	38.5±0.4	0	5.65±0.06	0	34.8	0
Dalbron (reference)	71.2±0.3	28.1±0.5	6.2±0.2	3.5±0.3	37.8±0.3	+0.7	38.6±0.5	+0.1	5.76±0.06	+0.11	34.7±0.3	–0.1
UZKHITAN (standard)	73.4±0.3	29.4±0.4	6.5±0.3	2.7±0.2	38.6±0.4	+1.5	38.5±0.4	0	5.72±0.07	+0.07	34.8±0.3	0
AChZ	74.3±0.3	29.7±0.5	5.9±0.3	2.9±0.3	38.5±0.3	+1.4	38.4±0.3	–0.1	5.76±0.06	+0.11	34.9±0.2	+0.1
PMC Cu^{2+}:Ag 7:3	76.6±0.3	32.1±0.5	6.1±0.2	2.6±0.3	40.8±0.3	+3.7	38.7±0.4	+0.2	5.83±0.10	+0.18	35.0±0.3	+0.2
PMC Cu^{2+}:Ag 8:2	77.1±0.4	33.4±0.5	5.8±0.3	1.9±0.2	41.1±0.3	+4.0	38.7±0.5	+0.2	5.84±0.07	+0.29	35.0±0.3	+0.2
Chitosan original	72.4±0.4	29.3±0.4	5.5±0.2	3.3±0.3	38.1±0.4	+1.0	38.5±0.4	0	5.87±0.06	+0.22	34.8±0.2	0
NanoChZ	75.5±0.2	32.2±0.4	5.2±0.3	2.1±0.2	39.5±0.4	+2.4	38.6±0.3	+0.1	5.71±0.05	+0.06	34.9±0.3	+0.1
NanoAChZ 0.5%	76.8±0.3	32.9±0.3	5.8±0.3	1.8±0.3	40.5±0.4	+3.4	38.6±0.4	+0.1	5.83±0.07	+0.18	34.9±0.2	+0.1
LSD_{05} = 2.34 c/ha												

N o t e. For a description of the preparations and the experimental design, see the Material and methods section.

The germination of the Andijan 36 cv. seeds treated with NanoChZ, PMC Cu^{2+}:Ag 8:2, PMC Cu^{2+}:Ag7:3, Chitosan original was higher by 15.3; 15.1; 13.6 and 13.0%, respectively, and exceeded the values obtained with the UZKHITAN standard by 11.9; 11.7; 10.2 and 9.6%. The best effect on growth and development were recorded when PMC Cu^{2+}:Ag 8:2, PMC Cu^{2+}:Ag 7:3 and NanoAChZ 0.5% were treated. On September 1, the plants were higher than the control ones by 7.4, 8.0, 7.7 cm and exceeded the control by 1.5, 1.2, 0.6 sympodial branches and 2.4, 1.8, 2.5 boxes per plant.

The yield forecast carried out on September 1 showed that PMC Cu^{2+}:Ag 8:2 and PMC Cu^{2+}:Ag 7:3 preparations had an advantage over the control. The yield upon the treatments was higher by 4.8 and 5.2 c/ha, respectively. It was also found that the yield of raw cotton is more affected by seed treatment with nanopolymer preparations than with polymer ones (Table 2). In almost all variants, where the seeds were treated with nanopolymer preparations, the yield was higher vs. control and vs. the standard. For example, with PMC Cu^{2+}:Ag 8:2, the yield exceeded the control by 4.0 c/ha, with the Dalbron standard by 3.3 c/ha, and with the UZKHITAN standard by 1.6 c/ha. It should be noted that a trend towards higher yields occurred from the first count, which indicates an earlier ripening of cotton when seeds were treated with nanopolymer preparations.

Based on test samples, economically valuable features of raw cotton were determined. The yield of fiber, the mass of raw cotton in one box and the length of the fiber corresponded to the author's description of the varieties. We did not find a wide variation in parameters between the tthes treanments (data not shown), that is, nanopolymer preparations did not affect the economically valuable traits but had a positive effect on the overall yield.

Our results are consistent with the data obtained by M. Wang et al. [33] on four irrigated and rainfed wheat varieties upon reatment with chitosan oligosaccharides (COS). The preparations were used for seed treated and foliar spraying at different stages of plant growth. In varieties of irrigated wheat, the number of grains per ear increased significantly when seeds were treated, and the number of spikelets when leaves were sprayed at tillering and heading. COS significantly affected the grain yield in all irrigated varieties, while the effect of chitosan oligosaccharides on rainfed varieties was insignificant [33]. D.-F. Zeng et al. [34] treated soybean seeds using a new chitosan-based preparation with carboxymethylchitosan as the main component supplemented with trace elements and growth regulators. Upon treatment, the soybean yield increased by 17.95%. The drug had an excellent antifidant effect.

Thus, cotton Andijan 36 cv. seeds treated with nanopreparations PMC (polymer metal complex) Cu^{2+}:Ag 7:3, PMC Cu2+:Ag 8:2 and NanoAChZ 0.5% 4:1 had a high laboratory germination rate and exceeded the control by 4-5% and Dalbron standard by 2-3%. High biological activity of these preparations at the first stage of seed germination also leads to an increase in the length of the aboveground and underground parts of the seedlings and activates plant growth. In field trials, plants from seeds treated with PMC Cu^{2+}:Ag 8:2 and PMC Cu^{2+}:Ag 7:3 outperformed the control by 4.0 and 3.7 c/ha, respectively, and the Dalbron standard by 3.3 and 3.0 c/ha. From the first record, there was a trend towards higher yields. Therefore, nanopolymer preparations PMC Cu^{2+}:Ag 7:3, PMC Cu^{2+}:Ag 8:2 which are recommended for all regions of the Republic of Uzbekistan, can be used to encapsulate cotton seeds.

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