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. 56, Issue 5
September-October

2021 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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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], 2021, Vol. 56, № 5

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2021, V. 56, Iss. 5, pp. 823-842
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reviews, challenges

UDC 631.522/.524:575:577.21

doi: 10.15389/agrobiology.2021.5.823eng
doi: 10.15389/agrobiology.2021.5.823rus

TRANSCRIPTION FACTORS OF THE MADS FAMILY IN PLANTS: RELATIONSHIP WITH DOMESTICATION TRAITS AND PROSPECTS FOR BREEDING

(review)

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The authors declare no conflict of interests
Acknowledgments:
Supported financially from the Russian Science Foundation (grant No. 21-16-0008), the Russian Foundation for Basic Research (grant No. 18-29-07007) and the Ministry of Science and Higher Education of the Russian Federation
Received June 9, 2021

Abstract

The traits of domestication, which are subdivided into three groups (productivity, adaptability, and reproduction) and together make up a domesticated syndrome that brings together taxonomically distant domesticated forms, remain economically significant in modern cultivated crops as well. A significant part of the genes that control domestication traits in plants are represented by the genes of transcription factors, in particular, those belonging to the MADS-domain family. MADS-domain proteins are key regulators of almost all aspects of plant reproductive development, including the determination of the flowering time, the inflorescence structure, the flower organ identity, the development of roots, fruits, and seeds, as well as the adaptive and stress response to adverse environmental conditions. The presented review describes the possible involvement of MADS-box genes in plant domestication and breeding. We discuss the role of MADS-box genes in the regulation of vernalization (plant response to prolonged cold treatment), bud physiological dormancy, inflorescence and flower structure, plant fertility and fruit qualitative traits (ripening characteristics, synthesis of carotenoids and anthocyanins, the number of seeds, fruit shuttering, fruit shelf life), as well as plant stress response (salinity, drought, temperature changes). The phenomenon of MADS-box gene functional pleiotropy and redundancy (due to the existence of paralogs) is considered. It has been supposed that MADS-box genes high structural and functional conservatism may indicate their high potential as tools for predictable fine tuning of crop phenotypes by combining (including dose-dependent) different alleles and paralogs of MADS-box genes. Another possible method is the separation of the pleiotropic functions of the MADS-box gene by introducing mutations in its coding or *cis*-regulatory sequence to alter specific protein-protein or protein-DNA interactions, as well as the pattern and/or level of expression, including in response to various external and internal signals. It is concluded that fundamental and applied studies of MADS-box genes in various plant species (both wild and cultivated) will not only lead to a deeper understanding of the evolution and development of modern plants, but will also greatly contribute to the improvement of crops, including using CRISPR/Cas and other modern technologies.

Keywords: transcription regulation, transcription factors, MADS-box genes, conservatism, pleiotropy, domestication traits, productivity, adaptation, reproduction, economically valuable traits, target genes

Crops have emerged on the course of domestication, in which wild plant species have adapted to cultivation by humans in the process of co-evolution with them [1]. At the same time, the domesticated forms developed traits useful for volume, quality, harvest and storage time of the crop, as well as for adaptation to the influence of the environment [1-4]. Together, they constitute a domestication syndrome that brings together taxonomically distant domesticated forms, and are subdivided into three groups - productivity, adaptability and reproduction [5, 6],

which remain economically significant in modern cultivated crops. According to various estimates, there are currently known from 1000 to 2500 semi- and fully domesticated plant species from 120-160 families [2, 7]. Thanks to intensive genetics, genomics and archeology research, a view on how domestication took place is gradually being formed [2, 3, 8-10], which is of interest both for deepening the understanding of evolutionary events and for modern breeding programs based on the knowledge of the molecular genetic characteristics of the regulation of economically valuable traits. Moreover, it is assumed that understanding the evolutionary origin and regulation of key features of domestication can help not only in the improvement of existing and breeding new varieties, but even in the domestication of new plant species [10].

Observations based on archaeobotanical studies, population genomic analysis, and the study of ancient DNA have shown that formation of the phenotype of various cultures with the fixation of key features takes about 2-3 thousand years [3, 10-14]. The main reasons for such a long process are considered to be the flow of genes between populations of nascent domestic plants and their wild ancestors [15, 16], as well as the polygenic nature of many traits [17]. Besides, it is assumed that, although some characteristics (for example, color and taste) are most likely due to a conscious choice of a person, most of the signs of domestication (resistance to shattering of seeds, synchronous germination, etc.) were initially the result of unconscious selection occurring naturally [10, 17]. Introgressive hybridization between wild relatives is considered as a mechanism of plant domestication [18], due to which the diversification of crops [19], for instance, the banana *Musa* spp. [20], wheat *Triticum aestivum* [21], rice *Oryza sativa* [22], corn *Zea mays* [23], barley *Hordeum vulgare* [24], apple *Mlus domestica* [25], and other perennial fruit crops [26] is happening today.

Interestingly, the genes underlying the traits of domestication and diversification in different plant species are in many cases the same or closely related [4, 5, 9, 27]. It forms the basis for the use of evolutionary homology in order to transfer the desired traits to many species, of which by using new technologies (for example, CRISPR / Cas) that make it possible to repeat the genetic stages of domestication [28-30].

A significant part of the identified genes associated with the traits of domestication are transcription regulator genes. Although they account for only about 5% of protein-coding genes in the plant genome, changes in them can affect a whole set of properties in a relatively short time [31-33].

Genes of transcription factors belonging to the MADS-domain family, which encode the conserved nucleotide sequence MADS-box (MADS-box genes), found in almost all eukaryotes are often considered the evolutionary targets. It is believed that duplication of precursors of MADS-box genes and subsequent diversification and neo- and subfunctionalization of duplicates played and play one of the key roles in the evolution and diversity of plants. [33-35]. While duplicates of most genes lose their functions, genes of transcription factors, including those that regulate transcription with the MADS domain, retain and renew their functions after duplication, which contributes to the expansion of genetic opportunities for the emergence of evolutionary innovations [36]. Moreover, analysis of the genomes of three pepper species - *C. baccatum*, *C. chinense*, and *C. annuum* showed that MADS-box genes are included in the top ten gene families with the largest mass duplication [37], which indicates the key positions occupied by these genes in evolution and diversification of plants. MADS-domain transcription factors are key regulators of almost all aspects of plant reproductive development, including the determination of the flowering time, the inflorescence and flower

structure, formation of pollen, seeds, fruits, as well as development of roots [38], and plant response to various stresses [39]. All this is another confirmation of the importance of MADS-box genes as objects of selection during the domestication of crops. Therefore, the data of the functional analysis of MADS-box genes in combination with the available biological resources can be used to improve various reproductive traits of crops using modern molecular breeding technologies.

This review is focused on the family of MADS transcription factors and their participation in the formation of characteristics of productivity, adaptability, and reproduction in plants.

Transcription factors of MADS-domain family. The abbreviation MADS comes from the names of the family founders: MINICHROMOSOME MAINTENANCE 1 (MCM1) (*Saccharomyces cerevisiae*), AGAMOUS (AG) (*Arabidopsis thaliana*), DEFICIENS (DEF) (*Antirrhinum majus*) and SERUM RESPONSE FACTOR (SRF) (*Homo sapiens*) (38). MADS-domain transcription factors are characterized by the presence of a highly conserved DNA-binding N-terminal MADS-domain [38].

In plants, MADS-domain transcription factors are represented by two structural types: type I — the protein including the MADS_SRF_like MADS-domain (NCBI: cd00266); type II, or MIKC, - the protein includes the MADS_MEF2_like MADS-domain (NCBI: cd00265), the interdomain I-site, the conservative keratin-like K-domain K-box (NCBI: pfam01486) and the variable C-region located sequentially (40). The first to isolate the MIKC genes, when knocked out, lead to a complete or partial homeotic transformation of some flower organs into others. Thus, the loss of the function of *DEF* or its ortholog *APETALA3 (AP3)* in *Arabidopsis* leads to the development of sepals instead of petals and carpels instead of stamens, and the *agamous-1* mutation causes the transformation of stamens into petals [41, 42].

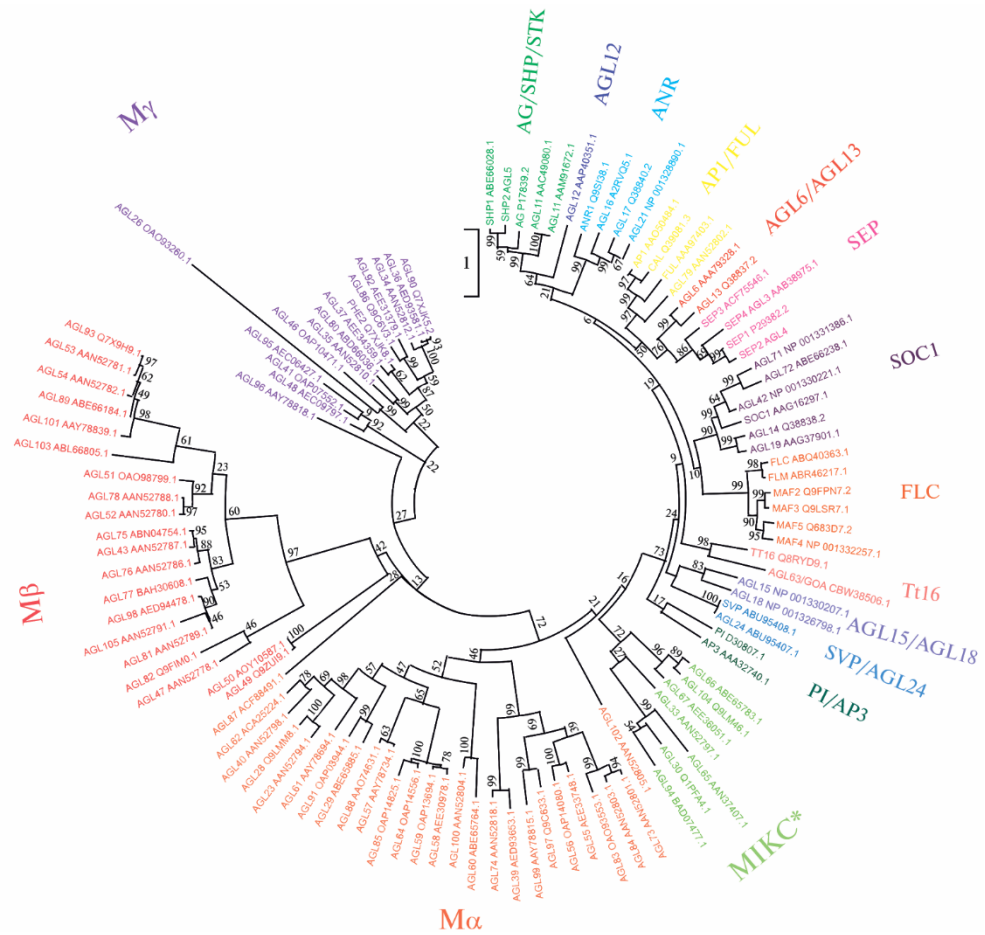
The number of MADS-box genes in the genomes of various plant species, including crops

Taxonomic group	Species	Gene number	Reference
Moss	*Physcomitrella patens*	23	[43]
Glycophytes	*Selaginella moellendorffii*	40	[132]
Gymnospermae	*Picea abies*	278	[33]
	Pinus taeda	367	[133]
	Gnetum gnemon	41	[133]
Angiosperm monocotyledons	Model plant for grain crops species *Brachypodium distachyon*	75	[33]
	Rice *Oryza sativa*	75	[33]
	Wheat *Triticum aestivum*	180	[134]
Angiosperm dicotyledons	Model species *Arabidopsis thaliana*	107	[40]
	Basal group of flowering plants *Amborella trichopoda*	33	[132]
	Brassica rapa	160	[135]
	Glycine max	106	[136]
	Malus domestica	146	[137]
	Citrullus lanatus	39	[138]
	Lactuca sativa	82	[139]
	Vitis vinifera	90	[140]
	Solanum tuberosum	167	[33]
	Solanum lycopersicum	131	[33]

In total, the genome of the *A. thaliana* model plant contains 107 MADS-box genes (40); genomes of other plant species, including crops, include from 23 to 367 of them (table). The MADS-box family is divided into subfamilies, most of which are preserved throughout the evolution of seed plants (see Fig.), and the functions of genes within each subfamily in different plant species are often homologous [40, 43]. For example, the genome of all flowering plant species contains

orthologs of the *AP3 / DEF* and *AGAMOUS (AG)* genes involved in the development of reproductive organs [35, 40, 43], as well as the flowering time gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1,* or *AGL20)* [44].

The presence in the plant genome of duplicates of MADS-box genes that have undergone subfunctionalization leads to redundancy of function [45]. Thus, *AG* is involved in the reproductive development of a flower, while its paralogs *SHATTERPROOF1 (SHP1)* and *SHP2* are involved in the development of ovules [46]. This is due to differences in the expression patterns of these genes, since overexpression of *SHP1* and *SHP2* in *Arabidopsis* plants with the *ag* mutation is able to restore the development of stamens and carpels [46].



Phylogeny of MADS-domain transcription factors in *Arabidopsis thaliana*. Subfamilies are highlighted in different colors. The Mα, Mβ and Mγ subfamilies include type I MADS-domain proteins, the rest of the subfamilies are MIKC type II MADS-domain proteins. Next to the name of each protein is its identification number in the NCBI database (https://www.ncbi.nlm.nih.gov/). Bootstrap values are indicated at the base of the branches. The dendrogram was built using the MEGA 7.0 program (https://www.megasoftware.net/) using the maximum likelihood method.

Two genes involved in the control of the identity of the flowering meristem, *APETALA1 (AP1)* and *CAULIFLOWER (CAL)*, on the contrary, have similar expression patterns, but differ in functionally, partially duplicating each other [47]. While plants with the *ap1* mutation show strong defects in the identity of the flowering meristem and flower organs, the *cal* phenotype is similar to the wild type, while the *ap1 cal* double mutation has the cauliflower phenotype [47, 48]. It has been shown that the functional difference between CAL and AP1 is partly due

to several substitutions of amino acid residues that alter the pattern of protein-protein interactions [49].

The results of intensive studies of the evolution of MADS-box genes and their contribution to the evolution and diversification of flowering plants allow us to make assumptions about the role of MADS-box genes in the domestication of flowering plants [33-35]. Next, we will consider the economically valuable traits of modern cultures and their relationship with the MADS-box genes.

Flowering time of plants. Vernalization. With the transition of a plant from vegetative growth to reproductive development, the apical meristem of the shoot becomes an inflorescence meristem, on the periphery of which flowering meristems are formed. Control of this process is one of the targets of adaptation mechanisms [50]. Analysis of natural variations, mutations, and transgenic *A. thaliana* plants that bloom later or earlier than the wild type revealed gene loci involved in the regulation of flowering time [50].

Today, six main signaling pathways are known, under the influence of which the transition to flowering occurs. Of these, three (the autonomous pathway, the age pathway, and the pathway mediated by gibberellins) are largely independent of external signals, and the fourth (the photoperiod pathway) starts or stops flowering in response to changes in day length. The remaining two paths are temperature-dependent. Together, signaling pathways control the main regulators of flowering time - the MADS-box genes *FLOWERING LOCUS T (FT), FLOWERING LOCUS D (FD),* and *SOC1*, whose products activate the transcription of genes for the identity of the inflorescence and flower meristems [44, 51].

The temperature-dependent signaling pathway of vernalization, reflecting the plant's susceptibility to prolonged exposure to cold, effectively uses the MADS-box genes [51-54]. The vernalization syndrome in plants probably arose as an adaptation to seasonal cold and local climatic conditions [52] and is important for growing crops: spring varieties insensitive to vernalization are sown in spring, while sensitive winter varieties - in autumn [55]. Thus, vernalization, obviously, was the goal of artificial selection during domestication of monocotyledonous and dicotyledonous crops, and the key targets in this case were the MADS-box genes.

One of them is the flowering repressor *FLOWERING LOCUS C (FLC*, or *FLF): in Arabidopsis*, the FLC factor suppresses the transcription of genes for the central flowering stimulators *SOC1, FT*, and *FD* [44, 51, 53]. Prolonged cold (vernalization) interferes with the expression of *FLC*, including epigenetic mechanisms, in particular, the modification of histones at the *FLC* locus, which, in turn, allows the activation of genes promoting flowering [54]. The genetic variability of *FLC*, which determines the amount and activity of the synthesized protein, can alter the need for vernalization in different *Arabidopsis* ecotypes [54]. Therefore, variations in *FLC* orthologues could play an important role in the adaptation of crops to different climatic conditions [56].

A striking example of the importance of the *FLC* genes is the species of the genus *Brassica L.* (*Brassicaceae* family) [57]. Thus, early flowering in Chinese cabbage *Brassica rapa* ssp. *pekinensis* (leafy vegetables) reduces the quality of the crop. Oilseeds (oilseed rape *Brassica napus* and field mustard *Brassica rapa* ssp. *oleifera*) have winter and spring varieties suitable for adapting reproductive development to various environmental conditions. Root vegetables (turnip *Brassica rapa* ssp. *rapa*) and, finally, cabbage (*Brassica oleracea*, varieties - cabbage var. *oleracea*, broccoli var. *italica*, cauliflower var. *botrytis*) are also subject to temperature-dependent regulation of flowering time.

Four *FLC* orthologs (*BrFLC1, BrFLC2, BrFLC3*, and *BrFLC5*) have been identified in the *Brassica* genome, variations of which determine the differences

in the flowering time of turnip cultivars [56, 58-60]. The *BrFLC1* gene is associated with late flowering of Chinese cabbage [58], and a mutation in the *BrFLC2* sequence is associated with accelerated flowering of rapeseed [61]. Variations in the *FLC* gene are responsible for differences between spring and winter rapeseed varieties [56] and changes in flowering time in broccoli [62]. Note that in the genome of *B. napus* resulting from the alloploidy between the paleopolyploid ancestors of *B. rapa* and *B. oleracea*, the flowering time genes are excessively represented; in particular, the *FLC* has nine identifiable copies [57]. Obviously, during the formation of *Brassica* species and their domestication, a number of molecular changes in the *FLC* orthologues and the presence of several *FLC* paralogs contributed to the differences in the sensitivity to vernalization and flowering time.

FLC orthologues have also been identified in cereals - barley (*H. vulgare*), wheat (*T. aestivum*), rice (*O. sativum*), and corn (*Z. mays*) [63]. Within the clade FLC of monocots, there are subclades *OsMADS51* and *OsMADS37*; the *OsMADS51* subclade is divided into two groups - *ODDSOC1* and *ODDSOC2* [63]. In wheat, the homologues of *ODDSOC2, TaAGL42* and *TaAGL33*, are characterized by different expression profiles in spring and winter varieties [63, 64]. This suggests that members of the *ODDSOC2* group were part of an adaptive mechanism through which different populations of cereals acquired different needs for vernalization [63, 64].

The *A. thaliana* genome contains five *FLC* paralogs: *MADS AFFECTING FLOWERING2 (MAF2*, or *AGL31), MAF3 (AGL70), MAF4 (AGL69), MAF5*, and *FLOWERING LOCUS M (FLM,* or *MAF1, AGL27*) [63]. The study of different populations of *A. thaliana*, representing the genetic diversity of the species, confirmed that the quantitative trait loci (QTL) of flowering include all three types of *FLC*-like genes (*FLC, FLM*, and *MAF2-5*) [65]. In addition, the MADS-box genes of the *StMADS11* clade, which in *A. thaliana* is represented by genes *AGAMOUS-like* 24 (*AGL24*) and *SHORT VEGATATIVE PHASE (SVP)*, are actively involved in the temperature-dependent regulation of flowering [34, 44].

Depending on the temperature, the *FLM* gene has different forms of splicing, two of which generate two different proteins, FLM-β and FLM-δ [34]. FLM-β is considered to be the main functional form of FLM responsible for temperature response [34]. It is assumed that the SVP/FLM-β complex binds to the promoters of target genes, such as the flowering inducer SOC1, repressing flowering, while the SVP/FLM-β complex cannot bind to DNA and, competing with SVP / FLM-β, acts as an indirect flowering inductor [34]. The amount of FLM-β increases at low temperatures, and elevated temperatures destabilizes the SVP protein, which implies that higher temperatures favor flowering due to a decrease in the formation of the SVP/FLM-β complex [34].

The tandem *MAF2-5* genes serve as flowering repressors [34]. The *MAF2* gene prevents early flowering in response to short periods of cold, thus avoiding the induction of flowering in the warm autumn period before the winter cold [66]. Like *FLM, MAF2* and *MAF3* are characterized by temperature-dependent alternative splicing [67, 68]. The low-temperature form of *MAF2* encodes a protein that interacts with *SVP* to inhibit flowering; at elevated temperatures, splicing shifts towards a variant that encodes a protein that does not interact with *SVP*; thus, at lower temperatures, *MAF2* and *SVP* suppress flowering simultaneously with *FLM* and *SVP* [68]. This may also be true for other *MAF* genes; however, the activity of *MAF* genes is not excessive, which is confirmed by the analysis of mutant phenotypes for individual genes [68]. Tandem genes are especially susceptible to sequence rearrangements during non-allelic homologous recombination [69]. Similar structural deviations in the *MAF2-5* cluster could serve as a tool for adaptation

of species to different climatic conditions.

Besides *FLC* orthologs and paralogs, wheat vernalization is largely regulated by three *VERNALIZATION (VRN)* genes, two of which, *VRN1* and *VRN3*, are MADS-box genes [63]. Vernalization leads to an increase in the expression of the flowering stimulator *VRN1* (ortholog *AP1*), the product of which suppresses the transcription of the flowering repressor gene *VRN2*, mitigating the repressive effect of this gene on the flowering stimulator *VRN3* (orthologue *FT*); *VRN3* then upregulates *VRN1* expression resulting in positive feedback and induces flowering [63]. Given that the *FLC* clade exists in monocots, it seems likely that both *FLC* and *AP1 / VRN1*-like genes were present in the genome of ancestral dicotyledonous and monocotyledonous species, and each group was replenished differently in development of susceptibility to vernalization [63]. A wide variety of responses to vernalization in wheat, barley, and ryegrass (*Lolium perenne*) samples is caused by various mutations in the regulatory regions of *VRN1* orthologs [70-72]. Interestingly, the wheat MADS-box *VRN4* gene, which appeared as a result of the *VRN1* gene duplication, is not present in all wheat samples; its activity reduces the need for vernalization, which can be used by breeders to modulate the vernalization response [73].

Physiological rest of buds. For agriculture, it is important to obtain fruit trees that are adapted to local climatic conditions in terms of the timing of recovery from dormancy. Induction of bud dormancy due to winter cold is an adaptive feature of perennial plants of a temperate climate, which provides optimal protection of vegetative and reproductive meristems from low temperatures [74].

Similar to the regulation of flowering by vernalization, the emergence of dormant buds of woody plants requires exposure to low temperatures during a certain period of time, and the *DORMANCY-ASSOCIATED MADS-BOX* genes (*DAM, SVP* and *AGL24* orthologs) are key regulators of this process [74, 75].

The genome of peach (*Prunus persica*) contains a cluster of six tandemly located *PpDAM1-PpDAM6* genes, which are considered one of the most important genetic elements underlying the response to vernalization [74, 75]. In apple (*Malus × domestica*) and pear (*Pyrus communis*), the main QTLs associated with the response to cold and dormancy in the buds are also associated with the *DAM* gene loci [75].

Thus, it is believed that the *DAM* genes played a key role in fine tuning the flowering time and adaptation to different climatic zones in cultivated plants. It was also shown that, in addition to the *DAM* genes, overexpression of the MADS-box gene *BpMADS4* (subfamily *FUL*) of birch (*Betula pendula*) in poplar plants (*Populus tremula*) leads to a delay in the winter transition of buds to the dormant state [76].

Inflorescence structure. Inflorescence structure was an important target trait for increasing yields during plant domestication [9].

The most vivid example is the head of cauliflower and broccoli, consisting of a dense mass of inflorescences with a delay in development, respectively, at a very early and later stage, as well as many varieties with an intermediate phenotype [77, 78]. The cauliflower phenotype in mutant *Arabidopsis* plants is explained by the *ap1 cal* double mutation [47]. Similarly, in cauliflower and broccoli cultivars, the structure and function of the MADS-box genes *BoCAL* and *BoAP1* are disrupted [79]. This indicates the selection of certain *BoCAL* and *BoAP1* alleles, as a result of which plants with modified inflorescences were obtained [78]. At the same time, the existence of several *AP1* paralogs in the *B. oleracea* genome can determine the differences between different phenotypes by inflorescences [78, 79].

Another example is the branched inflorescences in tomato (*Solanum lyco-*

persicum), the formation of which is regulated by MADS-box genes of the *SEPALLATA1 (SEP1)* subfamily: *JOINTLESS2 (J2), ENHANCER OF JOINTLESS2 (EJ2)*, and *LONG INFLORESCENCE (LIN)* [80]. Branching of tomato inflorescences is usually accompanied by a high percentage of barren flowers, and combinations of different mutant alleles *J2, EJ2* and *LIN*, depending on the gene dose, can reduce branching and simultaneously increase the fruiting rate, which increases the yield [80].

Another important target trait is branching of shoots (tillering) [81]. It was shown that the MADS-box gene *OsMADS57* (subfamily *AGL17*) affects the tillering of *O. sativa* rice plants. The mutation of the transcription factor *OsMADS57*, associated with the absence of the C-terminal region, significantly increases tillering of the rice plant and, thus, increases the grain yield [82].

The *OsMADS1* gene (subfamily *SEP1*), the overexpression of which leads to dwarfism of rice plants [83], can be another target for altering the inflorescence structure.

Flower structure. Sterility. According to the ABCDE model, the budding of flowering organs is determined by the combinatorial interaction of genes of five different classes of activity: the identity of sepals is determined by genes of classes A and E, petals - A, B, and E, stamens - B, C, and E, carpels - C and E, and ovules - C, E and D [35]. Almost all ABCDE genes encode MIKC-type transcription factors containing MADS domains. In *Arabidopsis*, this is *AP1* (class A); *AP3* and *PISTILLATA* (*PI*) (B); *AG, SHP1* and *SHP2* (C), *SEEDSTICK (STK)* (D); *SEP1, SEP2, SEP3* and *SEP4* (E) [35]. Loss of the function of these genes leads to homeotic transformations of the flower. For example, the *ag* mutation causes petals to replace stamens, and carpels to new flowers with the same developmental model [41]. Such phenotypes are attractive in view of breeding for ornamental plants. For example, *ag* mutations, including those in the cis-regulatory regions of the gene (changing the profile of its expression), cause double flowers to form in the ornamental varieties of Japanese cherry *Prunus lannesiana* and rose *Rosa* spp. [84, 85]. In the apple tree, suppression of the activity of *AG* orthologues (*MdMADS15* and *MdMADS22*) leads to the appearance of decoratively attractive flowering trees and a decrease in the number of seeds due to male sterility [86].

Seedlessness and male sterility of apple fruits are also observed in the case of knockout of the *PI, MdPI* orthologue, when flowers form sepals instead of petals and carpels instead of stamens [87].

Male sterility and prevention of outcrossing are desirable in many crops as these traits avoid gene flow between the cultivated plants and their wild relatives. A way to keep genes in check while maintaining male fertility is to prevent the flower from opening (cleistogamy). This is shown on the example of rice, flowers of which open under the influence of lodicules - organs homologous to petals. Mutant alleles of the rice *AP3* ortholog *SUPERWOMAN1 (SPW1,* or *OsMADS16*), depending on the allele strength, can cause a number of phenotypic changes, including male sterility and cleistogamy [88].

A number of studies have identified the key role of type I proteins containing MADS-domains in the regulation of plant reproduction (in particular, in determining the development of the female gametophyte, embryos, and endosperm) and their decisive importance for establishing reproductive boundaries between plant species [89].

Quality of fruits and seeds. Fruit quality is one of the main signs of plant domestication, including changes in the number and size of fruits, the number of seeds, the ability to crack, the rate of ripening, storage period and shelf

life, as well as the visual appeal and taste characteristics of the fruit. As the results of numerous studies show, the key regulatory role in the formation of these traits here also belongs to the MADS-box genes. Thus, in *Arabidopsis*, the *SHP1, SHP2*, and *STK* genes overemphasize the identity of the ovules; triple mutation *stk shp1 shp2* leads to abortion or lack of seeds [46].

Seedlessness refers to the desirable traits in the selective improvement of juicy fruits, when it helps to extend their shelf life, as well as the use for the production of juices. The reason for seedlessness in grape varieties (*Vitis vinifera*) is a decreased level of expression of the *STK* ortholog, *VviAGL11*, due to changes in its promoter, where the repeat length is inversely correlated with seed development [90]. The genetic characteristic of the *VviAGL11* locus allows winemakers and breeders to evaluate plants for the number of seeds in fruits before reaching the reproductive stage [91]. Expression levels of the *STK* ortholog in tomato - *SlyAGL11* positively correlate with the degree of seed development; knockout of *SlyAGL11* results in seedless fruit [92]. Suppression of the expression of *STK* orthologues in petunia (*Petunia* × *hybrida*) - *FBP7* and *FBP11* - led to the complete replacement of ovules by carpels-like structures [93]. Thus, *STK* orthologues in various plant species can be used in breeding in order to reduce the number of seeds.

Cracking of the fruit leads to problems in the harvest of grain and oilseeds. It is known that pod cracking in *Arabidopsis* is excessively regulated by *SHP1* and *SHP2* genes; in the case of a double mutation *shp1 shp2*, the ripe pod remains closed [46]. Possibly, *SHP* genes turned out to be a target in the selection of cereals for the sign of non-shedding grain. Knowledge of the function of these genes can be used to increase yields by reducing seed shedding. For example, suppression of the *SHP1* - *BnSHP1* ortholog in oilseed rape plants leads to an increase in pod resistance to cracking, thereby increasing crop yield [94].

The FUL transcription factor containing the MADS-domain is also involved in fetal development, which regulates the differentiation of fetal cells during development and serves as a negative regulator of *SHP1* and *SHP2* expression [95]. Overexpression of the *FUL* ortholog of mustard - *MADSB* in *B. napus* plants reduces pod cracking [96]. Interestingly, in the case of a juicy tomato fruit, suppression of the activity of two *FUL* orthologues, *FUL1* and *FUL2*, causes a strong delay in fetal maturation, presumably due to a decrease in the synthesis of ethylene and carotenoids [97].

Another important feature taken into account in tomato breeding is the absence of an articular area on the peduncle, which facilitates fetal shedding [98]. Several MADS-box genes are involved in the specification of the drop zone [98]. Among them, *J2* is considered the most suitable for plant breeding; the *j2* mutation is present in many tomato lines without an articular zone [80, 98]. A mutation in another MADS-box gene, *JOINTLESS1 (J1*, or *JOINTLESS*), which is homologous to the *SVP* flowering time gene, also leads to an arthritic phenotype [99]. However, its value for breeding is questionable, since *j1* inflorescences are prone to re-version to vegetative development after the formation of several flowers [80].

SEP orthologs have been shown to be involved in the ripening of juicy fruits. Suppression of the activity of *SEP* homologues of banana and apple trees, *MaMADS1 / MaMADS2* and *MdMADS8 / MdMADS9*, respectively, inhibits the ripening of climacteric fruits and increases their shelf life [100, 101]. *SEP* orthologs are also involved in the development of non-climacteric (ethylene insensitive) fruits such as strawberries [102]. It points to *SEP* orthologs as a versatile target in optimizing fruit ripening.

The MADS-box gene *LeMADS-RIN* (*SEP* subfamily), a mutation in

which leads to fetal immature, is considered a key regulator of the ripening of the juicy tomato fruit, as well as an important gene involved in domestication [103]. Green and hard *rin* fruits are characterized by the absence of an increase in ethylene synthesis and accumulation of pigments and aromatic compounds [103]. In the heterozygous state, the *rin* mutation is widely used in the breeding of tomato varieties, since it prolongs the shelf life of fruits [104]. However, in this case, the nutritional and gustatory value of the fruit is disturbed (due to the low content of likopin and other compounds) [103]. Using the *CRISPR / Cas9* approach, a number of tomato lines with different *SNPs* (single nucleotide polymorphisms) and short indels in the coding sequence of the *RIN* gene were created, leading to different degrees of manifestation of the fetal immature phenotype [105]. These lines are considered as candidates for use in breeding varieties with an extended shelf life of fruits [105].

It is worth mentioning the MADS-box genes involved in the initiation of pathways for the biosynthesis of metabolites of the succulent fruit. Succulent plant fruits (such as tomato and pepper) contain two important types of secondary metabolites — anthocyanins and carotenoids, which not only color the fruit, but also act as antioxidants [106].

The *LeMADS-RIN* gene is one of the key factors in the regulation of carotenoid biosynthesis in tomato fruits [107]. In this case, the genes of the key enzymes of carotenoid biosynthesis, phytoinsynthase 1 (PSY1) and phytoindesaturase (PDS), serve as the target of the *LeMADS-RIN* product, while the homologues of AG, TOMATO AGAMOUS (TAG1) and TAG-LIKE1 (TAGL1), regulate the biosynthesis of carotenoids genes for lycopene-β-cyclase (CYC-β) and carotenoid isomerase (CRTISO) [108, 109]. The MADS-box gene *SlCMB1* (*SEP* subfamily) is also involved in the induction of expression of the *PSY1* and *PDS* genes and inhibition of the transcription of lycopene cyclase genes (*CYCB, LCYB*, and *LCYE*) [110].

A lot of evidence has been found for the effect of MADS-box genes on the biosynthesis of anthocyanins in juicy fruits. Thus, the expression of *MrMADS01* (*SEP* subfamily) in the berries of the red gumboil (*Myrica rubra*) significantly increases at the last stage of maturation, which allowed the authors to suggest the participation of this gene in the biosynthesis of anthocyanins [111]. Silencing of the *PaMADS7* gene in sweet cherry (*Prunus avium*) inhibited fruit ripening and influenced, among other things, the content of anthocyanins [112]. In red pear (*Pyrus*) fruits, the *PbrMADS11* and *PbrMADS12* genes are involved in the activation of the expression of structural genes of the anthocyanin pathway, as well as in the regulation of the anthocyanin synthesis reaction in response to light and temperature changes [113].

Evidence that MADS-box genes were the target of selection during domestication was also obtained in studies performed on maize [114]. For example, the gene *ZEA AGAMOUS-LIKE1 (ZAGL1*), which is a homologue of the flowering time gene *SOC1*, during the domestication stage, apparently, not only influenced the timing of flowering, but also contributed to the increase in the number of rows of corn on the cob, thereby increasing the size of the fruits and yield [114, 115].

Plant response to stress. The signs of plant domestication include the mechanisms of resistance and adaptation to unfavorable environmental factors. The participation of MADS-box genes in the regulation of plant resistance to various stresses, such as dehydration, salinity, low and high temperatures, as well as oxidative and biotic stresses, has been recently noticed by researchers [39].

For example, in rice, the *OsMADS26* gene (ortholog *AGL12*) is known as a regulator of responses associated with the response to drought and disease caused

by pathogens [116]. Another gene, *OsMADS57* (clade *AGL17*), functions as a stimulator of resistance to cold stress; in addition to cold, gene expression is induced by exposure to salinity, drought, and abscisic acid [117].

The *Arabidopsis SVP* MADS-box gene causes modifications in some developmental processes and gas exchange functions in response to dehydration: plants with the *svp* mutation exhibit increased moisture loss and maintain a significant rate of photosynthetic CO_2 assimilation throughout the dry period [118].

In tomato, salt stress, dehydration, and injury induce the expression of the *SlMBP11* gene (ortholog *AGL15*) [119]. At the same time, a close homologue of *SIMBP11*, the *SIMBP8* gene, has the opposite effect on the resistance of tomato plants to salinity [120]. The *TOMATO APETALA3 (TAP3*) gene is induced under cold stress conditions [121], while the expression of the *TAP3, TOMATO MADS BOX GENE6 (TM6*), and *LePISTILLATA (LePI*) genes is suppressed in the anthers under high temperature conditions [122].

In response to cold, drought, and salt stress, expression of the *CaMADS* gene (clade *SEP1*) [123] is induced in pepper plants (*Capsicum annuum*) [123], and in *Ginkgo biloba — GbMADS9* (clade B-sister) [124]. Transcription of *AGAMOUS LIKE21 (AGL21*) in *Arabidopsis* is induced by various stresses (including osmotic stress) and phytohormones, which suggests the involvement of the gene in the regulation of the plasticity of the root system (its ability to change the structure under the influence of environmental factors) and seed germination [125].

Another important stress for plants is soil depletion in minerals such as phosphorus. It has been shown that nine MADS-box genes are differentially regulated in wheat (*T. aestivum*) under P-deprivation [126]. A functional analysis of one of them, *TaMADS51*, showed that its overexpression under conditions of phosphorus deficiency improves plant growth, as well as increases biomass, phosphorus accumulation, and increases antioxidant enzymatic activity [126]. Another example is the *ARABIDOPSIS NITRATE REGULATED 1* gene (*ANR1*, or *AGL44*), a well-known positive regulator of root development in response to nitrate availability [127].

Pleiotropy and redundancy of MADS-box genes. The above examples show that MADS-box genes, whose functions are pleiotropic and often redundant, were involved in the processes of plant domestication. It is worth noting that, in many cases, the gene networks were not completely disrupted - more subtle variations were introduced, which made it possible to fine-tune the phenotype [128]. An example is *Brassica* and tomato, where variations in *FLC*- and *SEP*-like genes lead to modulation, respectively, of flowering time and inflorescence structure [62, 80]. In many cases, certain traits are over-regulated by several paralogs of the MADS-box genes, highlighting their potential for fine-tuning the phenotype. For example, several *FLC* paralogs are present in the *B. rapa* genome [58-62], and the combination of different allelic variants of these genes can make it possible to adapt the flowering time to a wide range of climatic conditions. In the same way, the combination of different alleles of *SEP*-like genes makes it possible to customize the structure of the tomato inflorescence [80]. However, the pleiotropic effects of many MADS-box genes can create problems: while mutations in *SEP* genes *J2* and *EJ2* of tomato separately have a beneficial effect, a double mutation due to the redundancy of the function of these genes turns out to be harmful: although *j2 ej2* plants show increased branching of inflorescences, at the same time the number of barren flowers increases [80]. However, in many cases, this and similar effects can be mitigated by careful selection of combinations of alleles that affect one, but not another trait [80]. In addition, one

should take into account the dose-dependent effect of many alleles of the MADS-box genes [129], as well as paralogs of the MADS-box genes, for instance, the *SEP* genes in *Arabidopsis* [130], and this adds opportunities for fine-tuning the phenotypic result.

According to the "quartet" model, transcription factors of the MADS family perform their functions as part of tetramers and can have many overlapping DNA targets, some of which are regulated in the opposite way using protein complexes of different compositions [35, 38, 131]. Therefore, in the absence of functional redundancy, the introduction of mutations into the coding sequence of MADS-box genes can change specific protein-protein or protein-DNA interactions and, as a consequence, separate the pleiotropic functions of one gene [131].

In conclusion, it is worth noting that the high functional conservatism of the MADS-box genes and the detailed characterization of their homologues in model and cultivated plants make these genes perfect candidates for predictable manipulation of phenotypes [80]. This can, in particular, be achieved by changing the cis-regulatory elements of the MADS-box genes and, as a consequence, the level of their spatio-temporal expression (during a specific phase of development, in a specific tissue), including in response to various signals [128]. Modifications in the coding region can also be used to fine-tune the phenotype, since the function of proteins of the MADS family is largely determined by protein-protein and protein-DNA interactions, and, by changing partners and targets, the same protein can participate in different developmental pathways (determination of the identity of organs and other parameters of plant growth and ontogenesis; response to stress; formation of various economically valuable traits) [131].

Thus, in plants MADS-box genes are considered one of the key targets that were involved in the formation of domestication traits influencing such properties as productivity, adaptability, and reproduction, that remain economically significant in modern cultivated crops. The variability of the MADS-box homologues of the *FLC, SOC1*, *SVP*, and *VRN* genes determines the differences in the time of flowering initiation, including in response to low temperatures. Changes in the process of physiological dormancy of the kidneys are associated with the homologues *SVP, AGL24*, and *FUL*. Morphological diversification of inflorescence and flower is associated with homologues *AP1 / CAL, SEP, AP3, PI, AG*, and *AGL17*, while sterility and the number of fruits and seeds are associated with *AG, SEP, FUL*, and *SVP*. Homologues of the MADS-box genes of *SVP, SEP, AP3, AGL12, AGL15, AGL17, AGL21*, and *AGL44* are associated with differences in plant stress response. Considering the amount of accumulated qualitative and quantitative data, the prediction of specific phenotypic consequences of changes in MADS-box genes is much more realistic than in the vast majority of genes from other families. Continued fundamental and applied research on MADS-box genes in a wide variety of species will not only lead to a deeper understanding of plant development and evolution, but will also greatly contribute to crop improvement.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 843-867
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 633.11:632.112:631.8:579.64

doi: 10.15389/agrobiology.2021.5.843eng
doi: 10.15389/agrobiology.2021.5.843rus

ADAPTATION AND TOLERANCE OF WHEAT PLANTS TO DROUGHT MEDIATED BY NATURAL GROWTH REGULATORS *Bacillus* spp.: MECHANISMS AND PRACTICAL IMPORTANCE

(review)

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The author declares no conflict of interests
Acknowledgements:
Supported financially from the Russian Foundation for Basic Research, the Project No. 20-116-50151
Received April 7, 2021

Abstract

Environmental abiotic factors leading to water deficiency significantly limit the production of major crops worldwide (Z. Ahmad et al., 2018). In the face of rapid population growth and climate change, it is important to ensure food security, which is mainly possible by increasing the productivity of strategically important crops, including wheat, which is used for human consumption in many regions of the world and provides more than 50 % of food energy needs (S. Asseng et al., 2019). Application of beneficial growth-stimulating bacteria *Bacillus* spp. are effective, environmentally friendly and safe natural strategy for protecting plants from stresses resulting in water deficiency (M. Kaushal et al., 2019; A. Hussain et al., 2020; M. Camaille et al., 2021). To date, the growth-stimulating and protective effect of *Bacillus* spp. under various abiotic stresses are indicated for a wide range of plants (S. Moon et al., 2017; H.G. Gowtham et al., 2020; N. Shobana et al., 2020), including wheat (G. Sood et al., 2020; U. Rashid et al., 2021). The mechanisms of this physiological action of *Bacillus* spp. on host plants remain largely unknown. Presumably, it is due to i) competition for space and nutrients with plant pathogens and increased availability of macro- and micronutrients (S. Danish et al., 2019; D. Miljakovic et al., 2020; A. Kumar et al., 2021), ii) production of a wide range of bioactive components and protective compounds (M. Saha et al., 2016; R. Çakmakçı et al., 2017; N. Ilyas et al., 2020), and iii) induction of plant systemic tolerance to stresses (I.A. Abd El-Daim et al., 2019; C. Blake et al., 2021; U. Rashid et al., 2021). The efficacy of the same *Bacillus* strain may vary, depending on many factors including a spectrum of the synthesized compounds, strains, plant species, ecological and geographical origin, varietal characteristics, the types of stresses during the growing season, etc. (A. Khalid et al., 2004; G. Salem et al., 2018; O. Lastochkina et al., 2020). This review summarizes an information on the current state of research and the latest available information on plant-microbe interactions with a focus on protecting wheat against drought. In particular, the mechanisms underlying *Bacillus*-mediated adaptation and tolerance of wheat plants to drought are under consideration. It is shown that *Bacillus* spp. can induce wheat drought tolerance due to i) synthesis of compounds which provide protection against osmotic and oxidative stresses (D. Miljakovic et al., 2020; R. Çakmakçı et al., 2017), ii) intracellular transmission and enhancement of protective signals by a cascade of mediators, iii) regulation of the protective protein gene expression and interorgan transduction with the participation of the main phytohormones, their biosynthesis in the whole plant (U. Rashid et al., 2021), and iv) numerous compounds involved in increasing the bioavailability of macro- and microelements and productivity (A. Hussain et al., 2020; A. Kumar et al., 2021). *Bacillus* spp. can positively influence plant photosynthesis and water exchange (I.A. Abd El-Daim et al., 2019), as well as drought tolerance of wheat genotypes of different agroecological groups (L.I. Pusenkova et al., 2020). The joint use of *Bacillus* bacteria with other natural growth regulators enhance their effectiveness and stability of action (M. Zafar-ul-Hye et al., 2019). The listed commercial bacillary biologicals are effective on wheats. The review contributes to the understanding of the fundamental mechanisms of wheat—*Bacillus* spp. interactions under drought, the development of *Bacillus*-based biologicals and their use in ecologically oriented technologies for wheat growing under changing climate conditions.

Keywords: plant growth-stimulating bacteria, *Bacillus* spp., wheat, drought, defense mechanisms, plant-microbe interactions, induced systemic tolerance

Soft wheat (*Triticum aestivum* L.) is the main strategically important cereal crop used for human consumption in many regions of the world and playing a key role in meeting more than 50% of food energy needs [1-4]. Drought, which affects about 64% of the world's agricultural land, is among the dominant abiotic stresses that hinder the growth and loss of yield (up to 50-80%) of wheat [2-5]. Drought, adversely affecting the general metabolism of plants at the physiological, biochemical, and molecular levels, leads to damage to various cellular compartments, degradation of proteins, inactivation of enzymes, decreased absorption of nutrients, transpiration and rate of photosynthesis, closure of stomata, inhibition of growth, wilting and drying of plants [4-6]. Although plants have different defense systems to counter external threats, they are insufficient, due to which significant yield losses are observed for all types of crops, including wheat during drought [4]. According to forecasts, the severity of the drought will consistently increase, which, combined with the exponential growth of the world's population, only exacerbates this problem and requires an urgent solution to prevent an impending food disaster [2, 7]. Plant protection chemicals adversely affect the environment and human health due to high toxicity and the ability to accumulate in products and soils, which, combined with climate change and an increase in the world's population [1, 2], leads to the need to reduce the use of chemicals. This is a serious problem, for the solution of which measures are being taken to increase the stress resistance of plants through genetic modification and traditional breeding [8], which, however, requires a lot of time, significant financial resources and is associated with numerous regulatory restrictions. The use of beneficial microorganisms stimulating plant growth (PGPB, Plant Growth-Promoting Bacteria) [9-12], in particular the genus *Bacillus* spp., capable of activating the natural defense mechanisms of host plants without causing a negative effect on them, the environment and human health, are considered as an affordable, cheap, fast-acting, and environmentally friendly alternative biological strategy for increasing the adaptive potential [13-16] and plant productivity in changing environmental conditions with the simultaneous restoration of soil health [17, 18].

PGPB *Bacillus* spp. is a large and diverse group of useful non-pathogenic microorganisms that live freely in the soil or inhabit the surface (rhizosphere and phylosphere) or the inner part of tissues (endophytes) of host plants, capable of inducing plant growth and resistance to biotic stressors — plant pathogens [19-22], insect pests [20], nematodes [19], viruses [20] and abiotic stresses — drought [17, 24], salinity [11], temperature extremes [12], toxic compounds [14], and UV radiation [12, 13]. This has been demonstrated for many plant species [17, 25-28], including wheat [13, 29-31]. Although the fundamental mechanisms of physiological action of *Bacillus* spp. on plants, to a greater extent remain unclear, it is known that they directly or indirectly interconnected pathways modulate the hormonal background, affecting the architecture of the root system [32-35], improve the bioavailability of macro- and microelements and mineral nutrition of plants (biofixation of nitrogen, solubilization of phosphorus, potassium, zinc and other elements) [36-39], photosynthesis [40, 41], stomatal conductance [17, 42], and water status [17, 42, 43], regulate the production of phytohormones - auxins, cytokinins, gibberellins, abscisic (ABA), salicylic (SA), jasmonic (JA) acids, ethylene, and their accumulation in the plant [23, 44-46], activate the synthesis of various antioxidant and osmoprotective compounds [27, 47, 48],

expression of aquaporin genes sensitive to drought, dehydrins [25, 41], production of volatile organic compounds, exopolysaccharides [49, 50], 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) [44, 51-53], organic acids, spectrum of secondary metabolites [41], and signal for shield compounds that activate induced systemic defense in the host plant [25, 35, 42, 52], which leads to an increase in plant resistance and productivity under drought conditions [17]. Along with this, *Bacillus* spp., in particular *B. subtilis*, are generally recognized as safe microorganisms for use in the food industry (GRAS — Generally Recognized As Safe) [12, 13, 16]. In addition, they produce endospores that are extremely tolerant to various physical and chemical influences (heating, drying, organic solvents, UV irradiation, etc.), due to which they retain the ability to trigger defense reactions in host plants even under stressful environmental conditions [19]. All this is done by *Bacillus* spp. attractive agents for the development of commercial biofertilizers and plant protection products on their basis, the number of which is growing every year all over the world. However, in practice, their effectiveness often varies depending on many factors (characteristics of the microorganism strain, the type of plant, its place of growth, varietal characteristics) [13, 34, 54, 55]. For a fuller use of the potential of representatives of *Bacillus* spp. as inoculants that ensure sustainable productivity of crop production (especially against the background of constant climate change), it is extremely important to understand the features of these plant-microbial interactions and the mechanisms underlying the physiological effect that bacilli have on plants, in particular when protecting against dominant environmental stress factors.

The purpose of this review is to summarize the information on the fundamental mechanisms of adaptation and stress resistance caused by the action of *Bacillus* spp., and their role in the practical improvement of growth and maintenance of wheat productivity in drought conditions.

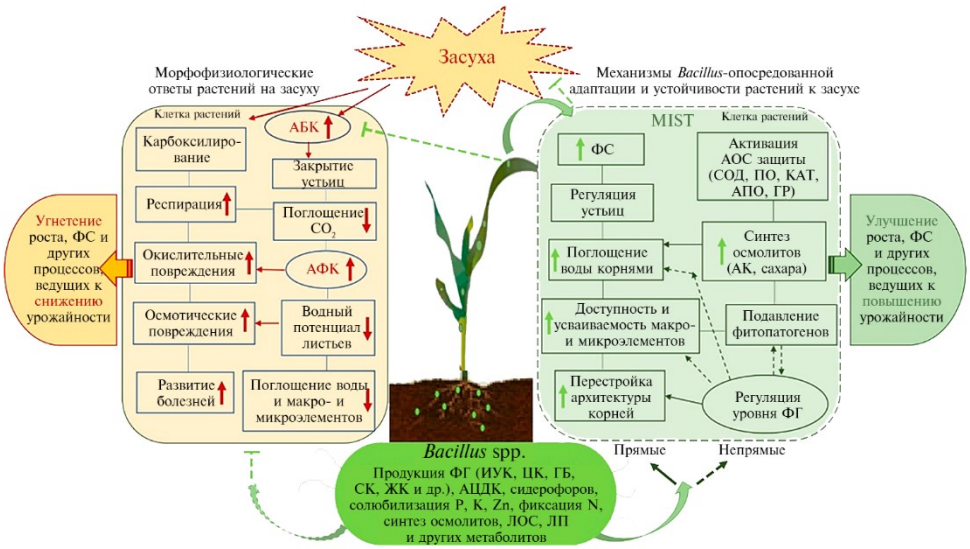
According to current theories, the bacteria *Bacillus* spp. contribute to a more effective resistance of plants to stress due to the development of microbe-induced systemic tolerance (MIST) [27, 42, 52]. It includes a wide range of direct and indirect complex interconnected mechanisms, the action of which causes various morphophysiological and biochemical changes and, reducing the effects of stress on plants, leads to an increase in productivity (see Fig.).

Modification of the root architecture and increasing the availability of elements of mineral nutrition. The main adaptive mechanism of *Bacillus*-mediated stimulation of wheat plant growth under drought conditions is the ability of bacilli, colonizing external (rhizobacteria) and (or) internal (endobacteria) tissues of host plants, to positively modulate plant metabolism and change the architecture of their root system (increase in the length of the main and lateral roots, the density of root hairs, root surface area, and the accumulation of their biomass) [23, 34], which improves the absorption of water and nutrients from the soil [28, 35, 40, 43]. *Bacillus* spp. increase the availability for plants and the assimilation of mineral nutrition elements due to the biofixation of atmospheric N applied with mineral fertilizers, which contributes to a decrease in the doses of fertilizers applied [3, 13, 28, 41]. The participation of *B. subtilis* in the stimulation of the activity of other rhizosphere nitrogen fixers has also been reported [23, 56]. Along with N, plant growth directly depends on P, a large amount of which (more than 80%) is fixed in the soil and is not available for absorption by plants due to adsorption, deposition or transformation. *Bacillus* spp. is able to dissolve water-insoluble and inaccessible to plants forms of soil phosphorus compounds due to the production of organic and inorganic acids,

siderophores, protons, hydroxyl ions and CO_2, which chelate cations or lower the pH to release phosphorus, as well as other biologically active compounds that solubilize P compounds and promoting their assimilation by plants [23, 57, 58]. It was shown that with the presence of N-fixing and P-solubilizing *Bacillus* spp. the absorption of nutrients and the subsequent stimulation of the growth of wheat plants are directly related [23, 58, 59]. Thus, inoculation with *B. subtilis* SIR1 improved the assimilation of NPK by wheat plants at different drought intensities and increased the content of these elements in plants [17]. The study by M. Zafar-ul-Hye et al. [44] showed that *B. amyloliquefaciens* solubilize P, K, which leads to the enrichment of shoots and wheat grains with these elements. In other studies, inoculation of wheat with *B. aryabhattai* strains MDSR7, MDSR11, and MDSR14 significantly increased the availability of Zn for plants, and their growth and accumulation of this element in grains increased [60]. For many *Bacillus* spp. the ability to form biosurfactants positively affecting plant growth was revealed - substances of a lipopeptide nature with surface-active properties capable of reducing the interfacial tension coefficient and emulsifying poorly soluble hydrophobic compounds, increasing their availability for plants [13, 16, 19]. To meet the Fe requirement of *Bacillus* spp. very specific pathways have developed with the participation of low-molecular-weight Fe chelates — siderophores, which, converting Fe into a form accessible to cells, increase its availability for plants and assimilability [38, 61].

Modulation of photosynthesis and water exchange processes. During a drought, the water potential of the soil dramatically decreases, photorespiration increases, stomata close, photosynthesis decreases, cell dehydration and hyperproduction of reactive oxygen species (ROS) occur, which ultimately causes secondary stresses - osmotic and oxidative, leading to inhibition of plant growth and even their death [4, 62] (see Fig.). For many strains of *Bacillus* spp. the ability to maintain the increased relative water content in plants during drought has been shown. This is an important physiological parameter that correlates with drought tolerance [17, 42]. The main physiological criteria for assessing the state of plants during a drought also include stomatal conductance, chlorophyll content, photosynthesis rate, the ratio of variable fluorescence (F_v) and maximum fluorescence (F_m) values, the content of biomarkers of the oxidative and osmotic status of cells, the end product of lipid peroxidation malondialdehyde (MDA) and proline osmolyte, respectively [17]. The positive effects of *Bacillus* spp. have been reported on chlorophyll content, stomatal conductance, and the efficiency of photosystem II in wheat plants during drought [17], primarily due to an improvement in the state of hydration and nutritional profile, which preserve cellular turgor and trigger plant defense mechanisms [42]. It has been shown that the *Bacillus*-mediated improvement of the water status in wheat under stress contributes to an increase in the rate of photosynthesis, since a higher stomatal conductance leads to an increase in CO_2 diffusion in mesophyll cells [40]. Bacterization with the *B. subtilis* strain LDR2 increased the drought tolerance of wheat, maintaining the increased photosynthetic activity of plants, promoting the accumulation of IAA, but reducing the level of ABA and ACC [63]. In the inoculated LDR2 and drought-exposed seedlings, the expression of *TaCTR1* and *TaDREB2* genes, which are responsible for the formation of wheat resistance to abiotic stresses, has increased [63]. According to other data, the manifestation of the protective effect of *B. velezensis* 5113 on wheat during drought was associated with the ability to maintain the photosynthetic apparatus of plants, maintaining a higher content of photosynthetic pigments

[41].



Main mechanisms of *Bacillus*-mediated adaptation and resistance of wheat plants to drought: PH — phytohormones, ABA — abscisic acid, AA — amino acids, AOS — antioxidant system, ROS — reactive oxygen species, ACCD — 1-aminocyclopropane-1-carboxylate deaminase, GB — gibberellins, JA — jasmonic acid, IAA — indolyl-3-acetic acid, VOC — volatile organic compounds, LP — lipopeptides, SA — salicylic acid, SOD — superoxide dismutase, PO — peroxidase, CAT — catalase, APX — ascorbate peroxidase, GR — glutathione reductase, PS — photosynthesis, CK — cytokinins, MIST — microbe-induced systemic tolerance.

A powerful defense *Bacillus*-mediated mechanism of overcoming the osmotic stress and maintaining the water status of plants under drought conditions is the production and accumulation of *Bacillus* spp. a number of polar metabolites, including many amino acids, for example, proline and glycine betaine, and sugars, such as glucose, sucrose, and fructose [26, 35, 41]. Osmolytes produced by *Bacillus* spp. are involved in osmotic regulation of cells and maintain the necessary cellular turgor through a decrease in the water potential of plants without a decrease in their actual water content [13, 26, 35, 42]. The amino acid proline belongs to the main osmolytes that are synthesized and accumulated in plants as a result of protein hydrolysis under the influence of drought. Proline has many functions, including regulating the acidity of the cytosol, minimizing lipid peroxidation by trapping free radicals, and stabilizing subcellular components and structures (proteins and membranes). Involvement of proline in the spectrum of mechanisms of the protective action of *Bacillus* spp. in various species of wild and cultivated plants, including wheat, has been demonstrated in a number of studies [3, 11, 16]. However, the question remains whether the *Bacillus*-mediated increase in proline content in plants is related to its more active absorption from the rhizosphere or to the regulation of biosynthesis in plants [35]. There is evidence on increase in wheat drought resistance under the influence of *Bacillus* spp. with the participation of various other osmolytes [26]. For example, a significant increase in plant drought tolerance was revealed upon inoculation with PGPB strains capable of overproduction of trehalose and / or enhancing its accumulation inside plants [64]. Trehalose is known to play a key role in cell signaling and stabilization of cell structures and proteins [42, 65]. An important place in the maintenance of some strains of *Bacillus* spp. osmotic balance and protection of external proteins in photosystem II during abiotic stress can be occupied by glycine betaine [35]. Thus, the ability of *B. subtilis* GB03 to enhance the biosynthesis of choline (the primary metabolite of glycine betaine biosynthesis) and the accumulation of glycine betaine

in plants was found, which was accompanied by an increase in the relative water content in leaves and the accumulation of dry mass of plants [35, 47]. *B. subtilis*-induced accumulation of glycine betaine (2-fold) and choline (5-fold) imparted drought resistance to *Arabidopsis*, while *B. subtilis*-induced drought resistance was lost in its mutant *xipotl* lines with impaired choline biosynthesis [42, 47]. A number of studies have confirmed the osmoprotective role of sugars in plants inoculated with PGPB during drought [42]. For some strains of *Bacillus* spp. the ability to increase the accumulation of polyamines (cadaverine, spermidine, spermine, and putrescine), the main metabolites that increase the osmotolerance of plants under drought conditions (in addition to the key role of these compounds in cell differentiation, root elongation, and transcriptional regulation), was revealed [35, 66]. Inoculation with *B. megaterium* BOFC15 increased the accumulation of polyamines in plants, which led to polyamine-mediated activation of signaling pathways that mitigated the damaging effect of drought by maintaining an increased amount of water in the plant and photosynthetic activity [66]. The role of cadaverine and spermidine in enhancing root growth in PGPB-treated plants during osmotic stress has been described [23, 35]. For example, an increase in the content of polyamines was observed in *Arabidopsis* plants treated with the spermidine-producing *B. megaterium* BOFC15 strain [66]. The plants were able to survive the drought due to a strong root system with longer primary and lateral roots compared to control samples [66]. Analysis of the filtrate of *B. subtilis* OKB105 culture confirmed that the main growth-stimulating compound was polyamine spermidine, the secretion of which involved the *yecA* and *speB* genes encoding polyamine permease and agmatinase [16]. OKB105-induced expression of expansion genes (*Nt-EXPA1, Nt-EXPA2*) inhibited expression of the ethylene biosynthesis gene *ACO1* [14]. Transcription profiles of *B. subtilis* showed that genes associated with acetylation, transport, and biosynthesis of polyamines are differentially expressed when grown under alkaline or acidic conditions. Literature analysis also indicates the existence of a relationship between polyamines and biofilm formation, which play an important role in the protection of host plants [16], as well as a correlation between the amount of polyamines, ABA, and abiotic stresses [66]. This research area is of interest for uncovering the mechanisms of MIST associated with drought [42]. Recent metabolomic studies have revealed the accumulation of proteins, dehydrins, and a number of other osmoprotective compounds in plants inoculated with bacilli under stress conditions [41]. In particular, the *B. amyloliquefaciens* GB03 strain mitigated osmotic stress by activating drought-sensitive genes for dehydrins and aquaporins, altering hormonal homeostasis, and enhancing the production of antioxidant enzymes and exopolysaccharides [27, 48]. It was reported that exopolysaccharides produced by bacteria are involved in root colonization, improve the water-holding capacity of plants and soil fertility, and also serve to protect the bacterial cells themselves from drying out [50, 67]. In addition, plants inoculated with exopolysaccharide producing PGPB showed higher accumulation of proline, sugars, and amino acids under water stress conditions [50, 67].

Activation of antioxidant defense systems. It is known that drought leads to an imbalance between the formation of ROS (superoxide radical, hydrogen peroxide, and hydroxyl radical) and their neutralization [4, 68]. Excessive amounts of ROS begin to spontaneously and nonspecifically interact with molecular cellular components, which lead to serious disruption of cellular structures, lipids, proteins, carbohydrates, and nucleic acids [62, 68]. The most important mechanisms of *Bacillus*-mediated drought resistance of plants include the participation of these microorganisms in the detoxification of ROS by modulating the natural antioxidant defense systems of plants - as enzymatic (superoxide dismutase, SOD; peroxidase, PO; catalase, CAT; ascorbate peroxidase, APX) and

non-enzymatic (ascorbic acid, AA; glutathione, cysteine, proline, flavonoids, carotenoids, and tocopherol), all of whose components are in complex functional interactions [6, 35] (see Fig.). An increase in the activity of antioxidant enzymes (SOD, PO, CAT) in plants has been reported upon inoculation with *Bacillus* spp. as the main mechanism of MIST protection against drought [13, 17, 35, 42]. Besides, treatment with *B. amyloliquefaciens* 5113 had a protective effect on wheat plants during drought by regulating the activity of the APX *APX1* gene and enzymes of the ascorbate-glutathione complex, which, as is known, due to the high intracellular content of AA and glutathione, provides a high buffer redox capacity of cells and serves as a key player in the antioxidant defense system [68]. Interestingly, under normal conditions, treatment with *B. velezensis* 5113 had practically no effect on the ascorbate - glutathione cycle in wheat, but significantly decreased APX activity under heat stress, while no significant decrease was found under drought and cold stress conditions [41]. Other authors reported that drought increased the activity of stress-related genes *APX1, SAMS1*, and *HSP17.8* in wheat leaves and increased the activity of enzymes of the ascorbate-glutathione cycle, while in plants treated with *B. amyloliquefaciens* 5113, the number of transcripts of these genes decreased, which indicates an improvement in the state of homeostatic mechanisms due to bacterial priming [68]. Obviously, different strains of *Bacillus* spp. can reduce the degree of oxidative damage in different ways with the involvement of various biochemical pathways, which may depend on many factors, including the characteristics of the strain itself, the type of plant, the type of stress, and its intensity. In general, all studies noted an improvement in the state of the entire pro-antioxidant system in plants inoculated with bacilli, which during drought was manifested in a decrease in the content of the end product of lipid peroxidation - MDA [13, 22, 28, 35, 52].

Regulation of phytohormone levels. An important mechanism of *Bacillus*-mediated increase in drought resistance and productivity of plants [16, 23], in particular wheat [13, 68, 70], is the production and (or) regulation of the amount of phytohormones [26, 63, 69, 72] as central connecting links, playing a key role in the reprogramming of ontogenetic and main signaling cascades involved in the formation of plant stress resistance.

It was shown that the filtrates of liquid cultures of *Bacillus* spp. contain phytohormones - auxins, cytokinins, gibberellins, ABA, SA, JA [71, 72], which play an important role in photosynthesis, plant growth, and the integrity of the plasma membrane, as well as in the development of MIST [13, 73-75]. Among the products of *Bacillus* spp. indole compounds, for example, indolyl-3-acetic acid, play a vital role in stimulating plant growth and development, being the main auxin that regulates cell division and elongation, their proliferation and differentiation, the development of vascular tissues, and apical dominance [13, 16]. *Bacillus* spp. use the IAA produced to interact with plants as an element of their colonization strategy, including phytostimulation and bypassing the main defense mechanisms of plants. Numerous studies have shown that IAA-producing bacilli significantly enhance the growth of wheat plants both under normal conditions and during drought [28, 40, 42, 63], causing an increase in the absorption of water and nutrients. An increase in the surface area and length of lateral and adventitious roots due to the high secretion of IAA by bacteria plays a vital role in improving the consumption and assimilation of micro- and macronutrients, in accelerating growth and the formation of drought resistance of plants [13, 42, 70, 76]. According to recent research, *Bacillus* spp. control the level of endogenous IAA in plant roots, regulating the expression of auxin-sensitive genes and thereby causing changes in root architecture

[23]. Drought reduced the IAA content and caused the activation of the *AUX / IAA1* gene in non-inoculated wheat seedlings, while the inoculation with the *B. subtilis* LDR2 strain increased the IAA content (by about 80%), and the *AUX / IAA1* gene expression was suppressed [63]. These data suggest that *B. subtilis* modulates the auxin signaling pathway to protect wheat plants from drought. Biochemical pathways and genes have been identified that control bacterial IAA synthesis; it is assumed that L-tryptophan is the main precursor for IAA formation in microorganisms [77]. However, there may be other pathways for IAA biosynthesis (indole-3-acetamide, indole-3-pyruvate, and tryptamine), and sometimes a bacterial strain possesses more than one pathway for IAA synthesis [77, 78]. It is believed that the ability to produce IAA is most widespread among soil bacteria [75] and is more common among endophytic bacteria than among epiphytic ones [77]. Thus, of the 363 epiphytic and 373 endophytic bacteria studied, the proportion of IAA-producing endophytes was 34%, and of epiphytes, 21% [79]. Another important group of phytohormones produced by *Bacillus* spp. are cytokinins that regulate seed germination, cell division in plant roots and shoots, stomata opening, and nutrient mobilization under drought conditions [23, 28, 31]. It is reported that 90% of P-solubilizing bacteria have the ability to produce cytokinins in vitro [23]. For many strains of *Bacillus* spp. the ability to synthesize gibberellins, which regulate various physiological processes (seed germination, stem elongation, flowering, fruit ripening, as well as leaf and fruit aging), was detected [35, 80]. However, the exact mechanism by which gibberellins promote plant growth during drought is not entirely clear. It is assumed that increased root growth and an increase in root hair density under the influence of gibberellin-producing bacteria are associated with an increased intake of nutrients and water by host plants [69].

Along with an increase in plant drought resistance as a result of microbial colonization, a change in the content of the phytohormone ABA [42], a key participant in the regulation of stress reactions, was recorded [69]. *Bacillus*-mediated shifts in the amount of endogenous ABA led to a change in the architecture of the root system due to an increase in the number of lateral roots and modification of the water status through regulation of the hydraulic conductivity of roots, a decrease in the rate of leaf transpiration, an increase in stomatal conductance, and the induction of the expression of genes involved in ensuring drought resistance [42]. It has been shown that almost 2/3 of 2000 drought-induced genes are regulated by ABA [81]. Treatment with the *B. subtilis* strain LDR2 promoted the maintenance of the photosynthetic activity of wheat plants under drought conditions, while the stress-induced accumulation of ABA and ACC decreased [63]. Since inhibition of ABA biosynthesis suppresses the expression of the aquaporin water transport protein gene *TaAQP7*, the transcriptional activity of which increases under water stress, a decrease in the amount of ABA caused by bacterization of *Bacillus* indicates that bacillus products modulate plant drought resistance through up-regulation of the *TaAQP7* gene. At the same time, seedlings treated with LDR2 were characterized by increased expression of the *TaCTR1* gene, which encodes a key negative regulator of ethylene signal transduction, as well as the gene for the transcription factor *TaDREB2*, which is involved in the regulation of the formation of plant resistance to a wide range of abiotic stresses [63]. According to the authors [63], an increase in wheat drought resistance under the influence of *B. subtilis* LDR2 is due to an increase in IAA and a decrease in ABA: ACC, as well as modulation of the activity of the regulatory component of ethylene signaling CTR1 and the transcription factor DREB2. It has been reported that *B. velezensis* 5113 can use ABA signaling to influence wheat drought tolerance

[41, 43]. In some studies, upon inoculation with bacilli in plants, simultaneously with a decrease in the amount of ABA, the content of SA and JA, which perform signaling functions, had increased [82]. The production of SA and JA by bacteria and (or) the regulation of their exogenous level in plants can play an important role in MIST, since to date there is no doubt about the key role of these phytohormones in triggering a complex chain of defense reactions leading to the formation of plant stress resistance [42, 69]. An increase in the accumulation of endogenous SA in wheat plants in response to inoculation with *B. subtilis* 10-4 correlated with drought resistance of plants, which indicates the involvement of SA-dependent signaling pathways in the implementation of effects mediated by *B. subtilis* [28, 34]. Phytohormones JA and ethylene also play an important regulatory role in the network of interconnected signaling pathways involved in MIST [42, 69]. Although there are reports in the literature on the bacilli-mediated activation of SA-, JA-, and ethylene-dependent signaling pathways involved in the development of a protective response in plants under biotic stresses [83], there is practically no information on the regulation of wheat drought resistance by bacteria with the participation of these signaling pathways. We did not find reports on the role of other phytohormones (for example, brassinosteroids) in microbe-induced systemic drought resistance in wheat plants, although the role of brassinosteroids in mitigating abiotic stresses has been confirmed [69].

Ethylene is another hormone important for plant growth and development, regulating cell growth, seed germination, leaf aging, ripening of flowers and fruits, but the effect of ethylene depends on its concentration in root tissues [69]. During drought, ethylene biosynthesis is enhanced, which negatively affects plant growth and development. ACC deaminase, a key enzyme in the system of ethylene-dependent plant growth regulation, participates in the decomposition reaction of ACC (the direct precursor of ethylene) [52]. Many strains of *Bacillus* spp. were registered as producers of ACC deaminase, and their use showed a decrease in stress-induced accumulation of ethylene, which could otherwise become a growth inhibitor [23, 52]. The use of ACC by bacteria as a source of nutrition leads to a decrease in its content in plants and, as a consequence, to the suppression of ethylene synthesis in the roots. The ethylene precursor ACC is released from the roots into the rhizosphere of the host plant and is cleaved by secreted *Bacillus* spp. ACC deaminase to ammonia and α-ketobutyrate. Ultimately, the ethylene content decrease, which, in turn, promotes plant growth, since with a decrease in ethylene concentration, the absorption of water and nutrients improves [44-46, 50]. The ability of PGPBs producing ACC deaminase, including *Bacillus* spp., to improve wheat growth during drought has been shown [35, 44-46]. For example, under drought conditions in plants inoculated with bacteria *B. amyloliquefaciens* producing ACC deaminase, the intensity of photosynthesis, transpiration, stomatal conductance, the content of chlorophylls a and b, and grain yield increased significantly [44, 46]. According to other studies, inoculation with bacteria producing ACC deaminase led to a noticeable increase in the relative water content in wheat leaves under water stress [54].

The study of the effect of *B. subtilis* LDR2 on the expression of the gene encoding CTR1 (a regulatory component of the ethylene signaling pathway that modulates stress-related changes in plants) showed that, under drought, in LDR2-inoculated wheat seedlings, *TaCTR1* gene expression was higher than in non-inoculated ones, which indicates the involvement of these bacteria in increasing the drought resistance of wheat [63]. Interestingly, although ACC deaminase activity has been described for many *Bacillus* strains, the ACC deaminase genes (structural gene *acdS* and regulatory gene *acdR*) were not identified in 271 strains with a fully sequenced genome [23]. These strains belonged to the

Bacilli class and represented many soil and plant-related species of the genera *Bacillus* and *Paenibacillus* [23].

Biocontrol of phytopathogens and production of metabolites. The indirect mechanisms of *Bacillus*-mediated improvement of wheat plant growth during drought include effective competition and suppression of pathogenic microorganisms (fungi, bacteria) using produced secondary metabolites with antibiotic properties, including ribosomal (bacteriocins) or nonribosomal (lipopeptides, polyketides) peptides with low molecular weight, enzymes that destroy the cell wall of phytopathogens (chitinase, cellulase, glucanase, protease, lipase), siderophores, volatile organic compounds, as well as due to a decrease in the formation of ethylene in plants and activation of MIST against stress [23] (see Fig.). Various *Bacillus* species, including *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. thuringiensis*, and *B. coagulans*, have been shown to synthesize bacteriocins and bacteriocin-like substances (amylolysin, amizin, subtilin, subtylosin A, subtylosin B, turicin) with antimicrobial properties against phytopathogens. However, bacilli producing nonribosomal lipopeptides and peptides exhibit much stronger antimicrobial properties [23]. Recent studies have shown that lipopeptides also affect the colonization and preservation of *Bacillus* species in the rhizosphere, which stimulates plant defense mechanisms [23]. The most important cyclic lipopeptides produced by bacilli are represented by the surfactin, iturin and fengycin families. It has been shown that *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and *B. coagulans* synthesize lipopeptides of the surfactin family (surfactin, lichenesin, pumilacidin, halobacillin, bamilocin) — heptapeptides that act as antifungal and antibacterial agents. The Iturin family consists of heptapeptides (iturin, mycosubtilin, bacillomycin, bacillopeptins, myxirin, moya-vensin, subtulene produced by *B. subtilis*, *B. amyloliquefaciens*, *B. circans*, *B. pu-milus* and *B. vallismortis*), which have a broad spectrum of fungal inhibiting but less active against bacteria. The production of lipopeptides of the fengycin family (decapeptides fengycin, plipastatin, maltacin), useful for protecting plants from fungal pathogens, was found in *B. subtilis* and *B. amyloliquefaciens* [23]. Non-ribosomal lipopeptides include kurstakins, bacitracins, polymyxins, gramicidins, and thyrokidines. Kurstakins, the cyclic or linear heptapeptides specific for *B. cereus* and *B. thuringiensis* can destabilize the biological membranes of both bacteria and fungi. Bacitracins are cyclic decapeptides produced by *B. licheniformis*, *B. subtilis*, and *B. sonorensis*, whose activity is primarily directed against gram-positive bacteria. Polymyxins are cyclic decapeptides produced by *Paenibacillus polymyxa* that inhibit the growth and reproduction of gram-negative bacteria. Gramicidins and tyrocidins, cyclic decapeptides synthesized by *B. brevis*, are active against a wide range of gram-negative and gram-positive bacteria. Some types of bacilli also produce other nonribosomal peptides (bacilisin, rhizocticin, amicumacin, mycobacillin, and diketopiperazines) and polyketides (bacillin, dihydrobacillin, dificidin, macrolactin) with various antifungal and antibacterial properties. In the most commonly used bioagents *B. subtilis* and *B. amyloliquefaciens*, a significant part of the genetic material (4-5% and 8.5%, respectively) is responsible for the synthesis of secondary metabolites with the potential for the production of more than 20 antimicrobial compounds of different structures. At present, clusters of genes encoding bacteriocins, as well as peptides and polyketides, are easily identified by genomic scanning. For 328 strains of 57 species of the order *Bacillales*, a total of 583 putative clusters of bacteriocin genes were identified; in addition, in 49 species of *Bacillales*, 1231 putative clusters of genes of nonribosomal antimicrobial compounds were found, which were combined into groups according to 23 types of peptides and five types of secondary metabolites of polyketid

nature. [23]. Many studies have shown that hydrolytic enzymes (chitinases, chitosanases, glucanases, cellulases, lipases, and proteases) synthesized by bacilli very actively destroy the cell walls of fungi and bacteria, and also increase plant resistance to stress [13, 23, 84]. The role of siderophores synthesized by bacilli in biocontrol based on competition for Fe in order to reduce its availability for pathogens has been described [23]. Most bacterial siderophores are catecholates, such as bacillibactin produced by some bacilli (eg, *B. subtilis, B. amyloliquefaciens, B. cereus, B. thuringiensis*). In addition, representatives of the genus *Bacillus* produce a wide range of sidero-phores, in particular pyoverdin, pyochelin, schizokinene, petrobactin [23].

Bacillus spp. also secretes extracellular polysaccharides and various volatile organic compounds that change the structure and morphology of roots and cause MIST in plants [85] (see Fig.). Volatile organic compounds are lipophilic in nature and act as signaling molecules for inter- and intraorgan communication and cell-to-cell signaling. Inoculation with *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 induced the production of volatile organic compounds - 2R, 3R-butanediol and 3-hydroxy-2-butanone in *Arabidopsis* plants, which modulated the expression of genes responsible for plant growth. Colonization of *Arabidopsis* roots by the *Pseudomonas chlororaphis* O6 strain induced the production of 2R, 3R-butanediol, which led to stomata closure and increased plant drought resistance; the role of various phytohormones (SA, ethylene, and JA) in the modulation of drought resistance was also established. However, in wheat seedlings treated with *B. thuringiensis* AZP2, an increase in biomass and an increase in plant survival during drought occurred, on the contrary, due to a decrease in emissions of volatile organic compounds, as well as an increase in photosynthesis [35, 40].

The emergence of new research methods allows a broader and more comprehensive assessment of the effect of microbial inoculations on all vital plant systems involved in the regulation of growth and development both under normal conditions and under stress conditions. Thanks to metabolomic studies, it was found that normally inoculated with *B. velezensis* 5113 and non-inoculated wheat plants significantly differed in the accumulation of 61 metabolites: for 36 it increased, for 25 it decreased [41]. In particular, inoculation with *B. velezensis* 5113 increased the content of the amino acids L-proline and L-glutamine, γ-aminobutyric acid (GABA), significantly influenced the metabolism of alanine, aspartate, glutamate and the biosynthesis of flavonoids [41] involved in defense reactions in plants and in interactions between plants and microbes. Drought caused a significant accumulation of metabolites (194 metabolites were found) in the leaves of non-inoculated wheat seedlings compared to control seedlings that were not bacterized and were not subjected to stress. In plants inoculated with *B. velezensis* 5113, drought caused the accumulation of only 139 metabolites. At the same time, under drought conditions, a significant difference between inoculated and non-inoculated wheat plants was noted only for 29 metabolites (the content of 10 increased, 19 decreased) [41]. In particular, treatment with *B. velezensis* 5113 reduced the accumulation of some metabolites associated with the biosynthesis of flavones and flavonols, as well as the intensity of flavonoid biosynthesis in leaves of unstressed and stressed wheat seedlings, which indicates a possible inhibitory effect of *B. velezensis* 5113 on the biosynthesis of flavonoids in plants [41]. The same authors, using proteomic analysis, showed that the treatment of *B. velezensis* 5113 in wheat leaves increased the content of proteins that are involved in the process of photosynthesis [41]. Interestingly, the formation of these proteins

was suppressed in all non-inoculated plants under drought conditions, which indicates an important role of *B. velezensis* 5113 in protecting the photosynthesis process in drought-exposed wheat plants. It was also found that in response to treatment with *B. velezensis* 5113, the synthesis of several proteins with unknown functions is activated in wheat leaves. Of particular note is the fact that wheat plants inoculated with *B. velezensis* 5113 significantly increased the amount of GABA, glutamine, and proline. The role of proline and glutamine in the *Bacillus-mediated* regulation of wheat drought resistance has already been described in the literature [34], but there are no such studies for GABA. There are few data on the participation of GABA in the development of microbial - plant interactions [86]. In particular, it was reported that GABA is synthesized inside legume nodules and is involved in the formation of symbiosis between bacteria and plants. With a moisture deficit under the influence of treatment with the endophytic bacterium *B. subtilis* B26, in the shoots and roots of timothy, the accumulation of GABA increased and the drought resistance of plants increased [87]. The non-protein amino acid GABA, which rapidly accumulates in plant tissues in response to biotic and abiotic stresses, plays a significant role in plant adaptation to stress and is involved in the regulation of physiological and biochemical pathways that ensure plant resistance to stress, including water shortage [78]. GABA is associated with the maintenance of carbon-nitrogen balance, with the metabolism of amino acids, carbohydrates, and growth regulation [86]. In addition, GABA can act as an effective osmolyte with no toxic effects and as an ROS scavenger in plants subjected to abiotic stress. It should be noted that relatively recently, GABA began to be considered as a secondary metabolite and signaling molecule involved in signaling and defense mechanisms in plants [86]. The revealed ability of *B. velezensis* 5113 to modulate the GABA content in unstressed wheat leaves may indicate that this strain is capable of influencing plant resistance to drought through priming, similar to the way it occurs when exogenous GABA is used [41]. Probably, under stress, the accumulation of GABA in plant tissues caused by bacterization contributes to an increase in their resistance, ensuring the formation of a critical link in a cascade of reactions from the perception of a stress signal to sequential physiological responses. This is evidence in favor of the important role of this metabolite in *Bacillus*-mediated drought resistance of wheat, which certainly deserves close attention and further research.

Drought tolerant strains of *Bacillus* spp. and their effectiveness. Particular attention is paid to the isolation of drought-tolerant growth-stimulating strains of *Bacillus* spp. from the rhizosphere of plants living in conditions of moisture deficit, since the growth and survival of bacteria under such conditions determines their ability to mitigate the damaging effect of drought on plants [50, 52]. Recent research by U. Rashid et al. [52] showed that drought-tolerant strains *B. megaterium* MU2 and B. *licheniformis* MU8 from the rhizosphere of plants growing in arid and semi-arid territories cause systemic wheat resistance to drought. In an in vitro experiment, the authors studied 90 isolates of rhizobacteria, of which 38 were found to exhibit one or more PGP properties, including solubilization of P, K and production of exopolysaccharides, but only two strains, *B. megaterium* MU2 and *B. licheniformis* MU8, had the best potential to increase drought resistance of plants (activity of ACC deaminase, IAA production and antagonistic activity against phytopathogens). In addition, when exposed to drought (-0.73 MPa), *B. megaterium* produced three new polypeptides with molecular weights of 18 kDa, 35 kDa, and 30 kDa [52]. Two selected drought-tolerant strains under normal irrigation and drought conditions increased the germination of wheat

seeds by 11-46%, respectively, the viability of seedlings - by 11-151%, the accumulation of wet mass by 35-192%, dry by 58-226%. Moreover, these strains effectively colonized wheat roots and increased the relative water content, the amount of photosynthetic pigments and osmolytes. Thus, after exposure to drought, the survival rate of wheat plants inoculated with *B. megaterium* MU2 increased due to an increase in the relative water content (by 59%), the number of chlorophylls a, b and carotenoids (by 260, 174 and 70%, respectively), protein content (by 136 %), proline (by 117%) and a decrease in MDA formation (by 57%) [52].

Genotypic specificity of the action of *Bacillus* spp. on wheat plants. In practice, the effectiveness of *Bacillus* spp. can vary depending on both environmental conditions and many other factors, including the genotype of plants, their ecological and geographical origin, varietal characteristics, characteristics of strains [34, 54, 55, 88]. The use of representatives of *Bacillus* spp. to increase the yield of field and vegetable crops is limited by the variability of the results obtained in the laboratory, in the greenhouse and in the field [23]. In fact, with repeated introduction (with inoculation of both plants and soil), only 1-2% of PGPB has a positive effect on plant growth comparable to the results of laboratory tests [23]. Analysis of long-term data on the effectiveness of the commercial biological product Fitosporin-M (base *B. subtilis* 26D, Research and Innovation Company BashInkom LLC, Ufa, Russia) in field conditions on spring wheat showed a higher responsiveness to seed treatment with a biological product in arid conditions of the Orenburg and Kurgan regions (increase, respectively, up to 43 and 24%) (55). The specificity of the interaction of Fitosporin-M with different wheat genotypes was revealed, depending on their resistance to drought and belonging to ecotypes. The highest efficiency of Fitosporin-M was noted under arid conditions on spring wheat varieties of the steppe ecotype [55]. The results of laboratory experiments also showed that, under the same growing conditions, wheat varieties belonging to different ecotypes exhibited unequal drought tolerance upon inoculation with *B. subtilis* 26D [88]. The *B. subtilis* strain 26D increased the germination of wheat seeds of the Saratovskaya 55 cultivar (Volga steppe ecotype) during drought, while practically no effect or even inhibited the germination of seeds of the Omskaya 35 cultivar (forest-steppe West Siberian ecotype) [88]. Inoculation with *B. subtilis* 10-4 strain led to similar results, which significantly mitigated the damaging effect of drought on seed germination and plant growth (length of roots and shoots, wet and dry weight) in wheat of the resistant cultivar Ekada 70 (Volga steppe ecotype) on early stages of ontogenesis and did not have a protective effect for the same parameters in relation to the susceptible cultivar Salavat Yulaev (forest-steppe West Siberian ecotype) [34]. Nevertheless, treatment with *B. subtilis* 10-4 induced (albeit to varying degrees) protective responses at the cell level in both varieties, which manifested itself in a decrease in the degree of oxidative and osmotic damage to cells caused by drought and in modulation of photosynthesis and water exchange [34]. Other authors reported that the growth response of wheat to inoculation with bacteria producing ACC deaminase in normal conditions and during drought depended on the plant genotype [46]. It was suggested that such differences are associated either with the composition of root exudates, which is unique for certain wheat genotypes, or with other differences between genotypes that affect their ability to maintain a relatively high number of ACC deaminase producers in the rhizosphere, which requires further study. Identification of features and differences between genotypes and ecotypes of wheat in reactions to inoculation with *Bacillus* spp. could lead to innovative selection strategies to improve plant drought tolerance. Successful use of *Bacillus* spp. in

the field largely depends on the interaction between plants and bacilli, which may be limited by poor colonization of the rhizosphere [23]. The bacillus takes 24 hours to form a biofilm, which promotes root colonization and prolongs the beneficial effects of *Bacillus* strains on the soil. However, colonization of roots with local (aboriginal) bacilli strains is more effective than laboratory or commercial ones. New strategies such as microbiome engineering and selective optimization of microorganisms are helping to detect, modulate and improve target traits, thereby increasing the effectiveness of *Bacillus* spp. and preparations based on them. It is necessary to understand how different strains regulate the growth and development of plants, taking into account the numerous factors affecting the effectiveness of *Bacillus* spp., to clearly determine the characteristics and method of choosing the most effective strains. Transcriptome analysis of the *B. amyloliquefaciens* genome revealed numerous genes involved in ensuring the engraftment of bacteria in the rhizosphere and control of traits useful for plants, including the use of plant polysaccharides, cell motility and chemotaxis, secondary synthesis of antibiotics, and clusters related to plant growth stimulation [23]. It has been shown that root and seed exudates serve as gene inducers, the products of which are involved in root colonization and interactions of plants with bacilli [49, 89, 90]. New studies of the interaction of plants and bacteria are revealing the ability of plants to form their rhizosphere and the microbiome of endoriza [91]. The results obtained to date indicate the existence of other yet unknown mechanisms of Bacillus-mediated drought resistance in wheat, which will be disclosed with the development of technologies for analyzing gene functions [41]. The use of Next Generation Sequencing (NGS) methods in combination with proteomics, metagenomics, and metabolomics will help clarify the details of these interactions, including how this relationship affects plant growth and drought resistance [23, 90].

The use of compositions of *Bacillus* spp. with other bioregulators and microorganisms. In order to improve the effectiveness of *Bacillus* spp. it is of interest to formulate their various combinations with other growth regulators, as well as consortia with other potentially useful microorganisms. For example, bacteria *B. amyloliquefaciens* producing ACC deaminase in combination with organic biofertilization biochar (wood biochar) more effectively mitigated the effect of mild and severe field drought on wheat plants, enhanced their growth and increased productivity compared to the use of *B. amyloliquefaciens* or biochar separately [44]. These results confirmed the data obtained by the authors earlier in laboratory conditions, where the use of B. *amyloliquefaciens* improved the morphological parameters of wheat under conditions of polyethylene glycol-induced drought [45, 46]. In other studies, inoculation with *B. subtilis* 10-4 in combination with SA also more effectively protected wheat plants from drought and the combined effects of drought and fusarium root rot, which manifested itself in a noticeable improvement in growth parameters (length of roots and shoots, accumulation of wet and dry biomass), in modulation of photosynthesis processes, water exchange, and a decrease in the degree of oxidative and osmotic damage [22]. A similar positive effect of the PGPB + SA combination on wheat plants with a lack of water was obtained by N. Khan and A. Bano [50]. During drought, wheat plants (varieties resistant and susceptible to drought) treated with the composition PGPB (*B. cereus* P2, *Planomicrobium chinense* P1) + SA were characterized by a significant increase in the amount of proteins and sugars in the leaves, the content of chlorophyll and the intensity of its fluorescence, as well as a lower synthesis of stress-induced proline, antioxidant enzymes, and a decrease in lipid peroxidation in comparison with control non-inoculated plants and plants inoculated with

PGPB alone [50]. The use of a combination of strains PGPB *Bacillus* spp. and SA appears to be a promising and environmentally sound strategy to reduce the damaging effects of drought on wheat plants. Several authors have reported on the promise of using PGPB in combination with exopolysaccharides to combat drought stress [67]. A study by Y. Li et al. [92] showed that the addition of a superabsorbent polymer (SAP) to a culture of *Bacillus* sp. L-56SAP significantly increased the survival rate of the inoculant and contributed to mitigating the effects of drought on wheat (improved seed germination and plant growth) and increased soil fertility (urease, sucrose and dehydrogenase activity). In addition, *Bacillus* sp. L-56 + SAP significantly increased the chlorophyll content in wheat plants. Real-time qPCR analysis showed that *Bacillus* sp. L-56 + SAP in wheat plants under drought conditions, the expression levels of genes involved in ROS capture (*TaCAT, CsCAT, TaAPX*, and *CsAPX2*), biosynthesis of ethylene (*TaACO2, CsACO1* and *CsACS1*), SA (*TaPR1-1a* and *CsPR1-1a*), and stress responses (*TaDHN3*, *TaLEA*, and *CsLEA11*), but transcription of the *TaNAC2D* and *CsNAC35* genes is activated [92]. Some researchers reported the effectiveness of using *B. subtilis* CP4 isolated from soil in combination with arbuscular mycorrhiza fungi to increase the accumulation of N, P, Cu, Fe, and Zn in wheat grain, which led to both the biofortification of grain products and increased growth, an increase in photosynthetic activity, yield and production of metabolites in wheat plants under field conditions [65]. Besides, the postharvest physicochemical analysis of soil samples showed that when *B. subtilis* CP4 and fungi of arbuscular mycorrhiza were introduced, the enzymatic status of the soil (invertase, β-glucosidase, dehydrogenase activity) improved in comparison with untreated plots, while it was the combined use of PGPB and fungi to the greatest extent contributed to an increase in soil fertility [65]. Experiments have been reported to study the effect of consortia of *Bacillus* spp. with other PGPBs on the growth and yield of wheat both in laboratory and in the field [39, 93]. Thus, treatment with tetra-combination *B. megaterium* + *Arthrobacter chlorophenolicus* + *Enterobacter* sp. + *Pseudomonas aeruginosa* significantly increased the height of wheat plants (in the greenhouse and in the field experiment by 24.56 and 47.06%, respectively), grain yield (by 75.80 and 40.09%), and straw (by 76.55 and 42.63%) compared to control. The same four-component and two three-component inoculations - *B. megaterium* + *A. chlorophenolicus* + *P. aeruginosa* and *A. chloro-phenolicus* + *Enterobacter* spp. + *P. aeruginosa* promoted an increase in the yield and absorption of nutrients by wheat [39]. It was reported that inoculation with the *B. megaterium* M3 strain and a combination of bacteria (*B. megaterium* M3 + *B. subtilis* 05U142 + *A. brasilense* Sp245) created a higher supply of wheat plants with nutrients than the application of mineral fertilizers [93]. Similar comparisons with chemically fertilized soils are given in other works [26, 37, 94]. It should be noted that some PGPBs, when inoculated, are capable of negatively affecting mutualistic associations between plants and native soil microorganisms, and this may be the reason for the lack of a favorable effect in some PGPBs [95]. The structure of the wheat rhizobacterium community is very dynamic and depends on various factors: cultivar, plant age, stage of growth, and distance of soil particles from root hairs, nature of root exudation, soil properties and agricultural practices [26, 90]. In general, the accumulated data indicate that the combinations of *Bacillus* spp. with other natural growth regulators and various microorganisms to enhance the growth-stimulating and anti-stress effects of microbiological preparations on plants and increase soil fertility.

Commercialization of bacillary biologics. Many beneficial strains of *Bacillus* spp. commercially available for use as biofertilizers and plant protection products in the form of finished preparations (see Table).

Examples of commercial biologics based on *Bacillus* spp. to stimulate the growth and protection of plants, including wheat

Basis	Biologic	Producer
B. subtilis 26Д	Fitosporin-M	BashInkom Research and Innovation Company, Russia
B. subtilis GB03	Quantum-400	Ecological Laboratories, Inc., USA
B. subtilis QST713	Serenade	AgraQuest, Inc., USA
B. subtilis улуч. GB03	Alinit, Kodiak	Gustafson, Inc., USA
B. subtilis MBI600	Subtilex	Becker Underwood, Inc., USA
B. subtilis Ч-13	BisolbiSan	All-Russia Research Institute of Agricultural Biotechnology, RAAS, Russia
B. subtilis 63-Z	Invivo, Russia	Invivo, Russia
B. subtilis ИПМ215	Baktofit	Sibbiofarm, Russia
B. subtilis B-10 ВИЗР	Alirin-B	AgroBio Tehnologia, Russia
B. subtilis M-22 ВИЗР	Gamair	AgroBio Tehnologia, Russia
B. subtilis BKM-B-2604D, *B. subtilis* BKM-B-2605D	Vitaplan	VISR, Russia
B. subtilis	Companion	Growth Products, Ltd., USA
B. subtilis	Cease	BioWorks, Inc., USA
B. subtilis	Pro-Mix	Premier Horticulture, Inc., Canada
B. velezensis ABi19	FZB24	ABiTEP GmbH, Germany
B. subtilis	Bio Safe	Lab. Biocontrole Farroupilha, Brazil
B. subtilis	Ecoshot	Kumiai Chemical Industry, Japan
B. subtilis	Biosubtilin	Biotech International, Ltd., India
B. amyloliquefaciens	BioYield	Gustafson, Inc., USA
B. amyloliquefaciens,	Rhizocell GC	Lallemand Plant Care, France
B. velezensis FZB42	RhizoVital®42	ABiTEP GmbH, Germany
B. velezensis FZB45	RhizoVital®45	ABiTEP GmbH, Germany
B. atrophaeus Abi05	RhizoFert®	ABiTEP GmbH, Germany
B. pumilus	Yield Shield	Bayer CropScience, USA
B. pumilus	Ballad Plus	AgraQuest, Inc., USA
B. pumilus	Sonata	AgraQuest, Inc., USA
B. licheniformis	EcoGuard®	Novozymes A/S, Denmark
B. velezensis	Botrybel	Agricaldes, Spain
B. megaterium	Symbion-P	T. Stanes & Co., Ltd., India
Bacillus sp.	Sublic	ELEP Biotechnologies, Italy
Bacillus spp.	Bacillus SPP	Bio Insumos Nativa, Chile

In the world, there is an annual increase in the number of developments of new commercial preparations containing useful strains of bacilli - *B. subtilis, B. megaterium, B. amyloliquefaciens, B. licheniformis, B. pumilus, B. cereus, B. thuringiensis, B. velezensis* [23]. In many works, attention is paid to individual stages of commercialization: the isolation of bacterial strains with useful properties, screening, fermentation methods, mass production, and determination of the viability of the formulation, toxicology, industrial relations, quality control and the effectiveness of practical application [13]. The success of the commercialization of useful strains is influenced by the market demand and popularization of drugs, their safety, stability, shelf life, low cost and ease of use, and the availability of carrier materials [96]. One of the most important aspects of the development and commercialization of effective biological products based on bacilli is the knowledge of the mechanisms of their interaction with plants in normal conditions and especially under stress. In addition, the result of developments largely depends on the effectiveness of interaction between scientific organizations and industries.

Thus, summarizing the literature data indicates the important role of *Bacillus* spp. in the regulation of growth, development and stress resistance of plants, which undoubtedly expands the significance of the practical use of bacilli for increasing drought resistance and productivity of the most important agricultural crops, including wheat. This is also evidenced by the annual increase in the number of basic research, development and distribution of commercial bacillary biological products. At the same time, the results obtained to date indicate the presence of as yet unrevealed mechanisms of *Bacillus*-mediated drought resistance in wheat. In future studies, it seems important to focus on the metabolic pathways

that provide bacilli-induced systemic plant resistance, on expanding knowledge about the spectrum of metabolites produced by bacilli, signaling molecules (phytohormones, amino acids, etc.) and their contribution to the functioning of vital plant defense systems, and also on the identification of new key components responsible for the regulation of wheat drought resistance by bacteria. For a more complete assessment and use of the potential of bacteria *Bacillus* spp. as inoculants that ensure sustainable wheat productivity, it is necessary to understand how different strains stimulate growth and protect plants from stress, and clearly identify the factors contributing to a more effective practical use of these strains, especially in arid conditions of a changing climate. Special attention should be paid to the study of the specificity of the interaction of bacillary strains with different varieties of wheat and the precise selection of their complementary combinations, taking into account the geographical factors of plant growth (climate, soil). It is also important to identify the most effective combinations of *Bacillus* spp. with other natural growth regulators and microorganisms (multistrain consortia), exhibiting an additive effect in protecting wheat from drought and intended for targeted use in various soil and climatic conditions of crop cultivation. In addition, future research should focus on the development of methods for preserving the ability of bacillary strains and (or) their combinations (consortia) with other growth regulators and microorganisms to influence the growth and drought resistance of wheat plants in the field as effectively as in laboratory tests. In-depth study of the mechanisms of interaction of bacilli with wheat plants during drought is based on the integration of modern approaches (NGS, proteomics, and metagenomics and metabolomics methods) in order to develop promising projects and strategies for the practical application of effective and environmentally friendly microbiological preparations.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 868-884
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 632.93

doi: 10.15389/agrobiology.2021.5.868eng
doi: 10.15389/agrobiology.2021.5.868rus

FUTURE DIRECTIONS FOR USE OF BIOLOGICAL AND BIORATIONAL HERBICIDES IN RUSSIA

(review)

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The authors declare no conflict of interests
Received July 6, 2021

Abstract

The emergence of weed populations resistant to chemical herbicides leads to a widespread decrease in the effectiveness of the chemical control. This fact, along with the currently increasing consumer demand for organic food, leads to an awareness of the need to develop research on the development of biological means of protecting crops from weeds. Despite the fact that biological (BLH) and biorational herbicides (BRH) are being introduced in the market of weed control products in the United States, Canada, China and South Africa, no such products has been registered in the Russian Federation to date. At the same time, the development of research on the development of environmentally friendly means of weed control allows to count on a change in the existing situation in the foreseeable future (A.O. Berestetskiy, 2017; M. Triolet et al., 2020). The purpose of this literature review was to analyze the current range of chemical herbicides allowed for use in Russia in order to identify market niches that BLH and BRH may occupy in the near future. To assess the prospects of these products, first of all, the spectrum of their action was taken into account, due to the species specificity of plant pathogens, which is significantly narrower than the activity spectrum of chemical herbicides (A. Berestetsky et al., 2018; A. Berestetsky, 2021). The analysis was based on a list of pest organisms that are particularly dangerous for crops prepared by the All-Russian Research Institute for Plant Protection (2013), in which the following types of weeds were indicated: perennial sowthistle (*Sonchus arvensis* L.), Canada thistle (*Cirsium setosum* (Willd.) Bess.), field bindweed (*Convolvulus arvensis* L.), couch grass (*Elytrigia repens* (L.) Nevski), and wild oat (*Avena fatua* L.). The list was supplemented with two quarantine weeds, common ragweed (*Ambrosia artemisiifolia* L.) and Russian knapweed (*Acroptilon repens* DC.), which are limited in the territory of the Russian Federation, but are problematic for a number of regions. These types of weeds have different degrees of harmfulness in different crops (A.M. Shpanev, 2011). The analysis involved the most significant agricultural crops from the point of view of the structure of the arable land of the Russian Federation. The use of BLH and BRH seems most promising in orchards and vineyards, where, due to the exclusion of glyphosate-based herbicides, only gufosinate-ammonium is allowed for use (A.S. Golubev et al., 2018; 2019). In addition, BLH and BRH, used in combination with some herbicides, would increase the effectiveness of weed control and the duration of the protective effect. The risks of using BLH and BRH in orchards and vineyards do not look significant due to the relative isolation of these agroecosystems. Forage crops and greenhouse vegetables do not have much potential as niches for the use of BLH and BRH, forage crops due to low economic returns, and vegetables in greenhouses due to the peculiarities of their cultivation technology. The use of BLH and BRH in fields intended for sowing agricultural crops in the autumn period and in fallow fields looks promising. In the conditions of crop rotations, BLH and BRH can be applicable against perennial root-sprouting weeds and Russian knapweed during the growing season of soybeans, sunflower, and potatoes. For the last two crops, the use of BLH and BRH against common ragweed looks promising as well. It will be possible to occupy a niche associated with the destruction of grass weeds (such as couch grass or wild oat), in the conditions of the existing range of chemical herbicides, only for the suppression of resistant weed populations.

Keywords: bioherbicides, cereals, corn, soybean, sunflower, potato, orchard, *Sonchus arvensis*, *Cirsium setosum*, *Convolvulus arvensis*, *Elytrigia repens*, *Avena fatua*, *Ambrosia artemisiifolia*, *Acroptilon repens*

Weeds represent a traditional challenge to cultivation of agricultural, medicinal, and ornamental crops. The main control measures include the use of chemical herbicides and, to a lesser extent, tillage. Due to a decrease in the effectiveness of the chemical control owing to the appearance of herbicide-resistant weeds, on the one hand, and owing to an increase in the share of consumption of organic food, on the other hand, there is a need to reduce the use of chemicals in the agricultural production. At the same time, for the control of weeds, certain hopes rest on the biological (BLH) and biorational (BRH) herbicides [1, 2].

There are many examples of widespread and commercially successful use of entomophages, microbiological preparations, and natural compounds to combat pathogens and phytophages. As practice shows, the effectiveness of BLH and BRH, as a rule, is significantly lower than chemical ones [3, 4]. In this regard, they are of limited use: in greenhouses, in organic farming, and in public places where the use of the chemical control methods is prohibited.

The purpose of this literature review was to analyze the current range of chemical herbicides allowed for use in Russia in order to identify market niches that BLH and BRH may occupy in the near future.

Bioherbicides are preparations used for weed control, consisting of live microorganisms and auxiliary components (surfactants, adjuvants, preservatives, water-retaining additives, and inert fillers). Since phytopathogenic fungi are mostly considered as active components of bioherbicides (BHB), preparations based on them are isolated into a separate group and called mycoherbicides (MHB). Plant or microbial extracts with phytotoxic properties, purified or semi-purified natural phytotoxins are classified as biorational chemical herbicides (BRH) [1].

The use of BHB is aimed at causing local epiphytotics in populations of unwanted plants and, as a consequence, reducing their competitiveness. BHB are designed for regular use, but their effect can be prolonged for several seasons. BHB are selective microbiological preparations suppressing one or several types of weeds. Some BHBs contain weakly specialized phytopathogens found both on target weeds and on cultivated plants. Such pathogens can be used in special situations when susceptible crops are not sown or are not included in the crop rotation where weed biocontrol is planned [5-7].

Herbicide preparations based on natural phytotoxins (biorational herbicides) have certain advantages over BHB: clear mechanisms of action and quality control, significantly less dependence of efficiency on external factors. Phytotoxic compounds are predominantly secondary metabolites of plants (allelopathy effect) and microorganisms (factors of plant pathogenicity or colonization), killing plant cells in small concentrations. In addition, some primary metabolites have phytotoxic properties: a number of amino acids, some organic and fatty acids [8, 9].

The existing biorational plant protection products, including BRH, can be conditionally divided into four groups: microbial preparations of toxin action; coarse extracts of plant or microbial origin; individual natural compounds (or mixtures thereof) of various degrees of purification; synthetic analogs of natural compounds [10]. The latter, strictly speaking, do not belong to the natural ones, therefore, in this review we are not considering chemical herbicides based on them.

The first group includes such preparations as Bioprotec Herbicide™ (AEF Global, Inc., Canada) containing lactobacilli, which form phytotoxic lactic and citric acids to inhibit the growth of clover on lawns [11]. For the control of the parasitic weed *Striga hermonthica* (Delile) Benth., a bioherbicide based on *Fusarium oxysporum*, a superproducer of tyrosine, an amino acid that is able to

inhibit the development of striga was developed in the United States [12]. MHB based on *Phoma macrostoma* fungus (Evologic Technologies GmbH, Austria) acts due to phytotoxins from the group of macrooxazoles [13, 14].

The next group of BRH includes essential oils, plant extracts, green manures, and food waste [15]. For instance, in the United States, corn gluten is used as BRH, the decomposition of which produces phytotoxic peptides [16]. Milled green mass of mustard and soy flour (application rate, respectively, about 1 and 4 t/ha) were effective for suppressing weeds in the crops of spinach *Spinacia oleracea* L. and broccoli *Brassica oleracea* L. var. *italica* in organic farming [17].

An extract from the legume plant *Canavalia ensiformis* (L.) DC showed high herbicidal activity in suppression of ivy *Ipomoea grandifolia* (Dammer) O'Donell and *Commelina benghalensis* L. in soybean crops [18]. Essential oils of about 20 plant species used to control various types of weeds were reviewed by R. Raveau et al. [19]. It seems interesting to use essential oils of ragweed weeds having phytotoxic properties [20]. Phenolic substances from *Ludwigia hyssopifolia* (G. Don) Exell provided significant suppression of shoot growth and biomass accumulation in *Amaranthus spinosus* L., *Dactyloctenium aegyptium* L., and *Cyperus iria* L. [21].

Several commercial and trial BRHs are known from the third group of biorational plant protection products. Acetic acid is used to control weeds on small gardens in the United States [16]. A herbicide mixture based on acetic and citric acids could be effective [22]. The phytotoxic glycoside tricolorin A has been isolated from the biomass of *Ipomoea tricolor* Cav., which Mexican farmers use as a cover crop on sugarcane to suppress weeds. This phytotoxin at a concentration of 60 μM acts as a nonselective inhibitor of seed germination and plant shoot growth and can be considered as an alternative to glyphosate [23]. Marrone BioInnovations (USA) has developed Opportune® preparation, the active ingredient of which is the bacterial phytotoxin takstomin A. Belchim Crop Protection (Belgium) offers BRH Katoun® Gold based on pelargonic acid for use in the organic farming [11]. Tenuazonic acid, produced by some fungi of *Alternaria* genus, is patented by Chinese scientists and is being studied as a natural herbicide with an original mechanism of action. Its chemical synthesis has been developed and the possibility of practical application in the field has been shown [24].

According to the literature analysis, new herbicidal compounds of natural origin are actively being sought in the world [25]. Officers of Dow AgroSciences LLC (USA) have identified a number of microbial phytotoxins which are perspective for creation of new BRHs: macrocidin [26], cinnacidin [27], albucidin [28], and mevalocidin [29]. Cordycepin (a metabolite of the fungus *Cordyceps militaris* (L.) Fr.) at a concentration of 0.04 mg/ml inhibited the growth of radish roots several times stronger than benzoic acid and glyphosate [30].

The natural compound plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was isolated from the leaves of *Plumbago auriculata* (Lam.) Spach, which was effective in field conditions against a number of monocotyledonous and dicotyledonous weeds [31]. Ailantone from *Ailanthus altissima* (Mill.) Swingle is perspective for the development of a new natural herbicide [32]. Asteric acid (one of the secondary metabolites of fungus *Aspergillus terreus* Thom) is a highly active dihydroxy acid dehydratase inhibitor and an effective post-emergence herbicide [33].

To increase the efficiency of weed control, various chemical herbicides, BRH and BLH can be used together to enhance their action. [34]. For a long time, mixtures of natural products, such as acetic acid, lemon extract and clover oil, have been used for organic farming and on lawns [35]. A composition increasing the effectiveness of glyphosate (0.8-1.2 l/ha), which includes a mixture of L-

2-amino-2-methyl-mercaptobutyric acid, L-α-diaminocaproic acid, and L-β-phenyl-α-aminopropionic acid (about 10 g/ha at a ratio of components 2:1:1) and ammonium nitrate (2 kg/ha) has been developed. An increase in the efficiency of glyphosate is achieved by more active absorption of the herbicide by weeds, which makes it possible to reduce the rate of its consumption. Microfield and field tests have shown the possibility of reducing the rates of glyphosate application by about 2 times when combined with a mixture of succinic and malic acids at a concentration of 10 - 11 M [36]. The mixture of manuka *Leptospermum scoparium* J.R. Forst. & G. Forst. essential oil and pelargonic acid was effective against three weed species (*Lolium rigidum* Gaud., *Avena sterilis* L., and *Galium aparine* L.) [37].

In the Russian Federation, there are no registered BLH and BRH, and there is little research in this regard. In particular, producers of herbicidal compounds (pheosferide A, stagonolide A, and herbarumina I) — strains of the fungi *Paraphoma sp.* and *Stagonospora cirsii* have been patented. Methods for their application have been developed, but there are no clear ideas about the prospects for their use; toxicology and methods of pilot production for field trials are poorly studied [38].

The effectiveness of BLH and BRH is lower than that of chemical herbicides, the area of treatment cannot be large, and the shelf life of such preparations is limited. However, they can be used in organic farming or as a component of an integrated control of particularly harmful weeds.

The registration stage following the creation of the preparation requires significant financial costs. Typically, such expenses are available to large chemical companies, whose marketing departments calculate an approximate return on the cost of bringing the bioherbicide to the market. At the same time, even considerable investments in registration of a preparation do not always repay by its high activity in the field [4].

In our opinion, the most important stage in planning a strategy for bringing scientific developments of biological and biorational herbicides to the end user should be a clear identification of market niches for their economically justified use, as well as an assessment of market prospects, taking into account the existing range of chemical herbicides for protection of major crops. It should be noted that this approach is especially significant in Russia, where no such preparation has yet been registered.

To assess the market prospects of BLH, it is important to take into account the spectrum of their action, due to the species specificity of phytopathogens. It requires a list of weed species, the use of biopreparations against which may be appropriate. It should also be noted that BRH based on organic acids, fats, and oils obtained from plants are less effective than chemical agents and require significant amounts of application, which complicates the use of BRH in industrial plant growing [39].

In 2013, All-Russian Research Institute for Plant Protection has prepared a list of pest organisms that are particularly dangerous for crops, in which the following types of weeds were indicated: perennial sowthistle (*Sonchus arvensis* L.), Canada thistle (*Cirsium setosum* (Willd.) Bess.), field bindweed (*Convolvulus arvensis* L.), couch grass (*Elytrigia repens* (L.) Nevski), and wild oat (*Avena fatua* L.) [40]. This list, in our opinion, can be taken as a basis and supplemented with two quarantine weeds, common ragweed (*Ambrosia artemisiifolia* L.) and Russian knapweed (*Acroptilon repens* DC.), which are limited in the territory of the Russian Federation, but are problematic for a number of regions [41]. These types of weeds have different degrees of harmfulness in different crops.

An analysis of the structure of the sown areas of the Russian Federation makes it possible to compile a list of the most significant agricultural crops. In

2019, cereals and legumes are 58.4% of the total sown area; industrial crops occupied 19.9%, fodder crops 19.3% [42]. It can be assumed that the latter group does not have significant potential as a niche for the use of microherbicides due to the low economic return. Greenhouse vegetables also do not look promising due to the peculiarities of their cultivation technology.

We will consider grain crops in aggregate, since most herbicides are allowed for use simultaneously on wheat (winter and spring), barley (spring and winter), and oats. As individual crops, we will take the most common: soybeans from the group of legumes, whose share is 2.7% of the total amount of all sown areas, corn (3.2%), sunflower (10.7%), and potatoes (1.6%) [42]. To systematize the approach to the analysis, we further described the type of weed plant, the degree of its threat to the crop, the range of herbicides that can effectively control the number of the object in the crops, and possible alternative solutions.

Perennial sowthistle, Canada thistle, and field bindweed belong to the group of perennial root weeds. The presence of Canada thistle and sowthistle among the dominant species is typical for most regions of Russia, whilst the field bindweed is more common in the middle and southern regions [43]. According to the research of A.M. Shpaneva (2011-2013), these perennial weeds in their harmfulness significantly exceed annual weeds [44-46].

Usually they are fought with the introduction of general exterminating glyphosate-based herbicides: Roundup Max, WS (450 g/l glyphosate/isopropylamine salt), Sprut Extra, WS 540 g/l glyphosate/potassium salt), Kileo, WSC (240 g/l glyphosate/isoprolamine salt + 160 g/l 2,4-D/3-alkylaminopropyl dimethylamine salt), etc. However, the use of these preparations, as a rule, is possible only in fallow fields and in fields intended for sowing or planting various crops in late summer or in autumn in the post-harvest period [47]. On individual crops (sunflower, soybeans), the preparations can be applied 2-5 days before sowing, on corn — 2 weeks before sowing, on potatoes — 2-5 days before the emergence of crop shoots.

Despite the high efficiency of general exterminating herbicides in the fight against perennial root-sap weeds, such treatments are becoming a preventive measure, which does not fully correspond to modern ideas about the ecological development of plant protection. In addition, in view of possible restrictions or even a ban on the use of glyphosate in our country [48], it makes sense to focus on drugs to combat perennial root weeds during the growing season.

A significant number of such preparations are among herbicides for protecting grain crops. The highly specialized herbicides against sowthistle and Canada thistle include preparations based on clopyralid, such as Lontrel-300, WS (300 g/l), Hacker, WSG (750 g/kg), Lontrel grand, WDG (750 g/kg); against field bindweed — the preparations based on fluroxipir: Demeter, EC (350 g/l), Starane Premium 330, EC (333 g/l). Preparations of a wider spectrum of action have been developed on the basis of proven and well-reputed active ingredients, such as 2,4-D acid esters — Esteron 600, EC (600 g/l), Drotik, CSC (400 g/l), Estet, EC (600 g/l); sulfonylureas — Laren Pro, WDG (600 g/kg metsulfuron-methyl), Tribun, DFC (750 g/kg tribenuron methyl), etc. In addition, there is a significant number of combined preparations that have high activity against perennial root-sapling weeds: Prima, SE (300 g/l 2,4-D/complex 2-ethylhexyl ether/ + 6.25 g/l florasulam), Bomb, WDG (563 g kg tribenuron methyl + 187 g/kg florasulam), Unico, CSC (100 g/l fluroxipir + 2.5 g/l florasulam), etc. [49].

The same preparations are often used on corn crops as on cereals [50]. Of the complex action herbicides specific for corn, one could use as an example the herbicide Modern, EC (412 g/l 2,4-D/complex 2-ethylhexyl ether/ + 80 g/l nicosulfuron + 8 g/l florasulam).

The number of herbicides for the control of perennial dicotyledonous weeds on soybeans, sunflowers and potatoes is significantly less. If we consider soybeans, herbicides Harmony Classic, WDG (187.5 g/kg thifensulfuron methyl + 187.5 g/kg chlorimuron-ethyl) and Fabian, WDG (450 g / kg imazethapyr + 150 g/kg chlorimuron ethyl). It is possible to use chemical preparations to suppress Canada thistle and thistle species on sunflower crops during the growing season of the crop only on special hybrids resistant to sulfonylureas, for example, tribenuron methyl [51]. For these purposes, Express, WDG (750 g/kg tribenuron methyl), Sanflo, WDG (750 g/kg), Prometheus, WDG (750 g/kg) are used. During the growing season of potatoes, some activity against perennial root-sprouting weeds is observed when using preparations based on rimsulfuron — Titus, DFC (250 g/kg), Cassius, WSP (250 g/kg), Escudo, WDG (500 g/kg), although it is difficult to solve the problem by limiting the use of only these preparations [52].

In our opinion, it is the niche of the fight against perennial dicotyledonous weeds that looks the most attractive for the use of bioherbicides. On the one hand, this is due to the existing assortment of chemical herbicides (and the possible exclusion of glyphosate from the number of preparations permitted for use), on the other hand, by the very number of pathogens of perennial root weeds. So, only for the fight against the field thistle *Phoma destructiva*, *Phoma hedericola*, *Phoma exigua*, *Puccinia punctiformis*, *Mycelia sterilia*, *Phomopsis cirsii*, *Sclerotinia sclerotiorum*, *Alternaria cirsinoxia*, *Stagonospora cirsii*, *Septoria cirsii*, and *Phyllosticta cirsii* were considered [53-55].

As of January 1, 2019, Russian centaury was found in 19 constituent entities of the Russian Federation, and the area of the established quarantine phytosanitary zones exceeded 1,885,590 hectares (56].

As a rule, the fight against the Russian centaury is most effective in fallow fields and fields intended for sowing grain crops. The following preparations are used: General Secretary, WSG (88.5 g/l dicamba + 88.5 g/l picloram + 177 g/l clopyralid), Gorgon, WSC (350 g/l MCA acid + 150 g/l picloram) [57]. During the growing season of the crop, you can use Octymet, EC (500 g/l 2,4-D acid + 5.5 g/l metsulfuron methyl) or Lancelot 450, WDG (300 g/kg aminopyralide + 150 g/kg florasulam).

The range of herbicides in this direction is small, and since preparations for the control of the Russian centaury usually have strict limitations on crop rotation [58], the development of alternative, including biological, means of control is potentially attractive. The fact that there are no herbicides approved for use on vegetative plants of corn, soybeans, sunflower, and potatoes to control the Russian centaury makes this area even more urgent.

At the end of the 20th century, attempts were made to use nematodes from the genus *Subanguina picridis* Kirj & Ivan as biogrebicides to control the Russian centaury. [59]. At beginning of the 21st century, it was proposed to use the allelopathic effect of essential oils of eucalyptus, Lawson's cypress, rosemary and white cedar for this purpose [60].

As of January 1, 2019, ragweed was found in 31 constituent entities of the Russian Federation, the area of the established quarantine zones exceeded 7,356,593 hectares [56]. It should be noted that ragweed not only worsens the growing conditions of cultivated plants, reducing their productivity, but also causes allergic reactions [61].

As in the case of perennial dicotyledonous weeds, herbicides based on clopyralid can be used to control this species on crops of grain crops, for example, Agron, WS (300 g/l), Agron Grand, WDG (750 g/kg), and also using the preparations with a wider spectrum of action, including the combined ones — Primadonna, SE (200 g/l 2,4-D acid + 3.7 g/l florasulam), Ballerina, SE (410 g/l

2.4-D acid + 7.4 g/l florasulam) [62]. These combined herbicides can also be applied to corn crops. In addition, maize crops use specific herbicides MaysTer, WDG (300 g/kg foramsulfuron + 10 g/kg iodosulfuron methyl sodium + 300 g/kg antidote isoxadifen ethyl) and MaysTer Power, OC (31.5 g/l foramsulfuron + 1 g/l iodosulfuron methyl sodium + 10 g/l thiencarbazone methyl + 15 g/l cyprosulfamide antidote) [63].

Soy is a leguminous plant and exhibits natural resistance to herbicides of the imidazolinone group, which can effectively destroy the ragweed plants. The examples of such preparations include Pulsar, WS (40 g/l imazamox) and Pivot, WC (100 g/l imazetapir). In addition, in the fight against this harmful object on soybeans, bentazone based herbicides — Bazagran, WS (480 g/l), Corsair, WSC (480 g/l), Benito, CSC (300 g/l) are successfully used, as well as combined drugs, for example Corum, WSC (480 g/l bentazone + 22.4 g/l imazamox) [64].

When cultivating sunflower hybrids that are resistant to imidazolinones, the introduction of herbicides of this group makes it possible to effectively destroy ragweed plants. Examples of such preparations include Euro-Lightning, WSC (33 g/l imazamox + 15 g/l imazapir), Pulsar, BP (40 g/l imazamox), Tapir Hybrid, OC (50 g/l imazethapyr + 20 g/l imazapir) [65].

Since clopyralid, bentazone and imidazolinones are not recommended for use in potato plantings, there are no highly effective means of combating ragweed during the growing season of this crop.

It should be noted that due to the significant stock of ragweed seeds in the soil, the second and sometimes the third wave of emergence of this weed plant can be observed in the agricultural crops. During this period, agrotechnical and chemical weed control is challenging due to the peculiarities of the biology of cultivated plants. As a result, ragweed plants go through the entire biological cycle of development, including seed maturation, which leads to their even greater distribution [66]. Therefore, despite the wide range of herbicides to control this object, biological products can be included in the system of protective measures against ragweed in addition to the chemical agents.

A promising direction is the use of essential oils *Nepeta rtanjensis* Dikli and Milojevi, as well as *N. cataria* L. [67]. Application of biofumigation based on allopathic mechanisms of the relationship between mustard *Brassica juncea* (L.) Czern. and this weed, allows reducing the number of seedlings of the latter [68].

Couch grass is one of the most vicious weeds, found everywhere. In the arid conditions of the southern steppes, semi-deserts and deserts, it loses its importance as a weed [69]. As a rule, it is not widespread in crops of grain crops, and its appearance in them indicates a low efficiency in agriculture.

In corn crops, couch grass in some regions (for example, in the Central region of the Non-chernozem zone) is quite common [70]. Weed control is carried out using rimsulfuron-based herbicides — Titus, DFC (250 g/kg), Cassius, WSP (250 g/kg); nicosulfuron — Nissin, SC (40 g/l), Ikanos, OC (40 g/l), Innovate, SC (240 g/l), DUBLON, SK (40 g/l) or combined preparations — Cordus, WDG (500 g/kg nicosulfuron + 250 g/kg rimsulfuron), Elumis, OC (75 g/l meso-trione + 30 g/l nicosulfuron) [71].

To protect soybean, sunflower and potato plantings, preparations based on fluazifop-P-butyl — Fuzilad Forte, EC (150 g/l), Fuzilad Super, EC (125 g/l); quizalofop-P-tefurila — Bagira, EC (40 g/l), Panther, EC (40 g/l), Heeler, EC (40 g/l); cellularhodima — Select, EC (120 g/l), Centurion, EC (240 g/l) could be used. At the same time, in addition to those listed, rimsulfuron based herbicides are used on potatoes [72].

According to the available data, most biological agents for suppressing couch grass are still too expensive and are associated with high labor costs [73],

therefore, there are few specific developments in this direction.

Common wild oats need a warm climate and dry soils. Its main habitat and harm zones are located in the southeast of the European part of Russia and the Southern Urals, where wild oats dominate in grain crops [43].

The range of wild oat herbicides recommended for these crops is extremely wide. It includes preparations based on fenoxaprop-P-ethyl — Puma Super 7.5, OWE (69 g l + 75 g/l of the antidote mefenpyr-diethyl), Ocelot, EC (100 g/l + 27 g/l of the antidote cloquintoset-mexil); clodinafoppropargyl — Topik, EC (80 g/l + 20 g/l of cloquintoset-mexil antidote); kick-sadena — Axial 50, EC (50 g/l + 12.5 g/l of cloquintoset-mexil antidote); flucarbazone sodium — Everest, WDG (700 g/kg), etc. With a mixed type of weediness, preparations are used for the complex suppression of dicotyledonous weeds and annual cereal weeds Alistair Grand, OC (6 g/l meso-sulfuron-methyl + 4.5 g/l iodosulfuron-methyl-sodium + 180 g/l diflufenican + 27 g/l mefenpyr diethyl), Ocelot Cross, EC (290 g/l MCA acid/2 ethylhexyl ether/+ 49 g/l fenoxaprop-P-ethyl + 15 g/l cloquintoset-mexil) [74].

To protect crops of corn, soybeans, sunflowers and potato plantings from annual cereal weeds, which include wild oats, the same preparations as for fighting wheatgrass are used. In addition to the listed funds, on planting potatoes, you can use herbicides based on metribuzin — Zenkor Ultra, SC [600 g/l), Lazurit, SP [700 g/kg), Soil, WDG (700 g/kg) [75].

It should be noted that the emergence of resistant populations of common wild oat might become an urgent problem in the future. Reports on the resistance of wild oats to fenoxaprop-P-ethyl were received from the Altai Territory [76]. The development of bioherbicides to combat resistant forms may be promising, despite the wide range of chemical herbicides.

There are no BLH and BRH against wild oats, although studies have been conducted abroad for a long time to identify and use its various pathogens, as well as searches for natural compounds that inhibit its growth. In particular, the mycobiota of wild oat seeds was studied in order to reduce their viability [77, 78], a rust fungus was tested under field conditions [79], the conditions of infection by *Drechslera avenae* and the range of plants susceptible to it were studied [80, 81]. In Australia, where wild oats turned out to be an invasive species, *D. avenae* was proposed to combat it, but in Russia this fungus serves as the causative agent of oat disease and therefore can hardly be used where this crop is grown. At the same time, a nonselective phytotoxin pyrenoforol A was isolated from the culture of *D. avenae*, which has herbicidal potential for combating wild oats and other weeds [82-84]. It has also been proposed to use eucalyptus essential oils [85, 86], bioactive ragweed sesquiterpenes [87], and even papaya extract [*Carica papaya* L.) [88].

Due to its special relevance [danger to humans and rapid spread), we cannot ignore the Sosnovsky hogweed (*Heracleum sosnowskyi* Manden.), which has a limited distribution on agricultural lands due to the complex of agricultural activities carried out on them. As a rule, the finding of Sosnovsky hogweed specimens on arable land indicates a very low level of farming. The main habitats of this species are non-agricultural lands, pastures and grasslands, ditches and roadsides, as well as areas occupied by forest vegetation.

The range of herbicides approved for use against Sosnovsky's hogweed on non-agricultural lands includes preparations based on glyphosate — Tornado, WS (360 g/l), Total, WS (360 g/l); sulfometuron-methyl — Ankor-85, WDG (750 g/kg); metsulfuron-methyl — Zinger, SP [600 g/kg); imazapira — Shkval, WC (250 g l), Arbonal, WC (250 g/l). Some of the preparations are combinations of the indicated active substances — AtronPro, WDG (250 g/kg imazapir + 75 g/kg sulfometuron-methyl), Gorgon, WSC (350 g/l MCA acid + 150 g/l picloram), Grunge, WDG

(525 g/kg glyphosate (potassium salt) + 105 g/kg sulfometuron-methyl + 20 g/kg chlorsulfuron) and General Secretary, WSG (88.5 g/l dicamba + 88.5 g l picloram + 177 g/l clopyralid). To combat Sosnovsky's hogweed on hayfields and pastures, it is allowed dicamba-based herbicides to be used, for example Banvela, WS (480 g/l), Dianata, WS (480 g/l).

On the one hand, a wide range of chemical herbicides on the market for the control of Sosnovsky's hogweed does not open up wide opportunities for the introduction of bioherbicides into it. On the other hand, a potentially interesting niche for the use of biological products can be their joint use with selective sulfonylureas or dicamba based herbicides. The fact is that one of the main conditions for achieving a long-term effect in the destruction of Sosnovsky hogweed is to prevent re-contamination of areas cleared of weeds. For these purposes, either "replacement plantings" in the form of lawn grasses are used, or the complete destruction of dicotyledonous weeds for the formation of "sod" by means of herbicides selective for cereals, which can potentially be supplemented with biological products. It should be noted, that research in this direction should include the study of the compatibility of sulfonylureas and dicamba with producers of bioherbicides.

An analysis of niches potentially attractive for the introduction of biological products in the agricultural production would be incomplete without mentioning orchards and vineyards. For weed control purposes, only general exterminating preparations based on two active substances are allowed here — glyphosate (in the form of salts) and ammonium glufosinate [89]. Currently, the use of preparations based on glyphosate in our country is significantly limited and the niche of operational means of weed control in orchards and vineyards is vacant (48]. It could be occupied by bioherbicides, which would be especially in demand in the context of combating all the problematic species of weeds indicated in the review, and especially with perennial species (it is known that favorable conditions for the growth and development of knapweed are formed in gardens and vineyards) [90].

Another advantage of using bioherbicides in orchards and vineyards is that they can be applied after the addition of glyphosate. With such technologies, a more prolonged action of the treatment is observed [91]. It is also possible to combine bioherbicides with chemicals, which increases the effectiveness of weed control [92].

An important advantage of bioherbicides in gardens is the ability to protect non-target objects from the negative effects of BLH in the long term. Unlike chemical herbicides, which have been widely used in production for more than half a century, the consequences of the use of bioherbicides can only be predicted empirically. This causes serious concern of scientists, since there are cases of unsuccessful introduction of such preparations [93-95].

Taking into account the possible long-term consequences of the use of bioherbicides, the sphere of a relatively closed agroecosystem of the garden looks most preferable for an attempt at the first approbation of such preparations in agricultural production. The second stage in the introduction of bioherbicides into production can be their inclusion in crop rotation systems in the fields intended for sowing agricultural crops in autumn. In this case, several months will pass from the moment of application of the preparations to sowing the crops. Subject to the successful completion of the first two stages, bioherbicides can be used in fallow fields. The final stage will be their application on sowing and planting of crops.

In conclusion, we give a list of the preparations mentioned in this review:

Preparation	Producer, country
Roundup Max, WS	Monsanto Europe S.A., Belgium
Sprut Extra, WS	AO Shchelkovo Agrokhim, Russia
Kileo, WSC	Nufarm GmbH & Co KG, Austria
Lontrel-300, WS	Dow AgroSciences Vertriebsgesellschaft m.b.H, Austria
Hacker, WSG	AO Firma Avgust, Russia
Lontrel grand, WSG	Dow AgroSciences Vertriebsgesellschaft m.b.H, Austria
Demetra, EC	AO Firma Avgust,, Russia
Starane Premium 330, EC	Dow AgroSciences Vertriebsgesellschaft m.b.H, Austria
Esteron 600, EC	Dow AgroSciences Vertriebsgesellschaft m.b.H, Austria
Drotik, CSC	AO Shchelkovo Agrokhim, Russia
Estet, EC	«Nufarm GmbH & Co KG», Austria
Laren Pro, WSG	OOO Dyupon Nauka i Tekhnologii, Russia
Tribun, DFC	OOO Agro Ekspert Grup, Russia
Prima, EC	Dow AgroSciences Vertriebsgesellschaft m.b.H, Austria
Bomba, WSG	AO Firma Avgust, Russia
Unico, CSC	AO Shchelkovo Agrokhim, Russia
Modern, EC	OOO GK ZemlyaFF, Russia
Classic Forte, WSG	OOO Dyupon Nauka i Tekhnologii, Russia
Fabian, WSG	AO Firma Avgust,, Russia
Express, WSG	OOO EfEmSi, Russia
Sanflo, WSG	AO Shchelkovo Agrokhim, Russia
Prometei, WSG	OOO Yarilo, Russia
Titus, DFC	OOO Dyupon Nauka i Tekhnologii, Russia
Cassius, WS	AO Shchelkovo Agrokhim, Russia
Escudo, WDG	AO Firma Avgust, Russia
Gensek, WSG	OOO Agro-Innovatsii, Russia; OOO Agruskhim, Russia
Gorgon, WSC	AO Firma Avgust, Russia
Oktimet, EC	OOO Alsiko Agroprom, Russia; OOO Agroimpeks, Russia
Lancelot 450, WDG	Dow AgroSciences Vertriebsgesellschaft m.b.H, Austria
Agron, WS	OOO Agro Ekspert Grup, Russia
Agron Grand, WDG	OOO Agro Ekspert Grup, Russia
Primadonna, EC	AO Shchelkovo Agrokhim, Russia
Balerina, EC	AO Firma Avgust, Russia
MaysTer, WDG	Bayer CropScience AG, Germany
MaysTer Power, OD	Bayer CropScience AG, Germany
Pulsar, WS	BASF Agrochemical Products B.V., the Netherlands
Pivot, WC	BASF Agrochemical Products B.V., the Netherlands
Bazagran, WS	BASF SE, Germany
Corsar, WSC	AO Firma Avgust, Russia
Benito, DF	AO Shchelkovo Agrokhim, Russia
Corum, WSC	BASF Agrochemical Products B.V., the Netherlands
Euro-Lightning, WSC	BASF Agrochemical Products B.V., the Netherlands
Pulsar, WS	BASF Agrochemical Products B.V., the Netherlands
Tapir Hybrid, OC	OOO Agro Ekspert Grup, Russia
Titus, CTC	OOO Dyupon Nauka i Tekhnologii, Russia
Cassius, WSP	AO Shchelkovo Agrokhim, Russia
Nissin, SC	ISK Biosciences Europe N.V., Belgium
Ikanos, OD	Nufarm GmbH & Co KG, Austria
Innovate, SC	Cheminova A/C, Denmark
DUBLON, SC	AO Firma Avgust, Russia
Cordus, WDG	OOO Dyupon Nauka i Tekhnologii, Russia
Elumis, OD	OOO Singenta, Russia
Fuzilad Super, EC	OOO Singenta, Russia
Bagira, EC	Arysta LifeScience Great Britain Ltd., UK
Pantera, EC	Arysta LifeScience Great Britain Ltd., UK
Healer, OEC	AO Shchelkovo Agrokhim, Russia
Select, EC	Arysta LifeScience S.A.S., France
Centurion, EC	Arysta LifeScience S.A.S., France
Puma Super 7.5, OWE	Bayer CropScience AG, Germany
Ocelot, EC	OOO Agro Ekspert Grup, Russia
Topic, EC	OOO Singenta, Russia
Axial 50, EC	OOO Singenta, Russia
Everest, WDG	Arysta LifeScience S.A.S., France
Alister Grand, OD	Bayer CropScience AG, Germany
Ocelot Cross, EC	OOO Agro Ekspert Grup, Russia
Zencor Ultra, SC	«Bayer CropScience AG», Germany
Lazurit, SP	AO Firma Avgust, Russia
Soil, WSG	OOO Agro Ekspert Grup, Russia
Tornado, WS	AO Firma Avgust, Russia
Total, WS	OOO Agro Ekspert Grup, Russia
Ankor-85, WSG	OOO Gerbivid Pervii, Russia
Zinger, SP	AO Shchelkovo Agrokhim, Russia
Shkval, WC	AO Shchelkovo Agrokhim, Russia
Arbonal, WC	OOO Novokemi, Russia

Continued list

AtronPro, WSG	OOO Agruskhim, Russia
Gorgon, WSC	AO Firma Avgust, Russia
Grange, WSG	ZAO Unaited Agro, Russia; ZAO NPF Golitsyno Agro, Russia
Gensec, WSG	OOO Agro-Innovatsii, Russia; OOO Agruskhim, Russia
Banvel, WS	OOO Singenta Russia
Dianat, WS	BASF Corporation, USA

Therefore, biological and biorational herbicides in the near future may occupy niches in the assortment of weed protection products in orchards and vineyards, in fields for sowing spring crops (when carrying out protective measures in the autumn) and in fallow fields. The development of bioherbicides against perennial root-sprouting weeds and Russian knapweed is promising for combating these species during the growing season of soybeans, sunflowers, and potatoes. For the last two crops during the growing season, the use of biological products against common ragweed looks promising. Such preparations are also of interest as additional methods of operative weed control along with chemical herbicides. The status of quarantine objects for wormwood-leaved ragweed and Russian knapweed should contribute to the interest of buyers in new environmentally friendly means of combating them, even in the presence of a large number of chemicals. In addition, existing chemicals against the Russian knapweed have side effects, which limit their use in industrial conditions. It will be quite challenging to occupy the niche associated with the destruction of cereal weeds (such as couch grass or wild oats) by bioherbicides in the conditions of the existing range of chemical preparations. A possible direction of their use may be the suppression of resistant populations of these weeds due to the large-scale use of chemical herbicides.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 885-898
[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 635.64:581.192:575:577.2

doi: 10.15389/agrobiology.2021.5.885eng
doi: 10.15389/agrobiology.2021.5.885rus

STRUCTURAL AND FUNCTIONAL ANALYSIS OF *GME1* HOMOLOGOUS GENES AND ASCORBATE ACCUMULATION IN CULTIVATED AND WILD TOMATO SPECIES

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The authors declare no conflict of interests
Received July 9, 2021

Abstract

Ascorbic acid (ascorbate, vitamin C) plays an important role in various metabolic processes both in plants and humans. Increasing the ascorbate content in plants using breeding approaches is important, both from the point of view of increasing the nutritional value of fruits, and from the point of view of plant resistance to stress. It is known that tomato has high potential as an ascorbate source in the human diet. Unfortunately, the ascorbate levels in ripe fruits of modern tomato (*Solanum lycopersicum*) cultivars and hybrids are low in comparison with relative wild tomato species. Use wild tomato accessions in breeding programs can significantly increase the ascorbate content of ripe fruits. However, for effective breeding for this trait, a more detailed study of the genetic determinants responsible for the ascorbate levels increase in in ripe fruits is necessary. In this study, we cloned and sequenced novel *GME1*gene homologous, which plays a key role in ascorbate biosynthesis in cultivated tomato and 11 wild tomato species. Structural analysis showed a low *GME1* variability level of in tomato species. In the *GME1* coding sequences, 28 SNPs were identified, of which only two led to nonsynonymous aminoacid substitutions (G2E and E281D) in *S. neorickii* and *S. peruvianum* var. *dentatum*. Analysis of GME1 motifs and domains did not reveal any specific motifs either at the interspecific level or at more distant taxonomic levels. The high *GME1* conservatism observed in quite evolutionarily distant tomato species is most likely due to the functional significance of this enzyme for the ascorbate synthesis and, indirectly, for protection from stress factors, primarily photostress. No correlation was found between amino acid or nucleotide substitutions and ascorbate levels in fruits. Expression analysis, including comparative interspecies organ-specific analysis and analysis of the dependence of the ascorbate content in mature fruits in tomato cultivars and wild species accessions and the *GME1* expression level, also did not reveal a relationship between transcriptional levels and ascorbate concentration. It can also be assumed that the final ascorbate content in the ripe tomato fruit may be influenced not by the intensity of GME1 expression at the last stage of fruit ripening, but by how this gene was active at earlier stages of ripening.

Keywords: *GME1,* gene expression, SNP, *Solanum lycopersicum*, wild tomato species, tomato cultivars, ripe fruits, ascorbate content

Ascorbic acid (ascorbate, vitamin C) plays an important role in various metabolic processes in plants in photosynthesis, photoprotection, resistance to stress, control of cell growth, biosynthesis of hormones and cell wall components [1-5]. In a plant cell, ascorbic acid acts as the main antioxidant due to its ability to reduce the content of reactive oxygen species, which are formed during photosynthesis and abiotic stresses (for example, at high light intensity, high temperatures, and strong ultraviolet radiation) [6, 7]. Humans and some primates are unable to synthesize ascorbate; therefore, plant foods rich in vitamin C are essential to maintain normal vital functions [8] and, thus, the search for new sources

of ascorbate is essential [9].

Tomato (*Solanum lycopersicum* L.) is one of the most commonly eaten vegetable crops with a wide cultivation area [10, 11]. Tomato varieties can serve as an additional source of vitamin C when consumed year-round [12]. However, in modern varieties and hybrids of cultivated tomato, the content of ascorbate in ripe fruits is low in comparison with that in related wild species [13]. Studies have shown that breeding programs involving wild tomato species, in particular *S. pennellii*, can significantly increase the ascorbate content in ripe fruits [14-18]. Nevertheless, effective breeding for this trait requires a more detailed study of the genetic determinants responsible for the ascorbate levels increase in ripe fruits.

One of the most important reactions of ascorbate biosynthesis in higher plant cells is considered to be the conversion of GDP-D-mannose to GDP-1-galactose, which is catalyzed by the enzyme GDP-mannose-3′, 5′-epimerase (GME; EC 5.1.3.18). Unlike most plants, which have only one copy of the GME gene, two copies have been identified in the tomato genome - GME1 and GME2 on chromosomes 1 and 9 [17, 19]. Analysis of QTL (quantitative trait loci) colocalization indicates that GME may serve as a genetic determinant of increased ascorbate levels in tomato fruits [17, 19]. This assumption is confirmed by work of L. Gilbert et al. [20], where the RNA silencing method was used to suppress the expression of both genes, *SlGME1* and *SlGME2*. Later C. Zhang et al. [21] demonstrated that overexpression of both genes significantly increased the ascorbate content in leaves and ripe tomato fruits. This directly affected the stress resistance of modified plants. A positive correlation between GME expression and ascorbate content was also shown for apple and blueberry [22, 23]. Analysis of the evolutionary patterns of the GME gene based on a comparison of 59 genomes of different species of higher plants and green algae revealed a high degree of conservatism of the sequence and structural organization of GME in higher plants [24]. However, the structure of GME gene homologous in wild tomato species has not yet been studied.

In this study, we were the first to identify and describe in detail *GME1* gene homologous in cultivated tomato cultivars *S. lycopersicum* and samples of wild species characterized by a high content of ascorbate in fruits. The variability of nucleotide and amino acid sequences, the composition of functional motifs in genes-homologs of *GME1* were determined. An analysis of the expression of such homologous genes in tomato samples with different contents of ascorbate in commercial products had shown that there is no relationship between this trait and the transcriptional activity of *GME1* in mature fruits.

Purpose of this study was to identify novel homologous GME1 gene and to conduct comparative analysis of their structure and transcriptional activity in different tomato species to identify possible correlations between the differences in these genes and the accumulation of ascorbate in fruits.

Materials and methods. Plant material included 15 tomato samples (section *Lycopersicon*, genus *Solanum*) — 11 wild species *S. chmielewskii* (VIR13725), *S. neorickii* (VIR5033), *S. chilense* (VIR4300), *S. corneliomulleri* (VIR4367), *S. peruvianum* (VIR4361), *S. peruvianum* var. *dentatum* (VIR3966), *S. arcanum* (VIR13958), *S. habrochaites* (VIR13964), *S. cheesmaniae* (VIR3969), *S. galapagense* (VIR3970), *S. pimpinellifolium* var. *racemigerum* (VIR1018), wild tomato specie *S. lycopersicum* var. *humboldtii* (VIR2912), tomato of *S. lycopersicum* Silvestre recordo (VIR1580), Bychye serdtse and Yellow belorus varieties (provided by the Vavilov All-Russian Institute of Plant Genetic Resources, Saint Petersburg and Federal Scientific Vegetable Center Gavrish LLC, Moscow Region). Plants were grown in a greenhouse (23 °C, day length 16 h, illumination 5 thousand lux/m^2).

When determining the content of ascorbate in fruits, the method of G. Giovanelli et al. [25] was used for its extraction with modifications. An aqueous solution of metaphosphoric acid (1 g/100 ml) was added to fresh tomatoes in a ratio of 1:10 (w/v) and homogenized in a Waring 8011 blender (Waring, USA). The resulting homogenate was quickly transferred into polypropylene tubes and centrifuged at 11000 g for 20 min at 4 °C. The analysis was performed by high performance liquid chromatography (HPLC, liquid chromatography with mass detector LC-QQQ Agilent 6460, Agilent Technologies, USA; ACE® 5 C18 column, Advanced Chromatography Technologies, Ltd., UK) (Shared-Access Equipment Centre Industrial Biotechnology of Federal Research Center Fundamentals of Biotechnology RAS, Moscow). The mobile phase was ultrapure water (pH 3), isocratic elution (flow rate 0.5 ml/min, temperature 35 °C). The supernatants were preliminarily filtered through membranes with a pore size of 0.45 rm (Amersham, USA). The volume of the analyzed samples was 20 ml. The concentration of ascorbic acid was expressed per 100 g of homogenized fruit pulp. The ascorbate content was determined in two biological replicates.

Genomic DNA was isolated from freshly harvested tomato leaves by method proposed by K. Edwards et al. [26]. The purity was evaluated and the concentration of the obtained DNA preparations was determined (spectrophotometer DU 530, Beckman, USA).

For the isolation of RNA with subsequent synthesis of cDNA, leaves, roots, flowers, and fruits (at the stage of full ripeness) of wild tomato species *S. lycopersicum* var. *humboldtii*, *S. peruvianum*, *S. habrochaites* and three tomato species var. *S. lycopersicum* Silvestre record, Bychye serdtse, and Yellow belorus were used. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations. The resulting RNA preparations were treated with DNase I (10 units) (Invitrogen, USA) in accordance with the attached description. The cDNA preparations were obtained using the GoScript™ Reverse Transcription System kit (Promega, USA) according to the manufacturer's method. The concentration of RNA and cDNA was determined on a Qubit 4 fluorometer (Thermo Fisher Scientific, USA) using the appropriate reagents (Invitrogen, USA). Additionally, the quality of RNA was checked by electrophoresis in 1.5% agarose gel.

PCR amplification was performed on a Veriti™ 96-well Thermal Cycler (Applied Biosystems, USA) in a 15 μl reaction mixture containing 1.5 μl Dream™ Taq buffer 10×, 20 mM dNTP, 10 μM of specially developed GME primers F (5′-CACTGTATTAGTGCC-TCATC-3′) and GME R (5′-CAATTACCAGAATCT-AACACATC-3′), 0.25 μl Dream™ Taq DNA Polymerase (5 UI/μl) (Fermentas, Lithuania) and ~ 100 ng of genomic DNA. Amplification conditions: 5 min at 95 °C; 15 s at 95 °C, 10 s at 58 °C, 1 min at 72 °C (40 cycles); final elongation for 4 min at 72 °C.

The resulting PCR products were analyzed by electrophoresis in 1.5% agarose gel in 1× TBE buffer stained with ethidium bromide. The results were documented using the Gel Doc™ XR + Imaging System (Bio-Rad, USA). Commercial standards were used to determine the size of amplified DNA fragments 1 Kb DNA Ladder and 100 bp DNA Ladder (Fermentas, Lithuania)

PCR products were cloned using the pGEM®-T Easy Vector plasmid vector system (Promega, USA) according to the manufacturer's procedure, with additional deproteinization with a phenol: chloroform mixture (1:1).

The analysis of the expression of homologous *GME1* gene was carried out in four organs of tomato - root, leaf, flower, and fruit in biological ripeness by quantitative real-time PCR (RT-qPCR) using the kit Reaction mixture for carrying out RT-PCR in the presence of SYBR GreenI and ROX (Syntol LLC, Russia)

on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The reactions were carried out in two biological and three analytical repeats. The following protocol was used to analyze the expression: 5 min at 95 °C; 15 s at 95 °C, 30 s at 60 °C (40 cycles), followed by reading the results. To obtain the melting curves of the amplification products, the temperature was increased from 55 °C to 95 °C (with a step of 0.5 °C every 5 s), followed by reading the results. To analyze the expression of homologous *GME1* gene, a pair of primers GMErnaF (5′-AGAATGGGAAGCTCTGGTGG-3′) and GMErnaR (5′-GGCTTCCAA-TTGAAATGATGACAG-3′) was developed allowing the amplification of a 185 bp fragment. During normalization, the expression value of the reference genes *Expressed* and *Actin 2/7* was used according to the description [27, 28].

Bioinformatic analysis of nucleotide sequences was performed using the MEGA 7.0 software [29] (https://www.kent.ac.uk/soft-ware/mega-7). Analysis of amino acid sequences and prediction of the presence of substitution sites that significantly affect the functionality of the enzyme were performed using the PROVEAN program [30] (http://provean.jcvi.org/index.php). The search for common hidden motives was carried out using the MEME 5.3.2 program [31] (https://meme-suite.org/meme/tools/meme). For additional analysis of amino acid sequences, the NCBI-CDD resource (https://www.ncbi.nlm.nih.gov/cdd/) and the UniProt database (https://www.uniprot.org) were used. For comparative analysis of nucleotide and amino acid sequences, sequences of *GME* genes-homologues from the NCBI database were also used, namely *S. lycopersicum* cv. Heinz 1706; *S. pimpinellifolium* (LA0480), *S. arcanum* (LA2157); *Oryza sativa* and *Arabidjpsis thaliana*.

The arithmetic mean values were calculated at determination of the content of ascorbate.

Results. Accumulation of ascorbate in ripe fruits. Table 1 shows the distribution of the studied samples by the content of vitamin C in fruits. Biochemical analysis showed that in the evolutionarily younger red-fruited tomato species *S. lycopersicum* and *S. pimpinellifolium* the accumulation of ascorbate occurs evenly as the fruit ripens, with a maximum in the fruit of biological ripeness. In the majority of green-fruited cross-pollinated tomatoes, the opposite dynamics is observed with the maximum content of ascorbate in the early stages of fruit development. Samples with the lowest and highest content of ascorbate in the fruit were identified at the stage of biological ripeness. Among the representatives of S. *lycopersicum*, the smallest amount of ascorbate was detected in the Yellow belorus (28.1 mg/100 g of homogenized fruit pulp) and Bychye serdtse (42.8 mg/100 g) samples, and the highest in *S. lycopersicum* var. *humboldtii* (120.1 mg/100 g). The obtained values slightly exceeded the data obtained earlier for cultivars and samples of wild species *S. pennellii* and *S. pimpinellifolium* [32], which may be due to the specificity of the manifestation of this trait in the studied cultivars and accessions, as well as the conditions for growing plants.

1. Vitamin C accumulation (*M*, mg/100 g pulp homogenate) **in tomato (*Solanum* L.) fruits at full ripeness**

Genotype	IG	MG	Br	Red ripe
S. lycopersicum Silvestre recordo (VIR1580) R/C	0.2	11.9	23.2	68.5
S. pimpinellifolium var. *racemigerum* (VIR1018) R/W	36.5	77.4	99.3	150.3
S. lycopersicum var. *humboldtii* (VIR2912) R/W	19.3	14.7	17.1	120.1
S. lycopersicum сорт Бычье сердце R/C	2.5	6.8	12.3	42.8
S. lycopersicum сорт Желтый белорус R/C	0.4	13.1	16.8	28.1
S. cheesmaniae (VIR3969) R/W	9.5	32.4	23.3	130.1
S. galapagense (VIR3970) R/W	34.2	46.4	65.7	84.2
S. chmielewskii (VIR13725) G/W	63.3	74.8	94.1	132.7
S. chilense (VIR4300) G/W	21.8	43.7	63.2	93.3

Continued Table 1

S. corneliomulleri (VIR4367) G/W	14.9	18.7	27.2	104.8
S. peruvianum (VIR4361) G/W	18.8	17.6	36.4	85.0
S. peruvianum var. *dentatum* (VIR3966) G/W	50.0	84.7	87.3	119.7
S. arcanum (VIR13958) G/W	66.9	65.2	37.8	44.7
S. habrochaites (VIR13964) G/W	0.2	0.2	0.3	0.3
S. neorickii (VIR5033) G/W	58.0	78.4	98.1	127.3

Note. R — red-fruited, G — green-fruited, W — wild, C — cultivated, IG — immature green, MG — mature green, Br — breaker. The measurements were carried out in two biological replicates (in generalized samples).

Identification and characterization of variability of GME1 homologous genes. When designing primer for DNA amplification of GME genes, being an enzyme involved in ascorbate synthesis, we used the genomes of tomato *S. lycopersicum* variety Heinz 1706 (NM_001247914.2), wild tomato *S. pennellii* (XM_015214839.2), and potatoes presented in the NCBI database. *S. tuberosum* (GCA_000226075.1) and mRNA sequences. As a result, a pair of flanking primers GME F–GME R was developed to amplify the *GME1* gene (Table 2). In addition, internal primers with localization in exon sequences were developed for sequencing. As a result, seven internal primers were obtained for amplification and sequencing of the *GME1* genes (see Table 2, Fig. 1).

2. Primers designed for amplification and sequencing of *GME1* homologous genes in the studied varieties and wild-growing tomato (*Solanum* L.) species

Primers	Sequences (5′→ 3′)
GME F	CACTGTATTAGTGCCTCATC
GME R	CAATTACCAGAATCTAACACATC
GME1exF	CAGCTTCAATATGATGGAAGCT
GME2exF	CAGCCTGAAAGAAGCTGATG
GME3exR	ACCTTTCCAGGTTCCAAAAGG
GME4exF	GCACAAACTGCAACGGATAAG
GME5exR	ATTGTTGGAGCCCAACCAAG
GME5exF	GCCAGTAAACATTGGAAGTGA
GME4inR	CTCCAGTAATTCAGAATAAGTGG

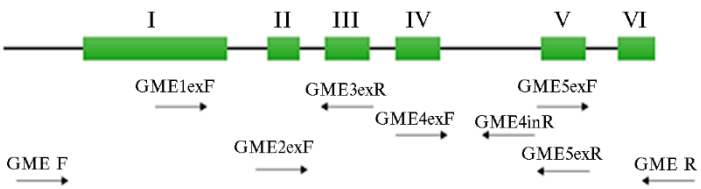

Fig. 1. Schematic representation of the structure of the *GME1* gene obtained during the analysis of *GME1* homologous genes in 11 wild tomato species and the tomato cultivar Silvestre recordo using the developed primers. The location of flanking and internal primers with localization in exons (green rectangles) and intron (lines) is marked. Exons are marked with Roman numerals. For a description of species and varieties, see the *Materials and methods* and Table 1.

Using the developed primers GME F–GME R, we have amplified the sequences of homologous *GME1* gene in 11 wild tomato species and the vegetable tomato cultivar Silvestre recordo as a representative of the *lycopersicum* species. The resulting fragments were cloned and sequenced using the internal primers.

Comparison of the structure of *GME1* homologous genes in the studied tomato samples showed that in all analyzed species this gene consists of six exons (see Fig. 1), which coincided with the data on the analysis of the exon-intron structure of the *S. lycopersicum GME1* gene obtained earlier [21]. The gene length varied from 1962 bp (*S. galapagense*) to up to 1985 bp (*S. peruvianum*). The size of the gene did not depend on whether the species was evolutionarily older (green-fruited) or younger (red-fruited). Comparison with the previously obtained data on the *GME* gene in the cultivated tomato cultivar Ailsa Craig also did not reveal a difference in the nucleotide sequences [21].

In the whole genome sequence of *GME1,* we found 139, or 6.9% single nucleotide polymorphisms (SNPs). Of these, 134 SNPs were found in green-fruited tomato species and only 5 in red-fruited species. The vast majority (108 SNPs) were found in intron sequences. Earlier, it was shown that the frequency of occurrence of nucleotide substitutions in intron sequences is on average 8±2% [33]. The level of intron polymorphism in *GME1* homologous genes described by us in this work generally corresponds to the given value. Also, as a result of the project The 100 Tomato Genome Sequencing Consortium, it was shown that the variability of gene sequences in red-fruited tomatoes is 20 times lower than in green-fruited species [33]. In our study, this difference for *GME1* turned out to be 26.8-fold, which is also consistent with previously published data [33].

In the coding region, 28 single nucleotide substitutions were found, the two SNPs in red-fruited species and 26 SNPs in green-fruited species (Fig. 2, see http://www.agrobiology.ru). The number of synonymous substitutions in the exon region ranged from 7-8 SNPs in *S. peruvianum* accessions to 12 and 13, respectively, in S. *arcanum* and *S. neorickii*. Only two substitutions, G2E and E281D, were found in green-fruited species *S. neorickii* and *S. peruvianum* var. *dentatum* were found to be nonsynonymous.

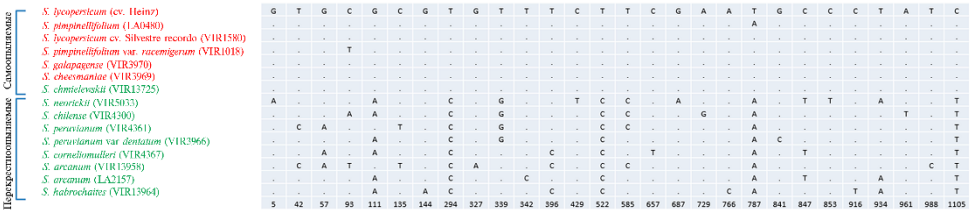

Fig. 2. Distribution of SNPs in the coding region of *GME1* homologues genes in the studied cross- and self-pollinated wild-growing species and varieties of tomato (Solanum L.). Numerical values indicate the location of single nucleotide substitutions. See the description of species and varieties in the *Materials and methods* and in Table 1.

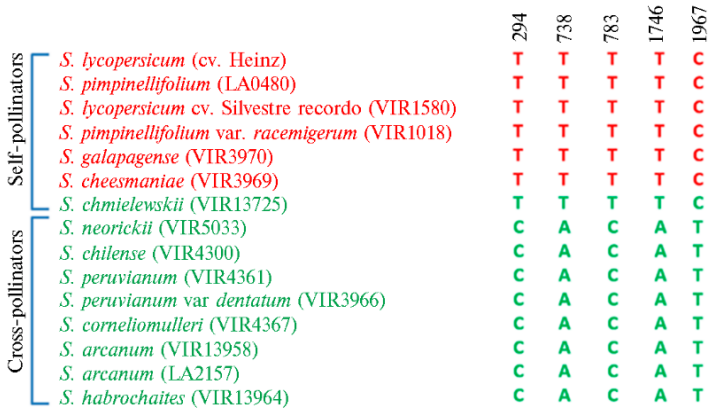

Fig. 3. Distribution of SNPs in *GME1* homologues genes in the studied cross- and self-pollinated wild-growing species and varieties of tomato (*Solanum* L.). The color indicates red (orange)- and green-fruited species. Numerical values indicate the location of single nucleotide substitutions. See the description of species and varieties in the *Materials and methods* and in Table 1.

In tomato, in addition to nucleotide substitutions in the analyzed sequences of *GME1* homologous gene, we found 22 indels ranging in size from 1 to 10 bp. All indels, as expected, were localized in introns, the maximum number of indels (seven) was in intron V. Interestingly, a 9-nucleotide indel (CCCTTGTA) was found in intron IV (at position 1237 from the start codon), which is present only in self-pollinated species, including the red-fruited *S. lycopersicum* (varieties

Heinz 1706 and Silvestre recordo), *S. pimpinellifolium* (LA0480), *S. pimpinellifolium* var. *racemigerum, S. cheesmaniae*, *S. galapagense*, and green-fruited *S. chmielewskii.* In addition to indels, in the analyzed *GME1* sequences, we found five SNPs, which are also characteristic of self-pollinated tomato species (Fig. 3).

3. Characterization of identified *GME1* homologous genes in the studied varieties and wild tomato (*Solanum* L.) species

Species	Gen-Bank NCBI record	Gene length, bp	Length			1	2	Isoelectric point, pI	Molecular weight, kDa
			introns, bp	cDNA, bp	protein, aar				
Red-fruited *Self-pollinated*									
S. lycopersicum cv. Silvestre recordo (VIR1580)	MK895092	1963	832	1131	376	0	0	5.65	42.456
S. pimpinellifolium var. *racemigerum* (VIR1018)	MK895094	1963	832	1131	376	1	0	5.65	42.456
S. cheesmaniae (VIR3969)	MK895095	1963	832	1131	376	0	0	5.65	42.456
S. galapagense (VIR3970)	MK895096	1962	831	1131	376	0	0	5.65	42.456
Green-fruited *Self-pollinated*									
S. chmielewskii (VIR13725)	MK895097	1966	836	1131	376	0	0	5.65	42.456
Cross-pollinated									
S. chilense (VIR4300)	MK895098	1973	842	1131	376	10	0	5.65	42.456
S. corneliomulleri (VIR4367)	MK895099	1981	850	1131	376	10	0	5.65	42.456
S. peruvianum (VIR4361)	MK895100	1985	854	1131	376	7	0	5.65	42.456
S. peruvianum var. *dentatum* (VIR3966)	MK895101	1982	851	1131	376	8	1	5.64	42.515
S. arcanum (VIR13958)	MK895102	1982	851	1131	376	12	0	5.65	42.456
S. habrochaites (VIR13964)	MK895103	1975	844	1131	376	10	0	5.65	42.456
S. neorickii (VIR5033)	MK895093	1981	850	1131	376	13	1	5.56	42.601

Note. 1 and 2 stand for the number of SNPs and amino acid substitutions compared to the reference sequence (*S. lycopersicum*, cv. Heinz 1706; GeneBank NCBI). The data for the Silvestre recordo cultivar are given as typical for the studied cultivars. See the description of species and varieties in the *Materials and methods* and in Table 1.

The coding sequences of *GME1* homologous gene were translated. The obtained protein sequences were of the same length, 376 amino acid residues, which corresponded to a molecular weight of 42 kDa (Table 3). In general, the amino acid sequences of the proteins were found to be similar. In GME1 proteins in green-fruited species *S. neorickii* and *S. peruvianum* var. *dentatum*, two amino acid substitutions were present, G2E and E281D. The analysis of these substitutions using the PROVEAN program showed that they are not radical and, presumably, cannot affect the functionality of GME1. The results obtained confirm the data that GME is one of the most conserved proteins involved in the biosynthesis of ascorbate [24, 34].

It should be noted that both samples in which nonsynonymous substitutions were identified, *S. neorickii* and *S. peruvianum* var. *dentatum* did not differ significantly from other samples in the content of ascorbic acid (see Table 1) and the dynamics of its accumulation.

In all studied tomato species, analysis of the amino acid sequences of GME1 using the UniProt database [35] revealed an epimerase domain (positions 30-269 aar). Within the domain, for all analyzed proteins, five binding sites for the GME substrate, GDP-mannose (G105, N204, K226, R307, and S357), as well as four binding sites for NAD+, the GME cofactor (D60, D80, Y175, and

K179).

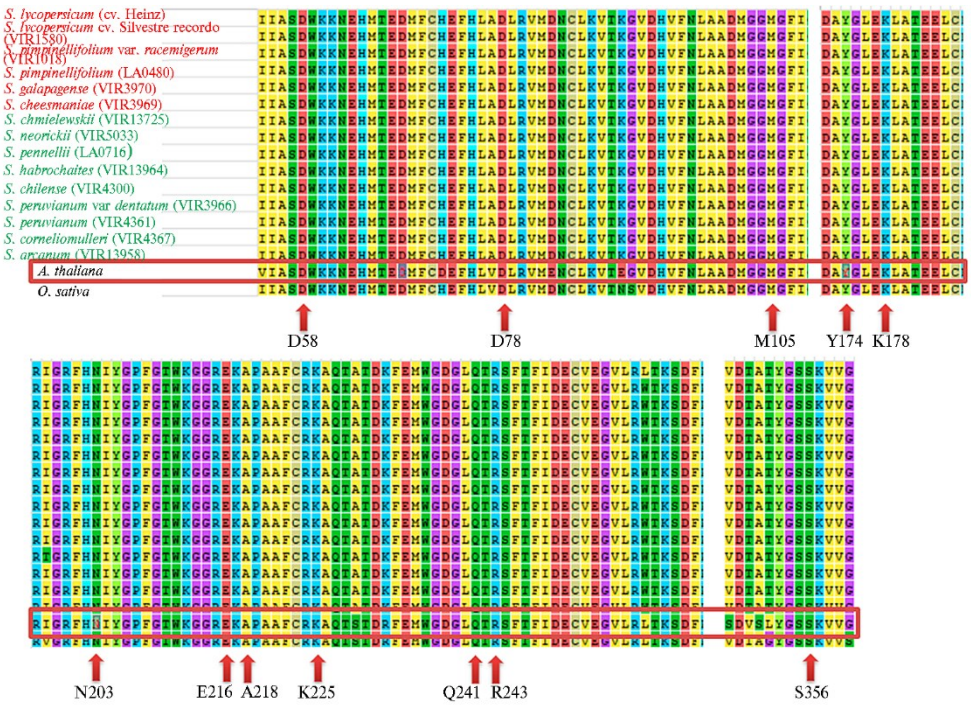

Fig. 4. Conservative amino acid residues in GME1 proteins in the studied cultivars and wild-growing tomato (*Solanum* L.) species, which are critical for the functionality of the GME enzyme (GDP-mannose-3′,5′-epimerase) (shown by arrows). For comparison, the amino acid sequences of the GME homologues of rice and *Arabidopsis* were taken from the NCBI database. *Materials and methods* and in Table 1.

The structure and function of GME in *Arabidopsis thaliana* was previously determined [36]. Our comparative analysis using the MEGA 7.0 software package showed a high degree of homology of this protein in *Arabidopsis* and tomato, which suggests a functional similarity of the amino acid sequence of GME1 in tomato and *Arabidopsis*. Thus, based on the data of site-directed mutagenesis of the GME gene in *Arabidopsis*, it was shown that amino acids C145 and K217 are responsible for the activity of oxidative and reductive epimerization [36]. These amino acid residues coincide with those in the tomato GME1 protein and, therefore, can also be decisive for its functional activity. Amino acids C145 and K217 were invariant in all GME1 sequences analyzed by us in the studied tomato samples (Fig. 4).

The analysis also showed that GME1 is a member of the extended short chain dehydratase/reductase (SDR) family, albeit with a modified glycine-rich nucleotide binding motif [37] (GAGGFIA instead of GXXGXXG) [36]. In the tomato species we studied, GME1 also revealed a conservative motif GAGGFIA at positions 34-40 aar. Such a high conservatism of this motif in GME proteins is most likely determined by its critical function, which is due to its participation in the formation of hydrogen bonds with NAD+.

Besides, we analyzed the amino acid sequences that we obtained for GME1 in the studied tomato species and in homologues of other species (*O. sativa, A. thaliana*) using the MEME 5.3.2 program, which made it possible to identify 10 conservative motifs. All detected motifs (see additional materials on the website http://www.agrobiology.ru) showed high conservatism in comparison with homologues in other representatives of the genus *Solanum* and with the GME homologue of *Arabidopsis*. Our results confirmed the data on the similarity of both

GME1 and *GME2* homologues in plants [24]. In the second motif (12-61 aar), the sequence GAGGFIA was identified, the nucleotide-binding functions of which were previously determined for *A. thaliana* [36].

We also compared the identified motifs using the NCBI-CDD resource, which made it possible to classify them as NAD-binding domains — NAD(P)(+)-binding Rossmann-fold (NADB_Rossmann). The NADB domain is characteristic of many metabolic pathway dehydrogenases and various redox enzymes. It is believed that the presence of this domain is due to the epimerase activity of GME1 [24].

Thus, the analysis of the motifs and domains of the GME1 protein in the studied tomato samples and the comparison with the homologous proteins of rice and Arabidopsis did not reveal any specific motifs either at the interspecific level or at more distant taxonomic levels. The observed low level of variability of GME1 proteins in evolutionarily quite distant tomato species, for example, *S. neorickii* and S. *lycopersicum* (cv. Silvestre recordo), and the presence of only two radical substitutions confirms its conservatism, shown earlier [34], and, most likely, indicates on the functional significance of this enzyme (as well as the biochemical reaction catalyzed by it) for the synthesis of ascorbate and, indirectly, for protection from stress factors, primarily from photostress.

Analysis of the expression of *GME1* homologous genes. Since the structural analysis of the sequences of *GME1* homologues genes did not reveal polymorphisms correlated with the accumulation of ascorbate, we assumed that the effect of the GME1 enzyme on the biosynthesis of ascorbate could occur at the transcriptional level.

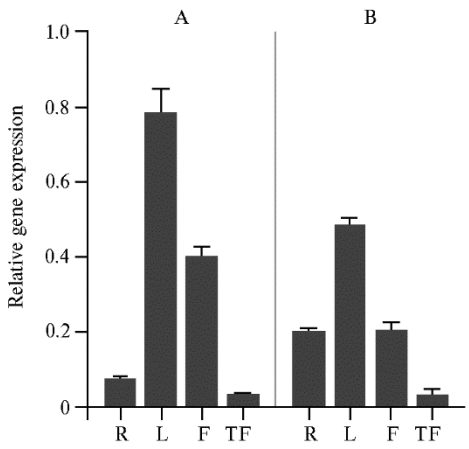

Fig. 5. Expression of homologous *GME1* genes in organs of two tomato species with a contrasting content of ascorbate in fruits : A — *Solanum lycopersicum* (var. Silvestre recordo), B — *S. peruvianum*; R — root, L — leaf, Fl — flower, Fr — fruit in biological ripening phase. Expression was normalized using two standard reference genes, the *Expressed* and *Actin 2/7*.

When studying the organ-specificity of the transcriptional activity of *GME1* homologous genes, we compared the transcriptional activity of this gene in four organs (root, leaf, flower, and fruit at the stage of biological ripeness) in *S. lycopersicum* plants of the Silvestre recordo cultivar and a sample of the wild species *S. peruvianum* with a contrasting content of ascorbate in fruits (see Table. 1, Fig. 5).

The analysis revealed the highest transcriptional activity of *GME1* in leaves regardless of the tomato species and the accumulation of ascorbate in fruits. The result is anticipated, since the leaves are most susceptible to photooxidative stress. The lowest level of *GME1* gene expression was observed in fruits (see Fig. 5).

However, it is the content of ascorbate in fruits (and in particular, the elucidation of the mechanisms that ensure the formation of this trait) that is of the greatest practical interest. To study in more detail the possible relationship between the accumulation of ascorbate in commercial products and the number of *GME1* transcripts, we determined the level of *GME1* expression in fruits of biological ripeness in five samples, the vegetable tomato *S. lycopersicum* var. Silvestre recordo, Bychye serdtse, and Yellow belorus, and samples of wild species *S. lycopersicum* var. *humboldtii* and *S. peruvianum* (Fig. 6, A). These samples are contrasting in the ascorbate content in fruits

and represented both varieties and a wild-growing sample of a cultivated red-fruited species and a wild-growing green-fruited species. In the same five samples, we annotated the expression of *GME1* in mature fruits (see Fig. 6, B).

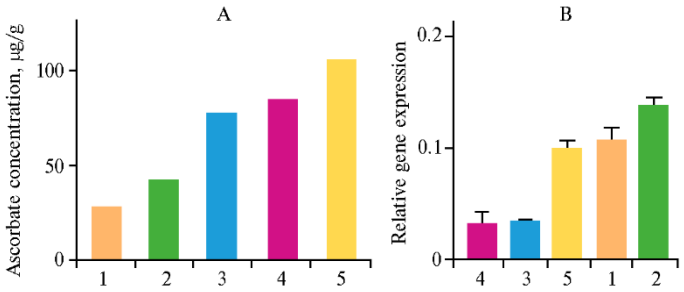

Fig. 6. Comparison of the ascorbate content (A) **and *GME1* expression** (B) **in fruits at the stage of biological maturity of tomato lines and cultivars *Solanum lycopersicum* Yellow belorus** (1)**, Bychye serdtse** (2)**, Silvestre recordo** (3) **and wild species *S. peruvianum*** (4)**, and *S. lycopersicum* var. *humboldtii*** (5). Expression was normalized using two standard reference genes, the *Expressed* and *Actin 2/7*.

Comparison of the results of *GME1* gene expression and biochemical analysis (see Fig. 6) does not reveal a clear correlation between the obtained indicators; we can only speak of a weak inverse relationship between gene expression in mature fruits and the content of ascorbate in them. It can also be assumed that the final ascorbate content in the ripe fruit in the analyzed tomato species may be influenced not by the intensity of *GME1* expression at the last stage of fruit ripening, but by the extent to which this gene was active at earlier stages of ripening, which was shown earlier for *S. lycopersicum* cv. West Virginia 106 [38]. To confirm or to refute this assumption, it is necessary to study the activity of *GME1* in the process of fruit ripening. In addition, possible alternative metabolic pathways for the accumulation of ascorbate in ripe tomato fruits should be considered, for example, the L-galactose pathway, which uses polysaccharides from the fetal cell wall as a substrate.

The biochemical analysis of the ascorbate content in mature fruits in the studied species of tomato did not show a correlation with the levels of *GME1* transcription (see Fig. 6) or identified SNPs. A possible explanation, in addition to the hypothesis formulated above that the transcriptional activity of the *GME1* gene can affect the ascorbate content in fetuses at stages preceding full maturity, is that *GME1*, in addition to biosynthesis of ascorbate, is actively involved in cell wall synthesis during fruit growth [38]. It should also be taken into account that, in addition to the L-galactose pathway, other alternative pathways of biosynthesis, for example, myo-inositol pathway can be used for the formation of ascorbate in tomato fruits [39].

Thus, in this work, we were the first to obtain and analyze the sequences of *GME1* homologous genes in 11 species of cultivated and wild tomato. The performed structural and functional analysis showed an extremely high conservatism of *GME1* homologous genes and proteins encoded by them both in the evolutionarily more ancient green-fruited species and evolutionarily younger red-fruited tomatoes. In the *GME1* homologous genes, 28 SNPs were found that are characteristic of self-pollinated tomato species, which can be used in breeding work. No correlation was found between the content of ascorbate in mature fruits, on the one hand, and the detected SNPs or levels of the *GME1* transcription, on the other hand. The results of this study show that the GME1 protein in the studied tomato species does not have a significant effect on the accumulation of

ascorbate in ripe fruits and indirectly confirm that the biosynthesis of ascorbate in tomato fruits can be provided by other alternative pathways, such as myo-inositol or L-galactose.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 899-909
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 635.21/.24:575.15:577.2

doi: 10.15389/agrobiology.2021.5.899eng
doi: 10.15389/agrobiology.2021.5.899rus

EXPRESSION OF THE α-AMYLASE GENE *StAmy23* IN PHOTOSYNTHETIC AND NON-PHOTOSYNTHETIC TISSUES OF POTATO (*Solanum tuberosum* L.) CULTIVARS

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The authors declare no conflict of interests
Acknowledgments:
Supported financially from the Federal Program for the Development of Agriculture of the Russian Federation for 2017-2025 (subprogram "Development of potato breeding and seed production in the Russian Federation").
Received June 26, 2021

Abstract

Potato (*Solanum tuberosum* L.) is the fourth most important agricultural crop after cereals. Almost every tissue of a potato plant contains starch, the regulation of metabolism and the physiological role of which depends on the type of tissue, the stage of plant development and external factors. Starch hydrolysis is catalyzed by α- (AMY) and β- (BAM) amylases. By degradation of cytosolic phytoglycogen, StAmy23 amylase regulates tuber cold-induced sweetening and physiological dormancy. Few available studies on *StAmy23* have focused on gene activity in potato tubers, including in response to cold stress. In this study, *StAmy23* expression pattern in photosynthetic and non-photosynthetic tissues of potato plants of three cultivars, differing in starch content in tubers, was determined for the first time. Structural and phylogenetic analyses revealed that the closest homologs of StAmy23 are the α-amylases of various potato and tomato cultivars. Analysis of the carbohydrate content in freshly harvested tubers of the studied potato cultivars showed a similar high starch content for cv. Gala and cv. Saturna and almost 2 times lower for cv. Barin (6.3 vs. 11.34 mg/g of tissue). The largest amount of reducing sugars was found in tubers of cv. Saturna; cv. Gala tubers contained 4.5 and 24.5 times less of glucose/fructose than cv. Barin and cv. Saturna tubers, respectively (0.016/0.000 vs. 0.056/0.016 and 0.217/0.175 mg/g of tissue). For the first time, the expression profile of *StAmy23* was determined not only in tubers, leaves and stems, but also in other organs and tissues of the potato plant. A high level of gene expression in stems and fruits was shown. In non-photosynthetic roots and stolons, *StAmy23* transcription level either corresponded (cv. Saturna) or significantly exceeded (cv. Barin, cv. Gala) that in tubers. In stems, the highest and lowest *StAmy23* transcription levels were observed in cv. Gala and cv. Saturna, respectively (0.58 and 0.13). Leaves and tuber peels showed similar, relatively low levels of *StAmy23* expression. In fruits, the highest *StAmy23* expression was found in cv. Barin (0.29), in the roots and tubers — in cv. Gala (0.55 and 0.17), and in the stolons — in cv. Barin and cv. Gala (0.31 and 0.33). A positive association was proposed between the level of *StAmy23* transcription and the starch content (but not the content of reducing sugars) in tubers. The transcriptional activity of the *StAmy23* gene in photosynthetic tissues of potato plants suggests the participation of encoded α-amylase in starch hydrolysis not only in storage organs, but also in vegetative organs to maintain physiological growth processes and plant stress response.

Keywords: *Solanum tuberosum*, potato, α-amylase StAmy23, starch content, reducing sugars, gene expression

Potato (*Solanum tuberosum* L.) is the fourth most important agricultural crop after cereals (rice, wheat, and corn) to ensure food security and economic development in the world. The main nutritional properties of potatoes are determined primarily by the quality and quantity of proteins, minerals and starch in the tubers.

The formation of plant storage organs depends on the import of carbon compounds from the initial photosynthetic tissues to provide substrates for the biosynthesis of all metabolites, including starch. After harvesting and during a certain period of storage, potato tubers are in a state of physiological dormancy, the violation of which negatively affects the consumer properties and technological characteristics of tubers [1, 2]. Tubers are stored at low temperatures (below + 4 °C) in order to slow down sprouting, moisture loss and pathogenesis. Such storage often leads to so-called cold induced sweetening (CIS), which manifests itself in the accumulation of reducing sugars (glucose, fructose) and, as a consequence, leads to browning of potatoes during frying with the formation of acrylamide [3, 4]. Understanding the mechanisms of regulation of tuber dormancy and their response to abiotic stress (for example, low temperatures) is very important both for potato seed production and for its subsequent processing.

Starch is found not only in storage organs. Starch granules can be found in almost every tissue of a plant at some stage in its life cycle. Starch metabolism is universal, and its regulation and physiological role vary depending on the tissue, the stage of plant development, and external factors [5-7]. In photosynthetic tissues, starch is subject to rapid degradation, which occurs at night, under stressful conditions, or during aging [7, 8]. Starch accumulates in plastids: spare - in amyloplasts of heterotrophic organs, transient - in chloroplasts of photosynthetic organs [9, 10].

Thus, starch can be a source of sugars when carbon is needed, or can serve as a kind of depot when sugars are present in excess, which allows optimal use of carbon stores [11, 12]. Decomposition of starch occurs hydrolytically or phosphorolytically. The hydrolytic pathway involves α-amylases (AMY, EC 3.2.1.1) and β-amylases (BAM, EC 3.2.1.2) [13, 14].

AMY is an endoamylolytic enzyme that specifically hydrolyzes α-1,4-glucan bonds to form various linear and branched maltooligosaccharides. Multiple α-amylase genes encode their different isoforms, which can play different roles depending on tissue localization and plant species. For example, suppression of α-amylase I-1 in rice leads to an increase in starch accumulation in young leaves [15, 16]. In contrast, in *Arabidopsis*, all single, double, and triple AtAMY knockout mutants exhibit normal starch degradation [17, 18].

Five AMY genes have been identified in the potato genome, the activity of the products of which is specific with respect to various substrates and in different cellular structures, for example, in chloroplasts and amyloplasts [4, 19]. Two of them, *StAmy1* and *StAmy23*, are expressed in tubers, but only *StAmy23* is induced by low temperatures [20]. The homologue of this gene in the apple tree, the *Amy8* gene, is also sensitive to cold; its expression is activated in berries at 0.5 °C [21]. Amylase StAmy23 is localized in the cytoplasm and regulates cold saccharification of tubers through the degradation of cytosolic phytoglycogen: silencing of *StAmy23* leads to an increased starch content and a decrease in the amount of reducing sugars in tubers stored at low positive temperatures [4]. In addition, StAmy23 is involved in the regulation of potato tuber dormancy, and suppression of the gene expression delays tuber germination, which is accompanied by a decrease in the amount of reducing sugars in the peel and core tissue under the tuber buds, as well as a slight change in the phytoglycogen structure and starch granule size [2]. Thus, StAmy23 can stimulate bud germination in dormant tubers by providing sugars through hydrolysis of soluble starch [2].

Interestingly, all (very few) studies of *StAmy23* focus only on the gene activity in potato tubers, including in response to cold stress.

In this work, the expression profile of *StAmy23* in various organs and tissues

of potato plants was determined for the first time. A high level of gene expression was shown in stems and berries, as well as in non-photosynthetic roots and stolons, where the level of *StAmy23* transcription either corresponded to that in tubers or significantly exceeded it.

The aim of this work was to compare the expression of *StAmy23* gene in photosynthetic and non-photosynthetic organs in three varieties of potatoes of Russian and foreign selection, differing in starch content in tubers.

Materials and methods. Comparative bioinformatic structural analysis of the mRNA nucleotide sequences encoding StAmy23 homologous proteins in different plant species, deposited in the NCBI Nucleotide collection, and the corresponding amino acid sequences of StAmy23 was performed using the NCBI-BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogeny of StAmy23 was assessed using the Fast Minimum Evolution method (Grishin distance matrix, https://www.ncbi.nlm.nih.gov/blast/treeview/). Based on the performed comparative structural analysis of the coding and complete sequences of β-amylase genes, homologous to *StAmy23*, found in NCBI, cDNA-specific primers were developed for carrying out quantitative real-time PCR (RT-PCR). Forward and reverse primers were selected in such a way that there was at least one intron between them.

To analyze the expression profile of *StAmy23* gene in various tissues of potato plants, as well as to determine the content of starch and reducing sugars (glucose and fructose) in tubers, we used the potato variety (*Solanum tuberosum* L.) (early) and Saturna (medium late) of foreign selection, differing, according to originators (https://reestr.gossortrf.ru/), in starch content in tubers: Barin is medium (13.4-14.6%), Saturna high (16.5-21.4%), and Gala low (10.2-13.2%) in the starch level. All three varieties (provided by Lorkh VNIIKH, Moscow Province) belong to canteens and are not used for the industrial production of chips. The plants were grown in 2020 in the field (Lorkh VNIIKH, Moscow Province). In September 2020, two plants of each variety were collected, tubers, tuber skin, pulp of tubers, roots, stolons, stems, leaves, and berries were separated for subsequent analysis of the expression of *StAmy23* amylase gene, tubers (whole with skin and pulp) were also used to determine the content starch and reducing sugars.

To isolate the total RNA, 50-100 mg of tuber tissue, tuber peel, pulp of tubers, roots, stolons, stems, leaves, and berries were used. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The resulting preparations were additionally purified from DNA impurities (RNase free DNasy set; Qiagen, Germany) in accordance with the manufacturer's recommendations. The synthesis of cDNA was performed using the GoScript™ Reverse Transcription System kit (Promega, USA) according to the attached protocol. The concentration of RNA and cDNA was determined on a Qubit 4 fluorimeter (Thermo Fisher Scientific, United States) using appropriate reagents (Qubit RNA HS Assay Kit and Qubit DS DNA HS Assay Kit, Invitrogen, United States). Additionally, the RNA quality was checked by electrophoresis in 1.5% agarose gel using a FastRuler Middle Range DNA Ladder (Thermo Fisher Scientific, USA).

The analysis of the expression of *StAmy23* gene in roots, stolons, tubers, tuber skin, pulp of tubers, stems, leaves, and berries of potato plants in all three cultivars (Saturna, Gala, and Barin) was performed using the RT-qPCR method. The relative level of *StAmy23* expression was determined in comparison with the transcription of the reference *ef1* genes (primers 5′-ATTGGAAACGGATATGCTCCA-3′ and 5′-TCCTTACCTGAACGCCTGTCA-3′) and *sec3* (5′-GCTTATCATACTTCCGATCTCGCA-3′) [22, 23]. For RT-PCR, we used 100 ng of cDNA template, a set “Reaction mixture for RT-PCR in the presence of SYBR

GreenI and ROX (LLC Syntol, Russia) according to the manufacturer's recommendations, and a thermal cycler CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The PCR assay was run in two biological and three technical repetitions under the following conditions: 5 min at 95 °C; 15 s at 95 °C, 50 s at 62 °C (40 cycles).

To quantify total starch, tuber material (including pulp and peel, 500 mg) was homogenized in 4.5 ml of a solution containing dimethyl sulfoxide (DMSO, 33%, v/v) and hydrochloric acid (0.44 M), incubated at 60 °C for 30 min in a water bath cooled to 25 °C, and diluted with water (mQ) in a ratio of 1:5. The pH was adjusted to 4.5 with 5 M sodium hydroxide. The suspension was filtered through Miracloth (Merck, USA); 100 μl of the filtrate was used to measure the starch content using the Starch enzyme test (Boeh-ringer Mannheim/R-Biopharm AG, Switzerland) according to the manufacturer's protocol (Eppendorf BioSpectrometer® basic spectrophotometer, Eppendorf, Germany; λ = 340 nm).

To assess the concentration of glucose and fructose, 1 g of tuber material (including pulp and peel) was ground in liquid nitrogen, suspended in 10 ml of 80% ethanol, and centrifuged at 16000 g for 15 min. The supernatant was analyzed by high performance liquid chromatography (HPLC) using a Varian ProStar chromatograph (Varian Inc., USA), a 102 M differential refractometric detector for a chromatograph (Styer model, ZAO SKB Khromatek, Russia) and an Agilent Pursuit column 200E PFP (150 mm × 4.6 mm, 5 μm, A3050150X046, Agilent, USA). Isocratic elution was performed using acetonitrile:water (75:25 v/v) as mobile phase; flow rate 1.5 ml/min, temperature 30 °C. Biochemical analysis was performed in two biological and three technical repetitions.

For statistical processing of RT-PCR results and analysis of starch and reducing sugars contents, we used the GraphPad Prism v.8 software (GraphPad Software Inc., USA; https://www.graphpad.com/scientific-software/prism/). Data were expressed as mean (*M*) with standard error (±SE) based on two biological and three technical replicates for each cDNA variant and each potato sample. To assess differences in gene expression and carbohydrate content, Welch's t-test (unequal variance) was used ($p < 0.05$ indicates the statistical significance of the differences).

Results. The first stage of the work was bioinformatic analysis of the data on potato amylase StAmy23 available in the NCBI database. Potato *StAmy23* gene, mRNA and protein sequences (2871 nt; 4 exons; LOC102598863 alpha-amylase-like [*Solanum tuberosum* (potato)]; Gene ID: 102598863, chromosome VI) were extracted from GenBank NCBI (https: // www. ncbi.nlm.nih.gov/).

Analysis using NCBI-BLASTP (in the NCBI Non-redundant protein sequences database) showed that the closest homologues of the StAmy23 amylase (Protein ID: XP_006354888.1) are the α-amylase proteins of different potato varieties (for example, XP_004238157.1, 98% identity) and tomato *Solanum lycopersicum* L. (for example, XP_004235226.1, 91%) (Fig. 1, A). Homologous β-amylase of a more distant solanaceous species *Capsicum annuum* L. (PHT84617.1) has a 91% identity to potato StAmy23 in the amino acid sequence. Phylogenetic analysis based on the amino acid sequence of StAmy23 and amylase homologues confirmed the revealed similarity (see Fig. 1, B)

Based on the performed comparative structural analysis of the sequences of genes and mRNA of β-amylases homologous to *StAmy23* in *Solanum* species found in NCBI, we developed cDNA-specific primers for RT-PCR: StAmy23-F 5′-ATGGCG-CTTGATGAAAGTCAGC-3′ and StAmy23-R 5′-CCA-GACTTTGCAATATCAGGAAC-3′.

The second stage of work was the analysis of the expression profile of

StAmy23 gene in various tissues of potato plants in comparison with the content of starch and reducing sugars (glucose and fructose) in the tubers of the same plants in order to search for a possible relationship between these characteristics.

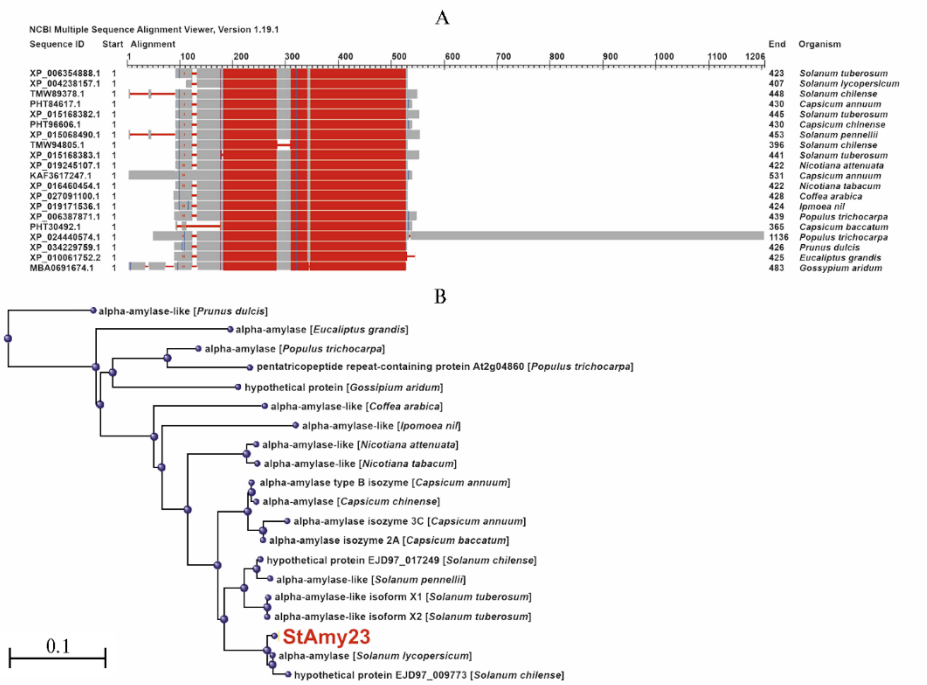

Fig. 1. Structural and phylogenetic analysis of potato α-amylase (*Solanum tuberosum* L.) StAmy23: A — the degree of conservatism of the amino acid sequence between species (highly conserved sequences are highlighted in red, less conservative in blue, variable regions in gray, deletions of variable regions — in white with a narrow red stripe); B — dendrogram of evolutionary relationships between potato StAmy23 and the nearest known homologues proteins (NCBI accession numbers are indicated on the left in the amino acid sequence alignment diagram). Structural comparison was performed based on the data of the search for the closest homologues of StAmy23 (Gene ID: 102598863) in the NCBI database using the BLASTP tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic dendrogram was constructed as a result of analysis and visualization of BLASTP data for structural comparison of StAmy23 homologues using the Fast Minimum Evolution method (Grishin distance matrix, https://www.ncbi.nlm.nih.gov/blast/treeview/).

The three chosen potato varieties (Saturna, Gala, and Barin) differ in the content of starch in the tubers (https://reestr.gossortrf.ru/). The samples were grown in the field conditions, and freshly harvested tubers (including pulp and peel) were used to determine the starch and reducing sugar content (Table). Biochemical analysis showed the highest and closest starch content in the tubers of the Gala and Saturn varieties, while in the Barin variety it was almost 2 times lower (see Table). The results obtained differed from the data of the variety originators who reported that the tubers of the Gala variety were the lowest in the starch content compared to the Saturna and Barin varieties, and the Barin tubers occupy an intermediate position between the Gala and Saturna varieties (see Table). The mismatch may be due to the influence of weather conditions during the growth of the samples analyzed in our work. The greatest amount of reducing sugars was found in the tubers of the Saturna variety, the smallest in the Gala variety, which coincided, respectively, with the highest and lowest starch content among the samples. The tubers of the Gala variety contained4.5 and 24.5 times less glucose and fructose, respectively, than the tubers of the Barin and Saturna varieties (see Table).

Content of starch and reducing sugars in freshly harvested tubers of the studied potato (*Solanum tuberosum* L.) varieties (M±SE)

Variety	Starch (as per the official characteristics of the variety), %	Starch, mg/g tissue	Reducing sugars (glucose/fructose), mg/g tissue
Saturna	16.5-21.4	1111.34±0.23	0.217±0.021/0.175±0.070
Barin	13.4-14.6	6.3±0.05	0.056±0.007/0.016±0.009
Gala	10.2-13.2	1111.34±0.34	0.016±0.001/0.000

In the same samples, we investigated the transcriptional activity of *StAmy23* gene using our developed primers StAmy23-F/StAmy23-R. Previously Hou et al. [4] and Zhang et al. [20] studied the expression of *StAmy23* in leaves, stems, and tubers of potato varieties resistant and sensitive to cold saccharification and showed its presence in all three types of tissues. The level of gene expression in freshly harvested tubers did not differ between cultivars, while in leaves, *StAmy23* mRNA was more actively transcribed in a resistant cultivar [20]. The expression of this gene in other parts of the plant was not evaluated. Therefore, it was of interest to evaluate the *StAmy23* transcription profile in six different organs of potato plants, i.e., in leaves, stems, berries, roots, stolons, and tubers (pulp and rind separately). In addition, a comparison of gene expression in three cultivars with different starch and reducing sugars content in tubers (see Table) could reveal a possible relationship between the *StAmy23* transcription level and carbohydrate accumulation at the time of potato harvest.

The results of RT-PCR assay showed an increased *StAmy23* gene transcription in tuber pulp compared to leaves in the cultivars Saturna and Gala, while it decreased in the cultivar Barin (Fig. 2). Together with the data of Zhang et al. [20] who reported that, during the harvesting period, the level of gene expression in tubers is either lower or the same as in leaves, it can be assumed that each potato cultivar has its own *StAmy23* transcription profile. The presence of *StAmy23* mRNA in all analyzed tissues and an unexpectedly high, in comparison with tubers, gene expression in vegetative organs and berries suggest the absence of a pronounced specificity of the work of the *StAmy23* enzyme in relation to certain plant tissues. The level of *StAmy23* transcription in non-photosynthetic roots and stolons either corresponded (cv. Saturna), or significantly (1.8-8.0 times) exceeded (cv. Barin, Gala) that in tubers. High expression also occurred in photosynthetic stems and berries, although its level in leaves was relatively low (see Fig. 2).

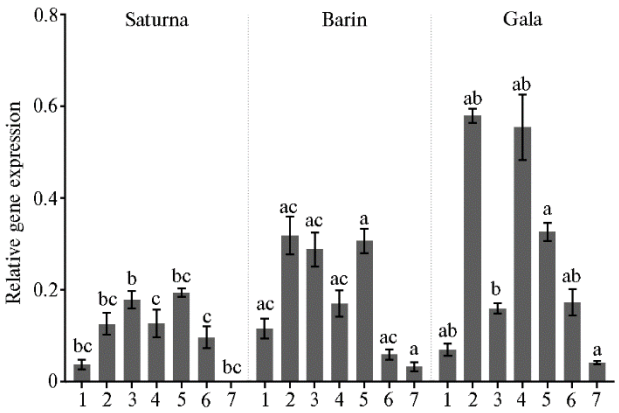

Fig. 2. Expression profile of the *StAmy23* gene in leaves (1), **stems** (2), **fruits** (3), **roots** (4), **stolons** (5), and **tubers** (underlined) **in pulp** (6) **and peel** (7) **in potato (*Solanum tuberosum* L.) cultivars Saturna** (a)**, Barin** (b) and **Gala** (c). *ef1* and *sec3* were the reference genes. The analysis was carried out in two biological and three technical replicates, the values of M±SE are given. Letters a, b and c denote statistically significant differences in gene expression between cultivars ($p < 0.05$). For example, bc means that the level of gene expression in the marked tissue in cv. Saturna (a) is significantly different from that in cv. Barin (b) and cv. Gala (c).

When comparing the expression levels of *StAmy23* between cultivars with

different starch and sugar contents, it turned out that cultivar Gala had the highest transcriptional activity of *StAmy23* in stems, while cultivar Saturna had the lowest transcriptional activity (see Fig. 2). In the studied cultivars, the leaves were characterized by a similar level of expression with a slight advantage of cultivar Barin. The *StAmy23* expression in berries was the highest in the Barin variety, in roots — in the Gala variety, and in the stolons — in the Barin and Gala varieties. In tuber, a fleshy storage organ the *StAmy23*expression level was the highest in the Gala variety and the lowest in the Barin variety. The studied cultivars did not differ in the transcriptional activity of the *StAmy23* gene in the tuber skin (see Fig. 2).

Comparing the data on transcriptional activity (see Fig. 2) and the results of biochemical analysis (see Table), it can be assumed that there is a positive relationship between the *StAmy23* transcription level and starch content, but not the content of reducing sugars in tubers. Thus, the level of *StAmy23* expression is the highest in cultivar Gala and medium in cultivar Saturna, while the starch content in these cultivars is equally higher than in cultivar Barin with the lowest gene expression level (see Table, Fig. 2). The highest amount of glucose and fructose was found in the Saturna cultivar with medium *StAmy23* gene expression level, and the lowest in the Gala cultivar with the highest *StAmy23* expression (see Table, Fig. 2).

However, based on the analysis of only three cultivars, it is too early to draw assumptions about a relationship between the transcriptional activity of *StAmy23* and the carbohydrate content in tubers. For strict conclusions, it will be necessary to study a sample of potato samples that are contrasting in the content of starch and sugars in tubers.

It should also be taken into account that the activity of β-amylase StAmy23 is supplemented by the action of β-amylases and starch phosphorylases and can be suppressed by inhibitors of amylases [24, 25]. Therefore, it would be most correct to look for correlations between the cumulative expression profile of genes encoding these enzymes and the starch content.

For example, the SbAI amylase inhibitor at the post-translational level regulates the activity of amylases [26, 27]. An inverse relationship was shown between the expression of the *SbAI* gene and the content of reducing sugars in tubers [28]. It was previously revealed that the level of *SbAI* expression in potato tubers of cv. Barin is significantly (approximately 3 times) lower than in cv. Severnoe siyanie, suitable for the production of crisp potatoes [25]. Based on these data, the unsuitability of tubers for frying in all three varieties studied by us in this work may be associated with a low level of *SbAI* transcription and, as a consequence, with sensitivity to cold saccharification.

In addition, if we talk about correlations with the content of glucose and fructose, then the activity of genes for the enzymes of sucrose hydrolysis (invertase, sucrose synthase) and inhibitors of invertase should also be taken into account [29-31]. The processes of glucose utilization in plant cells, including the synthesis of starch and sucrose, should also be considered. It should also be kept in mind that the source of glucose in tubers can be not only maltose resulted from the cleavage of starch by amylases [32], but also sucrose which is produced in photosynthetically active leaves and moves from them to tubers [33].

The way of decomposition of starch in storage organs differs from the way of decomposition of starch in leaves [7]; however, in both cases, amylases are involved in the process. It is known that the leaves of dicotyledons (including potatoes) are rich in starch, while the leaves of cereals (for example, barley) are rich in sugars [24, 34]. This suggests that the main role of transient starch in potato leaves is mainly to maintain growth processes at night, as has been shown for

Arabidopsis thaliana [35-37]. The presence of *StAmy23* gene transcription in photosynthetic tissues of potato plants indicates that the encoded enzyme is involved in the regulation of starch metabolism not only in storage organs (berries and tubers), but also in vegetative tissues to maintain plant growth and response to abiotic stresses.

So, in this work, we performed a bioinformatic phylogenetic analysis of α-amylase StAmy23, which determined the closest homologues of the enzyme. They are α-amylases of representatives of the *Solanaceae* family, primarily of the species and varieties of potatoes, tomatoes and peppers. Biochemical analysis of freshly harvested tubers of three potato cultivars (Barin, Saturna, and Gala) revealed differences between cultivars in the content of both starch and reducing sugars. Thus, in the Barin variety, the starch content (6.3 mg/g tissue) was 2 times lower than in the Gala and Saturna varieties, which have similar high starch contents (approximately 11.34 mg/g tissue). The amount of reducing sugars (glucose/fructose) was found to be the smallest in Gala tubers; tubers of varieties Barin and Saturna contained 4.5 and 24.5 times more glucose/fructose, respectively, (0.056/0.016 and 0.217/0.175 vs. 0.016/0.000 mg/g tissue in variety Gala). We have determined the expression profile of the *StAmy23* gene in the stems and leaves of the analyzed varieties, as well as for the first time in other organs and tissues of a potato plant (roots, stolons, berries; in tubers, separately in the peel and pulp). It was shown that the level of StAmy23 transcription in roots and stolons either corresponds (in the Saturna variety) or exceeds (in the Barin and Gala varieties) that in the tubers. The highest expression level of the *StAmy23* gene in berries was found in the Barin variety (0.29), in the roots and tubers in the Gala variety (0.55 and 0.17), and in the stolons in the Barin and Gala varieties (0.31 and 0.33). It is assumed that there is a positive relationship between the level of *StAmy23* transcription and the starch content (but not reducing sugars) in tubers. The transcriptional activity of the *StAmy23* gene in all analyzed tissues of the potato plant indicates the possible participation of α-amylase StAmy23 in the hydrolysis of starch not only in storage, but also in vegetative (including photosynthetic) organs to maintain the physiological processes of growth and plant response to stress. The data obtained are promising for expanding the understanding of the fundamental processes underlying carbohydrate metabolism of plant cells, including its changes in response to stress. From a practical point of view, the research results can be used in breeding potato varieties for the selection of donors of economically valuable traits (for example, resistance to cold saccharification of tubers, resistance to low cultivation temperatures, high or low starchiness of tubers). Continuation of research will be associated with the study of the cumulative effect of α- and β-amylases on various characteristics of plants of different varieties and types of potatoes.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 910-923
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 579.6:575

doi: 10.15389/agrobiology.2021.5.910eng
doi: 10.15389/agrobiology.2021.5.910rus

ON DDSL-BASED GENOTYPING OF POTATO BACTERIOSIS AGENTS, THEIR ANTAGONISTS AND MICROBIAL BIODESTRUCTORS FOR PLANT PROTECTION AND ECOTECHNOLOGIES

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The authors declare no conflict of interests
Acknowledgements:
The work was carried out in accordance with the State task, Section 5 "Plant protection and biotechnology" of the Federal Research Program of the State Academies of Sciences for 2013-2020, topic 15 "Molecular and nanotechnological bases for the development of biological and chemical new generation plant protection products for effective and safe use in integrated protection systems"
Received July 9, 2021

Abstract

Intensification of agricultural and industrial production necessitates environmentally friendly technologies to prevent human habitat from chemical pollutions. Microbial producers of biologicals for biocontrol of plant pathogens and hydrocarbon destructors for bioremediation are characterized by high spontaneous genetic variability which can lead to a change in their activity. Therefore, in stabilizing selection, it is necessary to confirm strain affiliation. Here, we presents data on the application of the double digest and selective label (DDSL) technique developed by us to study the genetic profiles of plant pathogenic agents of the genera *Pseudomonas*, *Pectobacterium*, their antagonists *Bacillus subtilis* (*Bs*), and the hydrocarbon destructors of the genus *Pseudomonas*. The study confirmed high biological efficiency of two selected *Bs* strains, the M-22 and I5-12/23 against bacterial diseases of stored potato tubers. In addition, destructors from the genus *Pseudomonas* were genetically identified. The aim of the study was to evaluate genetic diversity among *Pseudomonas*, *Pectobacterium*, and *Bs* strains to select effective microbial antagonists and hydrocarbon destructors. The DDSL technique uses two restriction endonucleases for bacterial genomic DNA digestion. Taq DNA polymerase supplemented into reaction mix provides simultaneous labeling DNA fragments by biotinylated deoxycytidine triphosphate (Bio-dCTP). Only fragments digested with one of the restriction enzymes producing fragments with 3′-recessed ends are subjected to labeling. The second restriction enzyme produces only blunt ends which can not bind Bio-dCTP tag. As a result of DDSL reaction 20 to 50 clear DNA fragments are visualized on the filter, and their quantity and distribution are characteristic for each bacterial strain. Genotyping allows generating genetic profile for each bacterial strain, i.e., assigning a "bar-code" to the bacteria that identifies a given microbe with confidence. Genotyping *P. atrosepticum* D822 и G784 allows for identification of about 50 DNA fragments more than 20 % of which were specific for only one of the compared strains. We used two pairs of restriction enzymes — XbaI/DraI and XbaI/Eco24I. Our results indicate on equal discriminatory ability of these two enzyme combinations when compared *P. atrosepticum* strains D822 и G784. We noted some advantage of XbaI/DraI enzymes because of its ability to identify differences in genetic profiles in a range of longer DNA fragments. The optimal enzymes for *Pseudomonas* genus genotyping were restriction endonucleases BcuI/Eco32I, for *Bs* the first restriction enzyme was SgsI (39 cleavage sites), the second was Eco32I reducing the size of the obtained DNA fragments. High antagonistic activity of *B. subtilis* strain I5-12/23 which belongs to I genotypic group was shown in laboratory experiments with artificial contamination of potato tubers with *P. atrosepticum* 1944 и *P. carotovorum* subsp. *carotovorum* 481. Index of development of bacterial soft rot disease after treatment of infected tubers by *B. subtilis* strain I5-12/23 was 0-0.02, in control

the index was 4.04. Biological activity of this strain after treatment of infected tubers was up to 100 % whereas chemical fungicide Maxim KS gave rise to only 77.7 % value. Experiments conducted in potato storage houses confirmed high biological activity of two selected bacillus strains against bacterial diseases during potato tuber storage. *B. subtilis* strain I5-12/23 demonstrated highly expressed antagonistic activity against causal agents of bacterial soft rot, ring rot as well as fusarium dry rot potato diseases. Significant suppression of potato tuber diseases in comparison with control after treatment by selected antagonist strains was demonstrated, and this effect was comparable with that of chemical standard Maxim KS. Percentage of healthy tubers treated by bacillus strains was in the range of 30.4-35.5 % whereas in control this value did not exceed 13.3 %. Thus, yield of healthy products compared to control was 2.7 times higher. The most efficient *B. subtilis* strain I5-12/23 effectively suppressed causal agents for ring rot and fusarium dry rot potato diseases. Prevalence of ring rot disease was 2.6-2.9 %, fusarium dry rot — 1.5-3.0 %, the values which are significantly lower than those in samples treated by *B. subtilis* M-22 and Maxim KS (4.8 and 3.5 %; 9.0 and 4.1 %, respectively). After genotyping, destructor strains of the genus *Pseudomonas* utilizing difficult-to-oxidize compounds, including heavy oil fractions and polyaromatic hydrocarbons (benzopyrene, chrysene, phenanthrene, anthracene, chrysene, naphthalene) have been deposited in the VIZR collection. The range of their activity is enough to compose associations for utilizing specific pollutants. Thus, DREAM genotyping identifies bacterial strains to confirm their origin in the course of development and use of biological products for various purposes.

Keywords: restriction endonucleases, genotyping, plant pathogens, microbial antagonists, biodestructors, *Pectobacterium*, *Pseudomonas*, *Bacillus subtilis*, *Solanum tuberosum* L., potato

Preservation of the environment in the intensification of agricultural and industrial production is one of the global challenges today. This problem could be addressed by using the environmentally friendly technologies, including biocontrol of plant pathogens and bioremediation.

Substantial crop losses from bacteriosis constitute a serious problem for potato growing in the Russian Federation. The most common disease is considered to be black stem, or soft rot. Its main pathogen is microorganisms from the genus *Pectobacterium* (syn. *Erwinia*) [1-3]. Semi-parasitic bacteria of the genus *Pseudomonas* (*Ps. fluorescens* and *Ps. marginalis*), producing pectolytic enzymes, also actively participate in damaging and subsequent decomposition of tubers, especially during the winter storage of crops.

The methods of phytosanitary monitoring at the population level make it possible to identify both the strains of pathogens that determine the intensity of the infectious process in the ecosystem, and suppressor microorganisms introduced to control it [4, 5]. Genotyping of microorganisms is used to study the genetic profiles of bacterial strains for their identification and individualization [3, 6, 7-9]. Based on genotyping results, each strain can be assigned a “bar-code”, which is extremely important for identifying the types of bacteria and their antagonists [6]. Following the introduction of an antagonist microorganism into the rhizo or phyllosphere of a plant, its presence in the microbial community is confirmed by molecular-genetic methods, with determination of the degree and duration of dominance, which will ensure effective suppression of the population density of phytopathogenic species and the dynamic stability of soil microbiocenoses. For in-depth identification of pathogens, it is important to carry out genotyping during the phytosanitary control of potato diseases caused by the bacteria *Pectobacterium* and *Dickeya* [10-12].

Studies have shown that genotyping allows quick and highly accurate identification of collection strains of the genus *Bacillus*, which are similar in morphological and cultural features, but differ in the composition of complexes of metabolic products. It can be used to recognize individual strains and groups of closely related *Bs* strains and identify introduced suppressor microorganisms, which provides grounds to recommend genotyping both for controlling the origin of strains and for phytosanitary monitoring [13-15].

Currently, antagonists of pathogens and biological products based on them

are successfully used to regulate the density of populations of phytopathogenic species in agrobiocenoses [16-18]. The high biological activity of biological products has been shown in many agricultural crops [19-22]. The selection of biological products based on phytosanitary monitoring data, taking into account biological characteristics and the spectrum of action of producer strains, largely determines the effectiveness of microbiological and integrated protection of potatoes from diseases [23, 24]. Methods of blocking chemical signals of communication (QS, quorum sensing) in pathogen populations due to the release of certain inhibitors by antagonist bacteria are of interest [25, 26].

The most effective and widely used (about 90-95% of the biopesticide market) means of combating diseases are preparations based on gram-positive spore-forming bacteria of the *Bacilliaceae* family, capable of synthesizing biologically active compounds of various chemical nature [27-29].

The density of populations of microorganisms — suppressors and antagonists of plant pathogens introduced into the soil microbiocenosis is of great importance [30-33]. In this regard, to predict the intensity of disease development, to assess the effectiveness of biological control of populations of phytopathogenic species, and to adjust the regulations for the use of biological products, it is necessary to determine and identify introduced strains of suppressor microorganisms.

Another important area of application of the genetic certification of microorganisms is associated with environmental pollution with hydrocarbons (leakage during oil production, oil spills after accidents at pipelines and oil refineries). The designated problem has been exacerbated in recent years, while bacterial hydrocarbon destructors consuming the carbon of such products in their metabolism are increasingly used. Selection and genetic certification of the most active destructor strains makes it possible to more effectively address such environmental issues.

Based on our previously proposed double digest and selective label (DDSL) technique, a method for genetic identification and certification of strains of *Bacillus subtilis* (*Bs*) and some representatives of the genus *Streptomyces*, being promising antagonists of phytopathogens, was developed [6]. This method was previously tested on strains that differ in physiological and biochemical characteristics and the composition of secondary metabolites, which determine the antibiotic activity. Its high resolution has also been proven.

Here, we present data on application of DDSL technique to study the genetic profiles of plant pathogenic agents of the genera *Pseudomonas*, *Pectobacterium*, and their antagonists (*Bacillus subtilis*). The outcomes can be used in the development of biological products against phytopathogens based on their antagonists and to control the origin of strains. The study confirmed high biological efficiency of two selected *Bs* strains (M-22 and I5-12/23) against bacterial diseases of stored potato tubers in production. In addition, we were the first to identify (certify) the strains of bacteria-destructors (genus *Pseudomonas*), suitable for addressing the environmental problems.

The aim of the study was to evaluate genetic diversity among *Bacillus*, *Pectobacterium* and *Pseudomonas* strains to select the most effective microbial antagonist destructor strains.

Materials and methods. The strains of plant pathogenic bacteria used in the work were certified and are included in the State collection of phytopathogenic microorganisms and their pests of the Vavilov All-Russian Research Institute of the Plant Industry (registered in WFCC WDCM 760, Japan, 01.28.1998). Strains *P. atrosepticum* D822 and G784 were isolated from the infected potato tubers in the Leningrad Province. Certified strains *P. atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481 were obtained from the State collection of phytopathogenic

microorganisms and plant varieties-identifiers (differentiators) of pathogenic strains of microorganisms of the All-Russian Research Institute of Phytopathology. The certified *Ps. fluorescens* 894 was obtained from the All-Russian collection of microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms (IBPM RAS). Strain *Ps. marginalis* (previously a member of the group *Ps. xanthochlora*) is a part of the collection of plant pathogenic microorganisms of Lorkh All-Russian Research Institute of Potato Farming.

Bacteria were isolated by methods generally accepted in plant bacteriology [34] from potato plants with symptoms of bacterial rot. Bacteria were identified using the conventional physiological, biochemical, and molecular techniques (polymerase chain reaction, PCR) [34]. For the genus *Pseudomonas*, the primary analysis was carried out using the LOPAT system (levan, oxidase, potato soft rot, arginine dihydrolase, hypersensitivity reaction), as well as for the fluorescent pigment on King B medium, for the genus *Pectobacterium* (pathotypes *carotovora* and *atroseptica*) by liquefaction of pectate potato tissue.

Double digest and selective label (DDSL) technique was used for genotyping of bacteria genus *Pectobacterium* and *Pseudomonas* [7, 8]. During preliminary application of this method on two strains of *Pectobacterium* and two strains of *Pseudomonas*, an in silico search was carried out (http://in-silico.ehu.eus/) showing that the best first restriction enzyme for microorganisms of the genus *Pseudomonas* is BcuI (A↓CTAGT, 78 cleavage sites in the reference genome of *P. fluorescens* Pf-5, Thermo Fisher Scientific, Inc., USA), and for representatives of the genus *Pectobacterium* XbaI (T↓CTAGA,, 87 cleavage sites in the reference genome of *P. carotovorum* subsp. *catovorum* , Thermo Fisher Scientific, Inc., USA). The first enzyme is characterized by a smaller number of DNA cleavage sites with 3'-truncated ends, capable of binding labeled Bio-dCTP. The second enzyme, which cleaves DNA at a greater number of restriction sites, produces only blunt ends which cannot bind the tag.

As a result, the reaction mixture after enzymatic digestion contains a limited number of labeled DNA fragments that can be separated and visually recognized. In relation to bacteria of the genus *Pseudomonas*, Eco32I should be considered the best second enzyme (GAT↓ATC, 1556 cleavage sites in the reference genome, Thermo Fisher Scientific, Inc., USA), for the microorganisms of genus *Pectobacterium* DraI (TTT↓AAA, 1332 cleavage sites, Thermo Fisher Scientific, Inc., USA). Enzyme pairs are compatible in the same reaction buffer Msp20I (TGG↓CCA, 1028 cleavage sites, Thermo Fischer Scientific, Inc., USA) and Eco24I (GRGCY↓C, 1314 cleavage sites, Thermo Fischer Scientific, Inc., USA) [6].

Bs strains were analyzed using a pair of enzymes SgsI and Eco32I. The *B. subtilis* genome has 39 cleavage sites (GG↓CGCGCC) for the first enzyme, thus forming sticky ends that are able to bind Bio-dCTP using Taq polymerase. This pair of enzymes is compatible in R buffer (Thermo Fischer Scientific, Inc., USA).

For the DDSL reaction, 15 μl of distilled water, 2 μl of bacterial DNA, 2 μl of a buffer suitable for both restriction endonucleases, and 1 μl of a preprepared mixture, including restriction endonucleases, a Bio-dCTP tag and DNA polymerase, for instance Taq-polymerase, were mixed in an Eppendorf tube [6, 12].

The reaction was carried out in a Termit solid-state thermostat (DNA-Technologies LLC, Russia) at 37 °C for 1-2 h. Tris-acetate buffer and 0.8% agarose gel were used for electrophoresis, the voltage was 1.5 V/cm. Vacuum transfer of DNA fragments onto a nylon filter was carried out immediately after electrophoresis in a VacuGene XL Vacuum Blotting System™ (GE Healthcare, USA). In this case, the transfer of DNA from a double-stranded state to a single-stranded state was not required, since there was no molecular

hybridization. The detection of DNA fragments on the filter was based on the detection of the enzymatic activity of alkaline phosphatase in the presence of substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (NBT, Thermo Fischer Scientific, Inc., USA). Unbound tag was washed in buffer with maleic acid and salt.

Bs strains M-22 and 5-I-12/23, used in this work, were previously selected based on high antagonistic activity and deposited in the State collection of plant pathogenic microorganisms and their pests of the Vavilov All-Russian Research Institute of the Plant Industry. The bacteria were grown at 28 °C for 72 h (a nutrient medium containing 30 g/l maize extract, 15 g/l molasses; pH 7.2; a Bio-san OS-20 laboratory shaker, Diaem, Russia, 220 rpm; 750 ml flasks with 100 ml of medium). Sampling and assessment of culture growth were carried out using an Axio Imager A-2 light microscope (Karl Zeiss, Germany) once a day. The titer of viable cells was measured by serial dilutions followed by inoculation on the SPA medium and counting the colonies.

To assess the biological activity of the *Bs* 5-I-12/23 strain with artificial infection, 10 intact visually healthy potato (*Solanum tuberosum* L.) tubers (cv. Sante) were selected in 5 replicates for each variant. Mechanical damages 10 mm deep were made to their surface with a scalpel, into which 0.3 ml of a mixture of 1-day-old cultures of *P. atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481 (10^6 CFU/ml) in sterile distilled water was inoculated. Then the tubers were treated with the culture liquid of the *Bs* 5-I-12/23 strain with a titer of 10^8 and 10^9 CFU/ml. In the control, the tubers were treated with water, in the standard with the contact fungicidal dressing agent Maxim, KS (0.2 l/t) (Syngenta AG, Switzerland). Development of infections was assessed on day 8-10. The lesion index was calculated using the formula:

$$x = \frac{dh}{100},$$

where d is the diameter of the decay zone (lesion), mm, h is the depth of the decay zone, mm.

The biological activity of *Bs* strains 5-I-12/23 and M-22 during storage in a pile of potato tubers was studied in the winter period of 2018-2019 in a potato storage facility (Lorkh All-Russian Research Institute of Potato Farming, Moscow Province, Kraskovo village) based on the dynamics of disease development. To assess the antagonistic activity of the strain, a preliminary psychopathological analysis of potato tubers was carried out (GOST 33966-2016 “Seed potatoes. Technical conditions and methods for determining the quality”. Moscow, 2020) [35]. To assess the biological activity of the strain under conditions that provoke rot development, tubers of cv. Udacha were used. Before placing for storage, the tubers were treated with the *Bs* 5-I-12/23 strain culture liquid (a titer of 10^8 and 10^9 CFU/ml). The control tubers were treated with water. The standards were treated with the *Bs* M-22 culture liquid (a titer of 10^9 CFU/ml) and the contact fungicidal dressing agent Maxim, KS (0.2 l/t) (Syngenta AG, Switzerland), recommended for the treatment of seed potatoes before storage to protect them from rot of various etiologies, including wet rot. Tuber sample weighted 5 kg, the working solution rate was 3 l/t, the experiment was repeated 10 times. After drying, the treated material in nets was placed for storage in a potato mound at a depth of 20-30 cm from the surface under high humidity and temperature to provoke the infection present in the tubers and to reveal the effectiveness of the studied strain. Tuberous infections were counted after 6 months of storage. The prevalence of common scab, ring rot, late blight and fusarium blight was assessed by the proportion (%) of diseased tubers. The biological efficiency was calculated by the formula:

$$BE = \frac{a - b}{a} \cdot 100\ \%,$$

where BE is the decrease in the prevalence or development of the disease to control, %; a is the prevalence or development of the disease in the control, %; b is the prevalence or development of the disease in the experimental variant, %.

Statistical data processing was performed by methods of analysis of variance using the Statistica 6.0 software package (StatSoft, Inc., USA). Methods of parametric statistics were used for statistical processing. Mean values (*M*), standard errors of means (±SEM) and their 95% confidence intervals were calculated by Student's *t*-test.

Results. Genotyping by DDSL-technique can be briefly described as the sequential execution of the following steps: 1) cleavage of genomic DNA by two restriction endonucleases simultaneously and labeling of individual DNA fragments (containing 3′-truncated ends) with a biotin residue; 2) electrophoretic separation of DNA fragments by length; 3) vacuum transfer of DNA in distilled water to a filter; 4) identification of fragments labeled with biotin in a color chemical reaction [6].

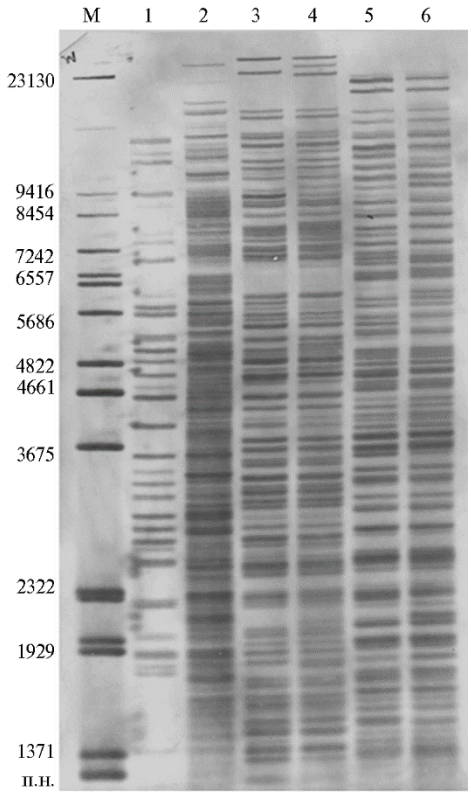

Fig. 1. Genotyping of strains of the genera *Pseudomonas* and *Pectobacterium* by double digest and selective label (DDSL) technique: 1 — *Ps. fluorescens* 894, 2 — *Ps. marginalis*, 3 and 5 — *P. atrosepticum* Д822, 4 and 6 — *P. atrosepticum* Г784. Restriction endonucleases are BcuI-Eco32I (tracks 1 and 2), XbaI-DraI (tracks 3 and 4) and XbaI-Eco24I (tracks 5 and 6). M is a DNA fragment length marker [12].

The DDSL-technique was successfully tested on *Ps. aeruginosa*, *Staphylococcus aureus*, *Salmonella* spp. and other causative agents of infectious diseases of interest in medical and veterinary practice [7, 8]. The use of the DDSL test also made it possible to identify genetic profiles in microorganisms of the genera *Pectobacterium* and *Pseudomonas*, which characterize the individuality of each of them (Fig. 1) [12]. For example, we noted significant differences between *Ps. fluorescens* and *Ps. marginalis*. *P. atrosepticum* strains D822 and G784 were compared based on two combinations of XbaI-DraI and XbaI-Eco24I enzymes in Tango™ reaction buffer (Thermo Fischer Scientific, Inc., USA). These pairs of enzymes helped us to detect about 50 DNA fragments. Despite certain interspecific differences in these species, genotyping revealed a group of identical DNA fragments, which indicates a generic phylogenetic closeness. Both combinations of restriction enzymes revealed a certain differentiation between *P. atrosepticum* strains D822 and G784 in terms of genetic profiles.

Therefore, genomic analysis by the DDSL-technique quantitatively shows both interspecies differences and genetic variations within a species at the level of individual strains [12]. For example, a comparison of microorganisms of the genus

Pectobacterium revealed more than 10 DNA fragments characteristic of each of them. Analysis of their genetic profiles allows us to conclude about the advantage of using the XbaI-DraI pair which detects, along with short fragments, longer ones, reaching 23000 bp. and grouped at the top of the filter (see Fig. 1).

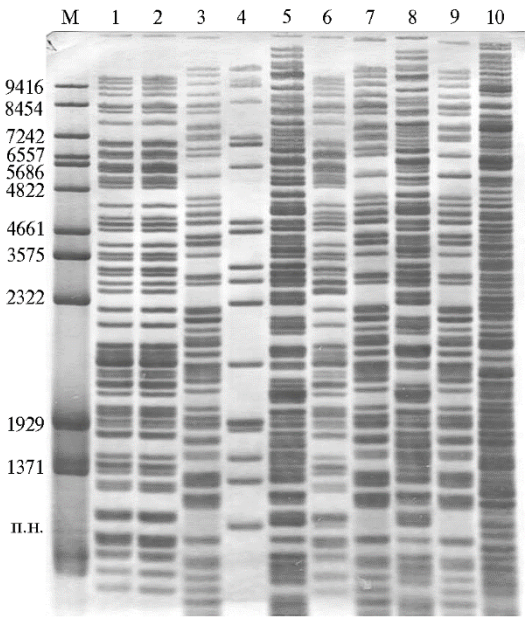

Fig. 2. Genotyping of strains destructors of hydrocarbons from the genus *Pseudomonas* by double digest and selective label (DDSL) technique using BcuI-Eco32I restriction enzymes: 1 — 1-1, 2 — 10-1, 3 — 14-2, 4 — ку-1, 5 — ко-1, 6 — Pp-2, 7 — lb 3-2, 8 — Pp-5, 9 — Pp-7, 10 — P.Pol Г+. M is a DNA fragment length marker [12]

The work was continued on a group of bacterial strains destructors of hydrocarbons from the genus *Pseudomonas* for their genetic certification. Earlier genotyping of clinical strains of *Pseudomonas aeruginosa* showed the effectiveness of the DDSL technique in identifying the pathways of infection and identification of bacterial strains circulating in products [7]. A pair of BcuI-Eco32I enzymes, compatible in the same reaction buffer, allows for clear visualization of more than 40 DNA fragments (Fig. 2).

Our study of bacteria destructors of the genus *Pseudomonas*, characterized by the ability to assimilate carbon from technogenically contaminated soil and water ecosystems in different climatic zones, revealed a significant genetic diversity of strains. On their basis, stable associations were created that effectively and quickly oxidize various toxicants in a short time in different environmental conditions. The formed collection of biodegradants (VIZR) includes strains that utilize difficult-to-oxidize compounds, including heavy oil fractions and polyaromatic hydrocarbons (in particular, benzopyrene, chrysene, phenanthrene, anthracene, chrysene, naphthalene), which makes it possible to compose bacterial associations for the utilization of various pollution.

The genotyping methodolofy we have developed based on DDSL technique was also successfully tested for the identification of 13 *Bs* strains (Table 1) [6], which have high antagonistic activity against a wide range of phytopathogenic fungi and bacteria and are part of the State collection of phytopathogenic microorganisms and their pests (Vavilov All-Russian Research Institute of the Plant Industry) [6, 8]. Currently, the collection includes more than 8000 strains of microorganisms, including more than 200 selected strains with high polyfunctional activity which are promising producers of biological products for various purposes [31].

Using the DDSL technique, we identified 7 groups of genotypically identical clusters and unique *Bs* strains: group 1 — 147/48/314, 110/723, 85/3/8, group 2 — 1-I, 2-I, 3 -I, 4-I, 5-I-12/23, group 3 — FR-318, group 4 — FR-327, group 5 — 1-K, group 6 — V-10, and group 7 — M-22. The noted distribution by genotypes is somewhat different from the previously obtained data, when we identified only six groups of genotypes [6]. In the genotyping performed

in this study, the M-22 strain, which is used in commercial biological products for plant protection, had a unique genotype that differs from all others. In previous work [6], this microorganism fell into a large cluster of genetically identical strains. Further re-culturing confirmed that there was a contamination of the M-22 strain with bacteria of group I. Otherwise, the genotypes of the analyzed bacteria coincided with those described by us in our previous work.

1. Characterization of *Bacillus subtilis* strains selected for genotyping by double digest and selective label (DDSL) technique [6]

Strain	Extraction site, source	Biological activity
B. subtilis B-10	Leningrad province (Russia), zoo-manure	Fungicidal, phytoregulatory
B. subtilis M-22	Ukraine, air medium	Fungicidal, bactericidal, phytoregulatory
B. subtilis 1-И	India, surface of cucumber seeds	Fungicidal
B. subtilis 2-И	India, surface of wheat seeds	Fungicidal
B. subtilis 3-И	India, surface of wheat seeds	Fungicidal
B. subtilis 4-И	India, surface of bean seeds	Fungicidal
B. subtilis 5-И-12/23	India, surface of tomato seeds	Fungicidal, bactericidal, phytoregulatory
B. subtilis 1-К	China, cucumber rhizosphere	Fungicidal
B. subtilis ФР-318	Collection of the All-Russian Research Institute for Agricultural Microbiology	Phosphate mobilizing
B. subtilis ФР-327	Collection of the All-Russian Research Institute for Agricultural Microbiology	Phosphate mobilizing
B. subtilis var. *niger* 147/48/314	Collection of the All-Russian Research Institute for Agricultural Microbiology	Fungicidal
B. subtilis var. *niger* 110/723	Collection of the All-Russian Research Institute for Agricultural Microbiology	Fungicidal
B. subtilis var. *niger* 85/3/8	Collection of the All-Russian Research Institute for Agricultural Microbiology	Fungicidal

Noteworthy is the group of strains 1-I, 2-I, 3-I, 4-I, 5-I-12/23 which did not differ genetically from each other, although they were isolated from different sources and had some differences in antagonistic activity. This could be due to the genetic and evolutionary proximity of the strains with the simultaneous presence of different genes that determine the biological properties. In other words, genomes can be similar, but differ in a small number of structural genes. With the appearance of an increasing number of changes in the genome in the process of evolution or selection for certain traits, visible differences appear in the genomic DNA. We compared the DNA fragments on the filter visually. Existing programs for comparing genetic profiles, for example BioNumerics™ (https://www.applied-maths.com/down-load/software) can be used when a large number of compared strains. According to the instructions for the specified program, the final stage of the computer comparison is the visual control of the coincidence of the fragments. In our case, given the small number of strains, preference was given to visual assessment. Differences in the number and distribution of DNA fragments suggest that the samples are genetically distinct strains. If the DNAs in the samples do not differ, there is a possibility that they may have a different genetic profile when using a different genotyping method.

Our studies have shown that the DDSL technique makes it possible to identify both strains of bacteriosis pathogens during the development of epiphytotics and antagonistic microorganisms introduced into the agrocenosis, which will allow us to further study the features of the parasite-host relationship in the plant-plant pathogen-antagonist system at the population level.

The study of *Bs* activity against pathogens of bacterial and fungal potato diseases in vitro on a wide range of test cultures showed that the *Bs* I-5-12/23 strain is the most promising for protection against bacterioses [32]. In our tests with artificial inoculation of tubers with *P. atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481, there was a significant decrease in the infection of potatoes

during storage after treatment of infected plant material with *Bs* I-5-12/23 (Table 2). The index of development of soft bacterial rot was 0-0.2 vs. 4.04 in the control. The biological efficiency of the chemical fungicide Maxim, KS was 77.7% while the treatment of tubers with a suspension of the *Bs* strain I-5-12/23 led to 100%.

2. Biological effectiveness of *Bacillus subtilis* I-5-12/23 strain against *Pectobacterium atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481 on potato (*Solanum tuberosum* L.) cv. Sante tubers after artificial inoculation ($N = 5$, $n = 10$, $M \pm$SEM)

Variant	Disease prevalence, %	Disease development index	Biological effectiveness, %
Control (nwithout treatment)	80.7±4.3	4.04±1.8	
B. subtilis, 10^9 CFU/ml	0	0	100
B. subtilis, 10^8 CFU/l	20.4±2.8	0.02±0.01	99.5±0.3
Maxim, KS, 0.2 l/g (standard)	80.1±6.6	0.9±0.1	77.7±0.2
LSD_{05}	9.8	0.1	0.5

Comparison of the effectiveness of *Bs* strains I-5-12/23 and M-22 in storage conditions showed that the former had a more pronounced antagonistic activity against ring rot, foot rot, and fusarium (Table 3). We did not observe any significant differences in the effectiveness of the preparations with respect to ordinary scab.

3. Biological effectiveness of *Bacillus subtilis* I-5-12/23 and M-22 strains against diseases of potato (*Solanum tuberosum* L.) cv. Udacha tubers during storage ($M \pm$SEM, Moscow Province, Kraskovo, winter of 2018-2019)

Variant	Portion of tuber, %					
	healthy	infected				
		total	ordinary scab	ring rot	foot rot	fusariosis
Control (water)	13.3±1.1	86.7±2.5	52.3±2.2	7.4±0.3	11.8±1.2	15.2±1.5
B. subtilis M-22, 10^9 CFU/ml	30.4±1.9	69.6±3.3	47.3±1.7	4.8±0.2	8.5±0.7	9.0±0.8
B. subtilis И-5-12/23, 10^9 CFU/ml	32.4±1.7	67.6±1.2	53.8±4.5	2.6±0.1	8.2±0.3	3.0±0.1
B. subtilis И-5-12/23, 10^8 CFU/ml	35.5±2.0	64.5±2.6	55.7±3.7	2.9±0.2	4.4±0.2	1.5±0.1
Maxim, KS, 0.2 l/g	30.0±1.5	70.0±4.3	54.1±3.3	3.5±0.1	8.3±0.4	4.1±0.2
LSD_{05}	2.3	5.4	6.6	0.4	1.4	0.2

Analysis of the obtained data indicates that the biological effectiveness of *Bs* strains against potato tuber diseases during storage is comparable to the effectiveness of a chemical fungicide. The rate of healthy tubers in the control did not exceed 13.3%, after treatment with *Bs* strains reached 30.4-35.5%, which increased the yield of healthy products by more than 2 times.

It should be noted that in the genotyping of plant pathogenic bacteria, antagonistic microorganisms and biodestructors by our proposed DDSL technique, the pairs of restriction endonucleases are used which require 1-2 hours to develop the reaction. In recent years, commercial preparations of similar enzymes with a shorter incubation time (up to 5 min, the so called fast digest), for example, restriction endonucleases from Thermo Fischer Scientific, Inc. (USA), have appeared. They give a faster result, but this is associated with a certain risk of incomplete cleavage of genomic DNA, which distorts the appearance of the genetic profile.

Various methods of genotyping pseudomonads are discussed in the literature. In particular, when genotyping 232 isolates using the pulsed-field gel electrophoresis (PFGE) method and multiple locus variable-number tandem repeat analysis (MLVA), the results were only 91% consistent [36]. These data once again emphasize the need for the use of high-resolution methods that allow the detection of a large number of DNA fragments in the analyzed genomes. Genotyping of species of the genus *Pectobacterium* is often carried out using multilocus sequencing of various numbers of housekeeping genes, from four [37] to thirteen [38].

So, the method of genetic certification developed by us (double digest and selective label technique, DDSL) helps to reveal and unambiguously identify strains of plant pathogenic bacteria of the genera *Pectobacterium* and *Pseudomonas* and their antagonists in the phytosanitary monitoring during epiphytoties of bacteriosis, which, in particular, is necessary for development of phytosanitary technologies for growing and storing potatoes. In addition, the DDSL technique genetically identifies strains of biodestructors from the genus *Pseudomonas*, which allows for identity control of strains during their commercial use (certification). Genotyping by the DDSL technique visualizes a large number of DNA fragments (in this work more than 40), the distribution of which characterizes and individualizes the bacterial strain. For each type of microorganism, a preliminary selection of restriction enzymes is required, which produce the optimal number and size of DNA fragments on the filter. The combination of the DDSL technique and the assessment of biological activity showed that of the studied *Bacillus subtilis* (*Bs*) strains, the *Bs* I-5-12/23 has the most pronounced antagonistic properties against the causative agents of ring rot, foot rot and fusarium disease. Postharvest treatment of potatoes with itaqueous suspension of *Bs* I-5-12/23 also revealed high biological effectiveness with a significant decrease in the damage to tubers during storage compared to the chemical preparation Maxim, KS. The DDSL technique can be also used to study populations of microorganisms in natural and artificial biocenoses.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 924-933
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

In vitro cultures

UDC 615.32:576.3/.7.086.83

doi: 10.15389/agrobiology.2021.5.924eng
doi: 10.15389/agrobiology.2021.5.924rus

HIGH EFFICIENT MICROPROPAGATION OF *Panax vietnamensis* Ha et Grushv. FROM IMMATURE ZYGOTIC EMBRYOS

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The authors declare no conflict of interests
Acknowledgments:
Funded by the Ministry of Science and Technology of Vietnam in the frame of project «Optimization of a protocol for micropropagation of Ngoc Linh ginseng (*Panax vietnamensis*) using a bioreactor culture system» (2018-2019)
Received June 9, 2021

Abstract

Despite its significant economic benefit as medicinal plant, cultivation of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) has not been enlarged due to limited source of planting material. Even though considerable research efforts to develop tissue culture systems have been made, micropropagation of Ngoc Linh ginseng is still considered difficult. Here, a micropropagation protocol was established for Ngoc Linh ginseng through somatic embryogenesis from immature zygotic embryos. Direct somatic embryogenesis from immature zygotic embryos was obtained at a rate of 44.33 % on Schenk and Hildebrandt (SH) basal medium with 7 % sucrose. Secondary somatic embryogenesis with proliferation coefficient of 26.71 was induced in liquid-shake culture for 4 week in $^1/_2$ SH medium with 0.1 mg/l kinetin and 0.1 mg/l thidiazuron. The SH medium supplemented with 1 mg/l α-naphthalenacetic acid, 0.5 mg/l benzyladenine and 5 mg/l gibberellic acid stimulated germination of somatic embryos and early formation of microrhizomes. On two next 10-week cultures on SH medium with 0.5 mg/l α-naphthalenacetic acid, 1 mg/l benzyladenine and 4 % sucrose, micropropagated plantlets were produced with well-developed root system and thickened microrhizome with a dormant bud. The acclimatization of micropropagated plantlets were successfully accomplished with survival rate of 91.67 % when using the soil mixing forest humus with sand in a ratio of 1:2. An efficient protocol developed for Ngoc Linh ginseng micropropagation from immature zygotic embryos comprised five steps, including embryogenesis induction, proliferation of somatic embryos, germination of somatic embryos, development of plantlets, and acclimatization of plantlets.

Keywords: *Panax vietnamensis* Ha et Grushv., Vietnamese ginseng, Ngoc Linh ginseng, in vitro culture, immature zygotic embryo, somatic embryogenesis, somatic embryo germination, microrhizome, micropropagation

Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.) belonging to the ginseng family *Araliaceae* is a valuable and endemic medicinal plant. It is thus so-called Ngoc Linh ginseng. In nature, Ngoc Linh ginseng grows only above 1.200 m of altitude and under the canopy of tropical moist forests with daytime temperature, ranging from 20 to 25 °C and at night from 15 to 18 °C. Ngoc Linh ginseng is a slow-growing, long-lived herbaceous perennial plant with a height from 40 to 100 cm. Its bamboo-like rhizomes are characterized by permanent scars caused by the annual loss of aerial stem. The rhizome of Ngoc Linh ginseng contains 52 saponins, among which 24 are not found in other ginsengs [1]. Saponin content

in the rhizome of Ngoc Linh ginseng is higher than that in other *Panax* species [2, 3].

Despite high economic value, cultivation of Ngoc Linh ginseng is difficult to expand to a large scale. Since 1983, many studies have aimed to develop Ngoc Linh ginseng as a medicinal plant [4].

Based on successful micropropagation of Korean ginseng (*Panax ginseng* C.A. Meyer) and other ginseng species, there are a number of studies on Ngoc Linh ginseng tissue culture, such as organogenesis [5], artificial seed formation [6], rhizome formation [7], somatic embryogenesis [8-12]. Some studies have focused on secondary and adventitious root culture [13] for biomass production of Ngoc Linh ginseng for production of ginsenosides [14-18]. Among different methods of micropropagation, somatic embryogenesis has been most studied in Ngoc Linh ginseng due to large quantity of somatic embryos produced and high frequency of plant regeneration. Truong et al. [11, 12] reported a potential of somatic embryogenesis and shoot or root morphogenesis from cell suspension culture of leaf explant-derived calli. In the study of Nhut et al. [9], a high-frequency somatic embryogenesis was also induced from the thin cell layers of main roots. However, since survival rate of plantlets after transferred to the nursery was quiet low, *in vitro* propagation of Ngoc Linh ginseng is still considered fractious

To date, no studies have been reported on the use of immature seeds of Ngoc Linh ginseng as an explant source for getting immature zygotic embryos (IZE) for in vitro culture.

In this study, we optimized a protocol for micropropagation of Ngoc Linh ginseng through somatic embryogenesis from IZEs. This protocol comprises five steps, including somatic embryogenesis induction, proliferation of somatic embryos, germination of somatic embryos, development of plantlets, and acclimatization of plantlets. The protocol will be useful for large-scale propagation of Ngoc Linh ginseng

Materials and methods. Immature seeds of Ngoc Linh ginseng were collected from fresh fruits, which were harvested from a research field in Ngoc Linh mountain (14°58′34″N and 107°54′41″E). The seeds were removed from capsules, and then sterilized by 1% NaClO for 15 min, treated with 500 mg/L streptomycin for 10 min followed by a brief immersion in 70% ethanol. Finally, the seeds thoroughly rinsed with sterile distilled water. After sterilization, IZEs were collected by removal of seed coat and $^2/_3$ endoderm that were used as explants for *in vitro* culture.

The collected IZEs were incubated on culture medium for induction of primary somatic embryos (SE). SH [19] and MS [20] basal media of different strength (full, half) supplemented with 7% sucrose were screened in combination with or without one of such plant growth regulators as 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2 and 0.5 mg/l), thidiazuron (TDZ) (0.2 and 0.5 mg/l), 1 mg/l α-napththaleneacetic acid (NAA) and 3 mg/l indole-3-butyric acid (IBA). The rate of explants induced primary SEs was scored after 10 weeks of culture.

First, the formed primary SEs were separated from embryogenic tissue masses. For that, embryogenic tissue masses were transferred to half-strength SH liquid medium supplemented with 3% sucrose followed by a ratio from $^1/_5$ to $^1/_{10}$ (mass/volume). The separated primary SEs were used for proliferation of secondary SEs. We investigated the effect of two type of culture media (liquid and solid media) containing half-strength SH medium with 3% sucrose and various combination of kinetin (0.1 mg/l) and TDZ (0, 0.05, 0.1 and 0.2 mg/l). The solid medium was supplemented with 7 g/l agar, and the liquid medium was prepared at a 1:20 explant:medium ratio with shaking at 100 rpm. The proliferation coefficient

was evaluated after 4 weeks of culture, and calculated as the ratio of fresh weight of secondary SEs after 4 weeks of culture to fresh weight of inoculated primary SEs.

For germination of the secondary SEs, solid SH medium supplemented with 1 mg/l NAA and 0.5 mg/l benzyladenine (BA) in combination with different concentrations of gibberellic acid (GA_3) (0, 1, 3, 5 and 7 mg/l) was tested. After a 10-week culture, the number of germinated SEs was recorded to compute the germination frequency.

After germination, the obtained small plantlets with roots and shoots were transferred to SH medium with 0.5 mg/l NAA, 1 mg/l BA and different concentrations of sucrose (2, 3, 4, 5 and 6%) for induction of microrhizomes. After 10 weeks, the plantlets were transferred to the same medium for an additional 10 weeks for initiation of dormant buds on the microrhizomes. After a total of about 5 months of culture, the plant biomass and length of shoots were measured. The presence of dormant buds was also observed.

Well-developed plantlets with microrhizomes and dormant buds were transferred to a research field at an altitude of 1,800 m. A two-level experiment was conducted. Before transplanting, the plantlets were soaked in 3% chitosan solution for 10 min. After that, the plantlets were transplanted to plastic bags containing various types of soil mixtures: (1) forest humus, (2) $^2/_3$ forest humus + $^1/_3$ sand, (3) $^1/_3$ forest humus + $^2/_3$ sand, and (4) $^1/_5$ forest humus + $^4/_5$ sand, and (5) $^1/_3$ forest humus + $^1/_3$ coconut fiber + $^1/_3$ perlite. After 3 weeks of planting, we scored percentage of plantlets sprouted, and the survival rate and different growth parameters of plants were assessed after 3 months of planting.

All experiments were conducted with 3 replicates and 20 explants for each treatment. All cultures were maintained in culture room at 23 °C with a 14 h photoperiod.

For comparison of the results, Tukey's multiple range test was applied using Statgraphics Centurion XVI (https://www.statgraphics.com/download-statgraphics-centurion-xvi).

Results. The effect of various basal media and different plant growth regulators on SE induction was observed. Somatic embryogenesis started initiating on IZEs on all examined media after 5 weeks of culture (Fig. 1, A). After 10 weeks of culture, of 10 media examined, SH medium with 7% sucrose exhibited maximal rate of SE initiation (44.33%), which was significantly higher than other basal media (i.e. $^1/_2$ MS, $^1/_2$ SH, and MS) and the media with plant growth regulators (i.e. 2,4-D, TDZ, NAA and IBA). After 10 weeks of culture on SH medium with 7% sucrose, we observed formation of somatic embryos at several developmental stages (Figs. 1, B-D). In addition, generation of abnormal somatic embryos were also observed, with high frequency on MS media.

After a brief shake of embryogenic tissue masses in liquid medium, SEs were definitely isolated from the parent explants which were then removed (Fig. 1, E). For proliferation of the obtained SEs, the solid and liquid $^1/_2$ SH media supplemented with 0.1 mg/l kinetin and different concentrations of TDZ were tested. After 3 weeks of culture, new embryos started generating surrounding the base of the primary SEs, then formed clusters of 4-12 single embryos which were observed at different developmental stages. The induced embryos were compact and light yellow within 4 weeks of culture (Figs. 1, F, G), and became greenish yellow when continuing the culture. The lowest efficiency of secondary embryogenesis was observed on $^1/_2$ SH medium with single addition of kinetin (0.1 mg/l) in both solid and liquid-shake cultures, with proliferation coefficient of 8.63 and 8.27, respectively. On the solid culture, coefficient of SE proliferation increased

gradually from 8.63 to 13.9 with the presence and increasing concentration of TDZ, especially, at 0.2 mg/l TDZ. Interestingly, secondary embryogenesis was significantly improved in the liquid-shake culture in comparison with the solid culture of the same medium composition. The highest proliferation coefficient (26.71) was achieved in liquid 1/2 SH medium with 0.1 mg/l kinetin and 0.1 mg/l TDZ.

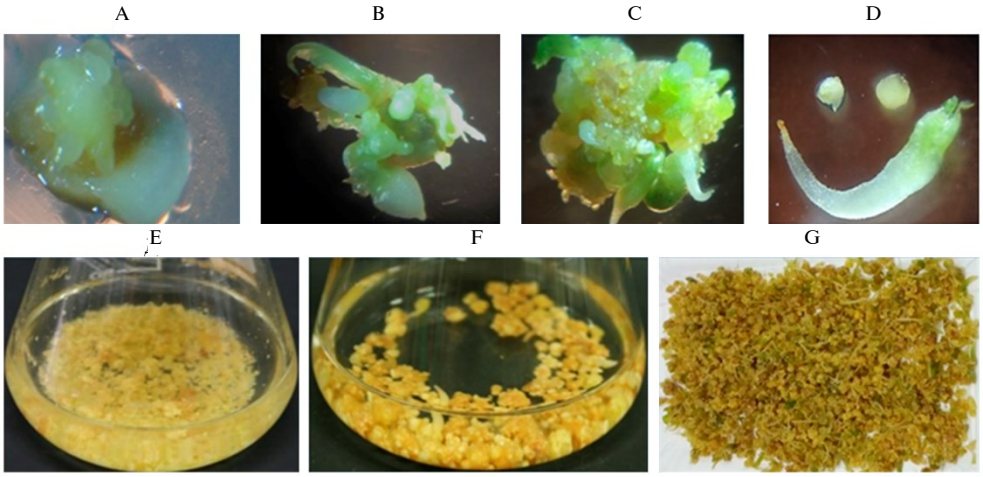

Fig. 1. Direct somatic embryogenesis in in vitro culture of immature zygotic embryos (A, B, C, D) **and proliferation of secondary somatic embryos** (E, F, G) **of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.):** A — somatic embryogenesis after 5 weeks of culture; B — somatic embryogenesis after 8 weeks of culture; C — somatic embryogenesis after 10 weeks of culture; D — primary somatic embryos at globular and bipolar stages; E — after isolation of primary somatic embryos; F — after 2 weeks of somatic embryo proliferation; G — after 4 weeks of somatic embryo proliferation

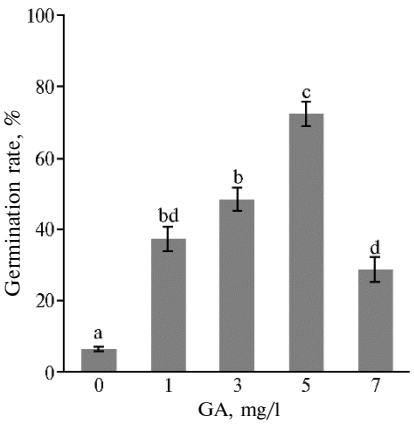

Fig. 2. Effect of GA_3 concentration on germination of somatic embryos of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) in in vitro culture. Differences between values marked with different letters are statistically significant according to Tukey's multiple range test at $p < 0.05$.

Germination of SEs occurred at very low frequency (6.33%) when inoculated on the GA_3-free medium (Fig 2). With GA_3 treatments, SEs rapidly germinated with an increase of frequency up to 72.28% on the medium with 5 mg/l GA_3. However, addition of higher concentration of GA_3 (7 mg/l) reduced the germination frequency of SEs by 28.67%.

The obtained results showed significant effect of sucrose on plantlet growth and microrhizome thickening (Table 1). Although on the medium with 2% sucrose, microrhizomes was thickened, but dormant bud was not produced. In higher concentrations of sucrose, the growth of plantlets was more intensive with greater plant biomass and plant height. Nevertheless, monitoring the experiment, we observed that, on media with 5% and 6% sucrose, leaf margins turned dry and whitish after 3 first days of culture; in next days, plants grew slowly; after 10 weeks of culture, induction of somatic embryos on microrhizomes was detected. The optimal sucrose concentration for growth of plantlets was 4%, on which plantlets was reached on average 1.60 g in biomass and 7.44 cm in height with visible microrhizome (about 5 mm) and dormant bud after approximately 5 months of culture.

1. Effect of sucrose concentration on the growth of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) plantlets in in vitro culture (*M*±SEM)

Medium	Sucrose concentration, %	Plantlet biomass, g	Plantlet height, cm	Presence of dormant bud
SH + 0.5 mg/l NAA + 1 mg/l BA	2	0.58±0.01[a]	4.35±0.03[a]	No
SH + 0.5 mg/l NAA + 1 mg/l BA	3	0.80±0.01[b]	6.12±0.06[b]	Yes
SH + 0.5 mg/l NAA + 1 mg/l BA	4	1.60±0.02[c]	7.44±0.09[c]	Yes
SH + 0.5 mg/l NAA + 1 mg/l BA	5	1.14±0.01[d]	6.24±0.06[b]	Yes
SH + 0.5 mg/l NAA + 1 mg/l BA	6	1.33±0.00[e]	5.62±0.05[d]	Yes

N o t e. SH — Schenk-Hildebrandt medium, NAA — α-napththaleneacetic acid, BA — benzyladenine.
[abcde] Values followed by different letters are significantly different according to Tukey's multiple range test at $p < 0,05$.

2. Effect of soil mixtures on acclimatization of micropropagated Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) plantlets (*M*±SEM)

Soil mixture	After 3 weeks of planting	After 3 months of planting		
	percentage of planlets sprouted, %	roots		survival rate, %
		number	length, cm	
Forest humus	10.00±4.08[a]	0.92±0.12[a]	0.49±0.00[a]	71.67±1.67[a]
$^2/_3$ forest humus + $^1/_3$ sand	38.33±6.39[bc]	2.58±0.14[b]	1.04±0.01[b]	73.33±1.67[a]
$^1/_3$ forest humus + $^2/_3$ sand	61.67±7.26[c]	5.05±0.20[c]	1.90±0.03[c]	91.67±1.67[b]
$^1/_5$ forest humus + $^4/_5$ sand	36.67±8.04[abc]	2.52±0.12[bd]	0.88±0.01[d]	80.00±5.77[ab]
$^1/_3$ forest humus + $^1/_3$ coconut fiber + $^1/_3$ perlite	20.00±6.07[ab]	2.00±0.10[d]	0.55±0.01[e]	70.00±2.89[a]

[abcd] Values followed by different letters are significantly different according to Tukey's multiple range test at $p < 0,05$.

Within 3 weeks of planting, the dormant buds started to sprout in the range from 10.00 to 61.67% depending on the types of soil mixture (Table 2).

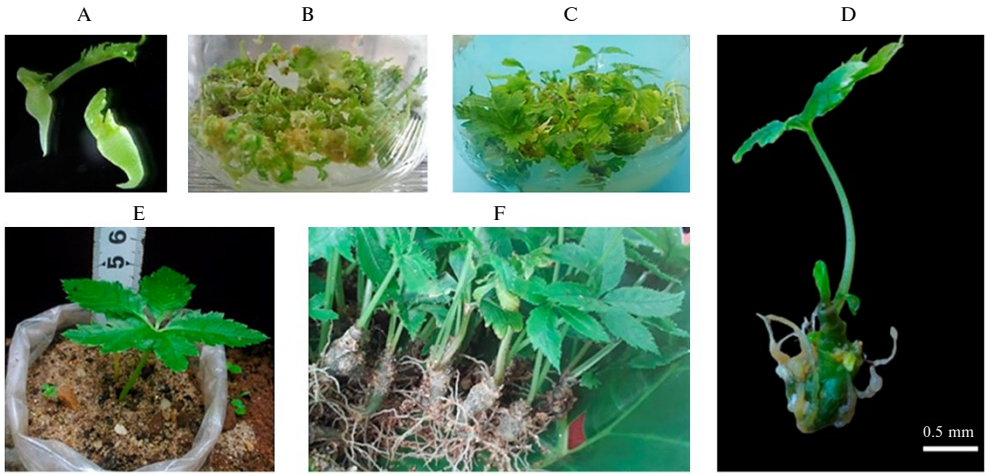

Fig. 3. Germination of somatic embryos and plantlet development in invitro micropropagation of Ngoc Linh ginseng (Panax vietnamensis Ha et Grushv.): A — germinating somatic embryos; B — after 4 weeks of culture on germination medium; C — after 10 weeks of culture on germination medium; D — plant with microrhizome thickened after culture on germination medium; E — plantlets after 8 weeks of planting; F — plantlets after 3 months of planting.

In next weeks, the sprouting continued to occur and was an indicator for predicting survival of micropropagated ginseng plantlets (see Table 2). As a result, the survival rate evaluated after 3 months of planting was directly proportional to the sprouting percentage at 3 weeks of planting. The tested soil mixtures were found to definitely influence the survival and growth of micropropagated plantlets. Plantlets were restarted in forest humus, in which sprouting percentage of plantlets was quiet low (10%) at 3 weeks of planting and reached 71.67% after 3 months with 0.92 roots of 0.49 cm length. Higher number of roots formed was obtained in the soil mixture containing $^1/_3$ forest humus + $^1/_3$ coconut fiber + $^1/_3$ perlite

(2.00 roots), followed by the soil mixture of $^1/_5$ forest humus + $^4/_5$ sand (2.52 roots) and the soil mixture of $^2/_3$ forest humus + $^1/_3$ sand (2.58 roots) (see Table 2). The maximal root growth (5.05 roots with an average length of 1.9 cm) as well as the maximal survival rate (91.67%) were recorded in the soil mixture containing $^1/_3$ forest humus + $^2/_3$ sand (Figs. 3, E, F).

The present report is the first tissue culture study using IZEs as initial explants to generate SEs from Ngoc Linh ginseng. In this study, IZEs were not completely separated from the endoderm but were cut together with a part of endoderm. It is because that IZE of Ngoc Linh ginseng and ginseng in general, are tiny [21, 22], and get injured easily when cutting off from the endoderm. After 10 weeks of culture, the best result for direct somatic embryogenesis was observed on SH medium with 7% sucrose without any plant growth regulators. Use of high concentration of sucrose (5%) for SE induction was also reported by [21-23] on cotyledons excised from immature or mature zygotic embryos of Korean ginseng. Similarly to [24, 25], addition of plant growth regulators resulted to indirect somatic embryogenesis. Interestingly, the highest rate (44.33%) of explants induced SEs in this study was recorded on SH basal medium, not on MS basal medium as identified in [22]. Moreover, SH basal medium induced formation of friable embryogenesis tissue masses, on which single somatic embryos were generated, meanwhile large number of somatic embryos emerged on MS basal medium was abnormal or conjoined, thus, leading to production of defective plantlets. In ginseng tissue culture, many studies have also reported high production of abnormal somatic embryos [23, 24, 26-28]. In in vitro plant propagation, it is important to produce morphologically normal plants, particularly from single somatic embryos in the case of this study. As that, we conducted harvesting of single primary SEs from formed embryogenesis tissue masses before proliferation of secondary SEs. The single primary SEs were easily isolated from the parent explants by a brief shaking in liquid culture. Types of culture and plant growth regulators both affected on proliferation coefficient of SEs that showed the best result in liquid culture on $^1/_2$ SH media supplemented with 0.1 mg/l kinetin and 0.1 mg/l TDZ. After 4 weeks of culture, primary SEs generated numerous new embryos, considered as secondary embryogenesis (see Figs. 1, E-G). Since formation of new embryos occurred asynchronous, the embryos obtained after 4 weeks of culture were at different stages of development, from globular to cotyledonary stages.

The frequency of SEs germinated was much greater on the media with addition of GA_3 than on the GA_3-free medium. In this study, we observed that, on the GA_3-free medium, the process of secondary somatic embryogenesis continued, leading to inhibiting the development of embryos. Whereas, on the media with addition of GA3, the embryo development occurred normally through globular, heart-shape, bipolar and cotyledonary stages, then proceeded to germination into plantlets (see Figs. 3, A, B). The best result, which gave the germination frequency of 72.28%, was obtained with 5 mg/l GA_3 (Fig. 3, C) as reported in [29]. It indicates that GA_3 stimulated germination of somatic embryos of Ngoc Linh ginseng. This finding is consistent with the results in other ginsengs reported by [23, 28, 30, 31]. After 10 weeks cultivated on germination media, plantlets with shoots of 2-3 cm height and microrhizimes of 3 mm diameter were obtained (see Fig. 3, D). This confirmed efficiency of the used medium, in which NAA and BA were supplemented with the aim of promoting the early induction of microrhizomes.

The growth of plantlets and microrhizomes was then enhanced on the media with different sucrose concentrations (2 to 6%) for approximately 5 months. Effect of sucrose on the growth of plantlets and root thickening in ginseng has been reported [32]. In the present study, the highest success was obtained using

the medium with 4% sucrose. After approximately 5 months of culture, dormant buds were produced in cultures supplemented with 3-6% sucrose. The presence of dormant bud plays an important role in the survival of *in vitro* ginseng plantlets when transferred to soil [32]. It can be explained as that, in the natural environment, roots begin growing after the first shoot emerged.

Before planting in soil, the plantlets were treated with 3% chitosan in order to control diseases [33-35]. In our study, without chitosan treatment, the proportion of micropropagation seedlings affected by root rot reached up to 90% (data not shown). Thus, chitosan treatment was applied to the plantlets before transplanting. We have found effect of soil mixtures on acclimatization of in vitro plantlets. The soil mixing forest humus with sand in the ratio 1:2 was significantly superior to the others due to desirable soil texture for potting in vitro plantlets. On the one hand, forest humus is enriched with nutrients, on the other hand, sand provides sufficient aeration and drainage. Using this soil mixture, the micropropagated plantlets reached a survival rate of 91.67%. The old foliage turned yellow and fallen off after 5-6 weeks of planting, but the plants grew well with new leaves and roots developed (see Figs. 3, E, F).

Thus, we have optimized the protocol for micropropagation of Ngoc Linh ginseng using somatic embryogenesis in vitro culture of immature zygotic embryos. The protocol includes induction of somatic embryogenesis, proliferation of somatic embryos, germination of somatic embryos, development of seedlings and acclimatization of seedlings. The frequency of direct somatic embryogenesis on Schenk and Hildebrandt (SH) basal medium with 7% sucrose was 44.33%. Secondary somatic embryogenesis with a proliferation coefficient of 26.71 was induced in liquid culture in $^1/_2$ SH medium with kinetin (0.1 mg/l) and thidiazuron (0.1 mg/l). SH medium with α-naphthylacetic acid (NAA, 1 mg/l), 6-benzyladenine (BA, 0.5 mg/l) and gibberellic acid (GA_3, 5 mg/l) stimulated the germination of somatic embryos and early formation of microrhisomes. As a result of micropropagation on SH medium with NAA (0.5 mg/l), BA (1 mg/l) and 4% sucrose, seedlings with a well-developed root system and a thickened microrhizome with a dormant bud were obtained. When micropropagated seedlings were acclimatized on a mixture of forest humus and sand in a ratio of 1:2, the survival rate was 91.67%. The micropropagation protocol we set-up allows the propagation process to be speed up and significantly improves multiplication coefficient and survival rate in field conditions. It is can be applied for large-scale production of Ngoc Linh ginseng.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 934-947
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Lighting for indoor gardening

UDC 635.63:581.132:631.588.5

doi: 10.15389/agrobiology.2021.5.934eng
doi: 10.15389/agrobiology.2021.5.934rus

THE INFLUENCE OF DIFFERENT LIGHT SOURCES ON PHOTOSYNTHETIC PERFORMANCE AND PRODUCTIVITY OF *Cucumis sativus* L. HYBRID TRISTAN F_1 IN AEROPONIC PHYTOTRON FACILITIES

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The authors declare no conflict of interests
Acknowledgments:
The work was carried out according to state order No. 0574-2019-0002.
Received August 25, 2021

Abstract

Global climate change and anthropogenic pollution of the environment pose serious problems for agricultural producers. Drought or flooding of fields, the emergence of new diseases and pests, and reduction of agricultural land pose serious problems in providing food for the growing population. Moreover, more than half of the world's population lives in cities, and this proportion is expected to increase to 67 % at 2050. To meet the growing needs of the population of megacities, new non-standard approaches and technologies are needed to increase the production of fresh vegetables, fruits, and berries. Vertical plant growing in the so-called "city farms" is a promising resource-saving method of compact multi-tier cultivation of various plants, especially greens, vegetables, medicinal and ornamental plants. The use of hydroponics and aeroponics allows a new type of agriculture that combines biotechnology, industrial architecture, design and successfully integrates into urban infrastructure. A significant increase in the production and yield of basic food vegetable crops, especially in "city farms" necessitates understanding needs of plants for light, mineral nutrition and other equally important factors, e.g., temperature, humidity, CO_2 content. Under the conditions of a phytotron that imitates a "city farm" model, we compared the effects of high-pressure sodium lamps (HPSLs) DNaT-600 traditional for greenhouse plant lighting and alternative light-emitting diode phytolamps (LEDs) on photosynthesis and, ultimately, the production process in *Cucumis sativus* L. Tristan F_1 hybrid as a cucumber crop usually cultivated in greenhouses. In treatments 2 and 3, LED irradiators and DNaT-600 lamps at a radiation intensity of 305 and 413 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and a temperature of 25 and 26 °C, respectively, provided formation of an effective photosynthetic apparatus capable of performing at an increase in light intensity up to 1200 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The LEDs of treatment 2 can serve as a single light source when growing cucumbers in a "city farm". These irradiators are characterized by a smaller proportion of blue (λ_{max} = 450 nm) and far red (λ_{max} = 730 nm) light and a larger proportion of red (λ_{max} = 660 nm) light in the spectrum. However, for early harvesting, the DNaT-600 lamps with the standard plant lowering method are preferable. The period of growing plants under DnaT-600 irradiation in the "city farm" simulating aeroponic phytotron with a limitation of the phytolamp height of 1.5 m without plant lowering, ended 12 days earlier than under LED irradiators. Nevertheless, the yield during the growing season was higher for DNaT-600 than for LED irradiators with the same energy consumption. The data obtained are helpful in the design and creation of modern biotechnological enterprises, such as vertical "city farms" for the food production and biotechnological enterprises for production of biopharmaceuticals.

Keywords: *Cucumis sativus* L., photosynthetic apparatus, LED phyto lamps, growth processes, aeroponic phytotron, city farms

The right choice of the light regime for growing plants, taking into account

their species characteristics, development stage, and physiological state, is important for obtaining products under photoculture conditions [1-3]. In this case, one of the main light sources is LED irradiators (LEDs) [4-6]. By using the programs for controlling the light conditions of growing, they allow to targetedly influence on the production process by accelerating or slowing down certain phases of plant growth and development [7-9].

Currently, LEDs with peak emission wavelengths in all spectrum ranges are commercially available. The combination of red and blue LEDs is most often used to ensure the growth and development of many plants, especially green crops [10, 11]. The use of these LED irradiators positively influences on the increase in the rate of photosynthesis and the accumulation of plant biomass [12, 13]. Red light spectrum (RS) range is important for the normal growth and development of plants, the formation of the photosynthetic apparatus and its further activity, the synthesis and accumulation of photoassimilates [6, 14, 15]. Blue light spectrum (BS) range is necessary for the formation of chloroplasts, accumulation of chlorophyll, opening of stomata, and photomorphogenesis of plants [16]. For different crops, the ratio of red and blue spectrum fluctuates in a significant range - from 2:1 to 9:1 [8, 17]. Besides RS and BS, other ranges should also be present in the irradiation spectrum of plants. Previously, the following ratio of the spectral regions of the irradiators was considered the most effective: 25-30% in the blue region (BS), 20% in the green light (GL), and 50-55% in the red light (RL) [18]. Such irradiation ensures the growth, morphogenesis, and productivity of plants. The presence of other spectral regions is important, i.e., a small fraction of ultraviolet (UV), far-red light (FRL) and infrared (IR) light. In a study of Qian et al. [2], UV light was used as a growth regulator in cucumber plants, the presence of UV radiation led to an increase in the degree of stomata opening, the rate of photosynthesis and transpiration. Pretreatment with UV-A (long wavelength radiation) produces robust tomato seedlings that are suitable for transport to production nurseries and greenhouses [19].

A change in the RL/FRL ratio can have a significant effect on plant morphology [20] and the activity of a number of physiological processes [21, 22]. When plants are illuminated with RL (λ_{max} = 660 nm), the stable form of phytochrome (Ph_r, phytochrome red) is converted into Ph_{fr} (phytochrome far red). An increase in the proportion of far-red light (λ_{max} = 730 nm) leads to the transformation of the Ph_{fr} back into the Ph_r form. As a result, various changes occurs, for example, lengthening of the hypocotyl, an increase in the length of internodes, and the distribution of assimilates.

The importance of certain areas of the spectrum is evidenced by studies [23-25], showing that a change in the maximum irradiation of plants even within the same spectral regime (red, green, blue, significantly affects the characteristics of the production process.

For different crops, the ratio of spectral irradiation ranges which are most favorable for growing plants is not the same [25]. Additional spatial restrictions for vertical plant growing arising in the context of multi-tier aeroponic “city farms” should also be taken in account [26]. Therefore, physiological processes that make it necessary to change the irradiation spectrum depending on the culture, optimization of the light regime for plant growing continue to be an area of primary focus [27, 28] and require further research.

In this work, we have shown that LEDs could be successfully used to regulate growth processes, but the energy efficiency of their industrial use remains questionable. In our study, irradiation with sodium lamps, the spectrum

of which is close to that of the sun, promoted the acceleration of plant growth and development more than irradiation with LEDs of the same power. An exception was one option, in which the combination of the spectral characteristics of the LEDs and temperature turned out to be optimal for the growth, development and formation of plant productivity, comparable to that when using high pressure sodium lamps (DNaT-600). It is worth mentioning that DNaT-600 has a high proportion of infrared radiation in the spectrum, which under conditions of "city farms" can lead to a heat shock to plants. This can be avoided by lowering into the gutters of the aeroponic-hydroponic installation (patents RU 88246 U1, RU 131569 U1).

The purpose of this work is a comparative study of the effect of LED irradiators (LEDs) of different spectral composition and intensity and high-pressure sodium lamps (DnaT-600) (at the same energy power of all irradiators) on the parameters of the production process in cucumber plants in aeroponic cultivation conditions according to the “city farm” model.

Materials and methods. The studies were carried out on cucumber (*Cucumis sativus* L.) plants of the Tristan F1 hybrid (Enza Zaden, the Netherlands). Hybrid Tristan F1 forms balanced plants of the generative type with stable (without pronounced interruptions) fruiting. The hybrid is demanding on the relative humidity of the air during all periods of development and fruiting. Fruits (length 22-25 cm, weight 260±20 g) are characterized by a very high uniformity and marketability, which is close to 100% [28].

Seedlings were obtained from previously prepared seeds (patents RU 180527U1, RU 2675932C1, RU 2708829C1) in an aeroponic installation developed by us (RU 199457U1) under the LEDs at a light intensity of 100±5 μmol photons $m^{-2} \cdot s^{-1}$ (16 h day/8 h night). On days 19-20 (the phase of 3-4 true leaves), the plants in holders were transferred to the phytotron and placed in the troughs of our aeroponic modules (patents RU 88246U1, RU 131569 U1) under the LEDs developed by us with different spectral characteristics (variants 1, 2, and 4 of the experiment) and under the DNaT-600 lamps (MASTER GreenPower 600W 400V, Philips, the Netherlands; variant 3 of the experiment). The phytotron room with a volume of 120 m^3 was divided into four identical compartments (30 m^3 in each variant of the experiment) using opaque reflective screens. The distance from the illuminators to the surface of the gutters (planting field) was 1.5 m (accounting for restricted location of illuminators height wise in the conditions of the “city farm”), the density of the placement of plants in the gutters of aeroponic modules was 4.8 per 1 m^2 (12 plants per variant).

The set ambient temperature regimes in the phytotron room corresponded to those recommended for the Tristan F_1 hybrid and depended on the phase of plant development and the cultivation technology. In all variants, the first 2-3 days after planting, the temperature remained low (20-22 °C), then they switched to a constant temperature regime 25±1 °C (day)/20±1 °C (night) with a photoperiod of 16 hours (day)/8 hours (night). The ambient temperature regime was maintained in an automatic mode using the air conditioning and ventilation systems, the root zone (21±1 °C) was maintained by cooling the nutrient solution in an automatic mode using a G 30 Polar device (UBC Group, China). The humidity in the phytotron room (75±5%) corresponded to the optimum for the hybrid. The concentration of CO_2 in the air was 420±22 μmol $CO_2 \cdot mol^{-1}$.

Temperature, relative humidity, and CO_2 concentration were monitored using an E + E EE244 wireless sensor (E + E Elektronik, Austria) which was integrated into the process control system in the phytotron. Spectral parameters

were monitored using an ASENSEtek PG100N spectrometer (UPRtek Corp., Taiwan).

The nutrient solution was prepared based on commercial fertilizers (Yara International ASA, Northway). The used fertilizer was Kristalon cucumber ($N_{14}P_{11}K_{31}$), calcium nitrate, magnesium nitrate with the concentration of the main fertilizer 1 g/l, 0.8 g/l $Ca(NO_3)_2$, 0.3 g/l $Mg(NO_3)_2$, the pH of the solution was corrected with orthophosphoric acid (chemically pure grade, OOO KhimMed, Russia). A finely dispersed aerosol obtained from the solution containers was supplied directly to the root zone using high-pressure pumps and special nozzles (each variant of the experiment had an individual nutrient solution supply scheme). Then the condensed solution flowed down the chute back into the containers. The periods of feeding the nutrient solution alternated with periods without feeding, during which aeration of the plant root system took place. The hybrid has a powerful and active root system, but, according to the originator (Enza Zaden, the Netherlands), it does not tolerate high salt concentrations, therefore, in the experiment, the optimal values of the conductivity of the nutrient solution were controlled (2.3±0.2 mS/cm, pH 5.8±0.2). We used ST320 pH electrodes, STCON3 conductometric electrodes (OHAUS Corp., USA), which were integrated into the plant cultivation process control system in the phytotron.

The intensity of photosynthesis was determined on day 10 of the growing and before the onset of the fruiting period on day 20 of the growing; on day 20, the parameters of the variable fluorescence of chlorophyll a (Chl a) were also measured. Measurements were carried out in the leaves of the 2nd and 3rd upper tiers on plants with 10-14-tiered leaves..

The activity of photosynthesis under LEDs and DNaT-600 lamps was assessed by the rate of CO_2 exchange of leaves using a portable infrared gas analyzer Lcpro+ (ADC BioScientific, Ltd, Great Britain) at a light intensity of 300 μmol photons $\cdot m^{-2} \cdot s^{-1}$, as well as at light saturation 1200 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The dependence of the photosynthesis rate on the light intensity was taken into account in the range from 0 to 1200 μmol photons $\cdot m^{-2} \cdot s^{-1}$ at a CO_2 concentration in the air of 420±22 μmol $CO_2 \cdot mol^{-1}$. For this, the level of light intensity was sequentially increased from 0 to 1200 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The light curve was fitted using the Prioul and P. Chartier model [29] with Photosyn Assistant software (http://www.ddsci.com/) [30].

To study the reactions of the light stage of photosynthesis, we used the method of variable fluorescence Chl a, which characterizes the activity of photosynthetic system II (PS II) [31]. Variable fluorescence was recorded using a portable PAM fluorimeter (PAM-Junior, Heinz Walz GmbH, Germany). Leaves were kept in the dark for 20 min, after which the level of minimum (F_o) and maximum (F_m) fluorescence was measured. The potential quantum yield of PS II was found as $F_v/F_m = (F_m - F_o)/F_m$, where F_v is variable fluorescence. The real quantum yield of PS II Y(II) was calculated by the formula $Y(II) = (F'_m - F_t)/F'_m$, where F'_m is the maximum fluorescence of Chl a in light-adapted samples, F_t is the stationary level fluorescence of Chl a in light-adapted samples. The values of the coefficient of non-photochemical quenching of fluorescence Chl a of PS II (NPQ) were determined by the formula: $NPQ = (F_m - F'_m)/F'_m$. The relative rate of electron transport through PS II was calculated as $ETR = Y(II) \times PPFD \times 0.5$, where PPFD is the flux density of quanta of photosynthetically active radiation (PAR).

Plants were grown in one stem, all side shoots were removed. To maintain

uniformity in planting, the plants were not lowered (unlike the technology commonly used in greenhouses). The position of the stem was kept strictly vertical to avoid mutual shading of the plants. The first harvest was obtained 38-42 days after planting. Fruits were harvested with a weight of about 220 g. The yield was uniform throughout the growing season and was greatest from the middle layer (at a height of about 80-100 cm).

Three cycles of plant cultivation were carried out. Since the general patterns did not differ in the series of experiments, the data for one cycle are presented (the average data for the three cycles are given for the yield). When determining the activity of photosynthesis and the variable fluorescence Chl a, the sample size (n) was 8, the analytical repetition of measurements in the experiments was 4-5 times. Statistical processing was performed using the Statistica Base software (StatSoft Inc., USA). The tables show the arithmetic mean values (M) with standard error (±SEM). The significance of the differences was determined by the Student's t-test at $P = 0.95$.

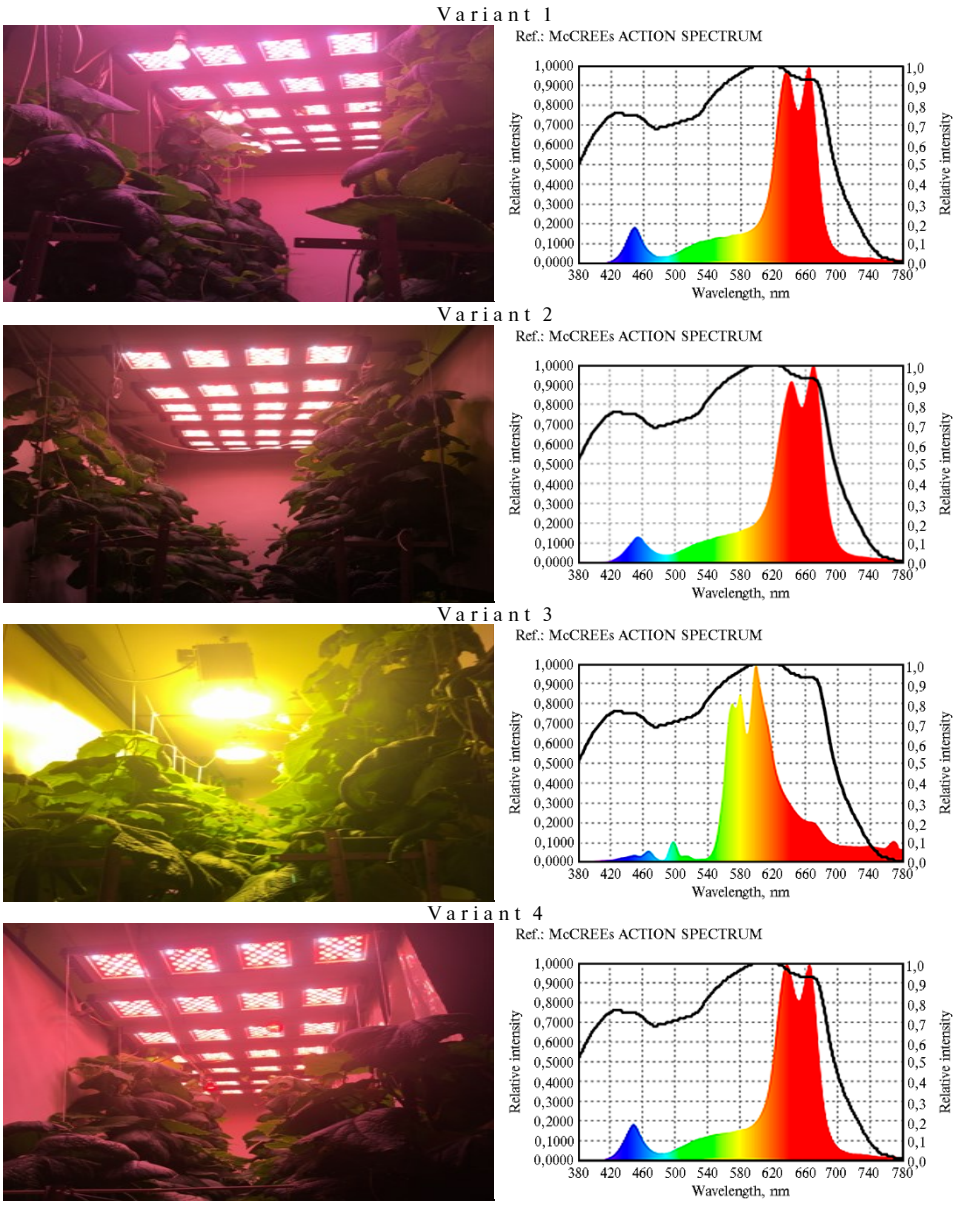
Results. The objective of this work was to assess the intensity of CO_2 gas exchange, the activity of the light stage of photosynthesis (according to the indicators of the variable fluorescence Chl a) and to determine the yield of cucumber plants grown for a long time by the aeroponics method in a phytotron under LEDs or DNaT-600 lamps with specified intensity and spectral composition of irradiation (variants 1-4 of experiment, Fig.).

According to the variants (1, 2, 3 and 4), the light intensity levels in the range of 400-780 nm were 193±7.0, 305±12.5, 418±47.6, 309±11 μmol photons $\cdot m^{-2} \cdot s^{-1}$, respectively (Table 1). The energy power of all irradiation sources was 1200 W, but LED irradiator in variant 1 was dimmed to 75% of the initial power (see Table 1). For LEDs, the spectra in variants 1 and 4 differed from the spectrum in variant 2 in the near infrared region of 701-780 nm (a decrease in irradiation by 12%) and in the region of 400-499 nm (an increase of 12%). As a result, in variants 1 and 4 the portion of blue light was slightly higher than in variant 2.

At a light intensity of 300 μmol photons $\cdot m^{-2} \cdot s^{-1}$, the rate of photosynthesis as per variants was 4.1±0.2, 5.2±0.3, 5.6±0.5, and 4.7±0.4 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively. At 1200 μmol photons $m^{-2} \cdot s^{-1}$, plants in variants 2 and 3 showed higher gas exchange rates (7.9±0.5 and 7.6±0.6 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$) than plants in variants 1 (75% of the initial LED power) and 4, the 4.4±0.3 and 5.1±0.4 μmol $CO_2 \cdot m^{-2} \cdot c^{-1}$, respectively. Analysis of the light curves of photosynthesis (Table 2) assesses the balance between the absorption of CO_2 and its release during dark respiration.

In all variants of the experiment, the plants had a positive carbon dioxide balance. In absolute terms, the difference between the absorption and release of CO_2 in the processes of photosynthesis and dark respiration was 3.1, 7.0, 5.1 and 3.1 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ as per the variants. According to the gas exchange data, it could be expected that the productivity of plants in variants 2 and 3, as a result, would be higher than in the other two variants of the experiment.

Other indicators of the light curve of photosynthesis provide additional information about the efficiency of the use of light energy by plants. In variants 1, 2 and 3, the quantum yield of photosynthesis turned out to be below 0.04, although in natural conditions of plant growth the average value is 0.04-0.07 [31]. Only in variant 4, the quantum yield was equal to 0.046 (see Table 2). We associate the observed low values of the quantum yield with the formation of a large leaf surface in plants.



Spectral characterization of LEDs and DNaT-600 lamps used for growing cucumber (*Cucumis sativus* L., hybrid Tristan F_1) plants by aeroponic technology in a phytotron facility. For the spectral composition of phyto-luminaires, see the Table. 1.

1. Energy power (P) and spectral characterization of phyto-luminaires for growing cucumber (*Cucumis sativus* L., hybrid Tristan F_1) plants by the aeroponic technology in a phytotron facility as per test variants

Spectral range, nm	Light intensity for each variant, μmol photons $m^{-2} \cdot s^{-1}$			
	1 (P = 945 W)	2 (P = 1200 W)	3 (P = 1200 W)	4 (P = 1200 W)
PPFD (400-700)	193,00	304,86	412,98	309,02
PPFD IR (701-780)	7,227	12,432	47,650	10,955
PPFD R (600-700)	153,03	245,59	207,05	245,94
PPFD G (500-599)	28,224	43,831	190,58	44,329
PPFD B (400-499)	11,748	15,437	15,348	18,749
PPFD UV (380-399)	0,0579	0,1420	0,6102	0,1087
Total PPFD (380-780)	200,28	317,34	461,43	320,08

N o t e. PPFD — photosynthetic photon flux density.

2. Analysis of light curves of CO_2 gas exchange in leaves of cucumber (*Cucumis sativus* L., hybrid Tristan F_1) plants in growing by the aeroponic technology in a phytotron facility depending on the light and temperature conditions as per test variants ($n = 8$ with 5-fold analytical repeatability of measurements, $M \pm SEM$)

Parameter	Variant 1, 75 %, 23 °C	Variant 2, 100 %, 25 °C	Variant 3, 100 %, 26 °C	Variant 4, 100 %, 24 °C
Maximum CO_2 absorption rate, μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$	4.4±0.4[a]	7.9±0.5[b]	7.6±0.6[b]	5.1±0.5[a]
Dark respiration rate, μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$	–1.3±0.3[a]	–0.9±0.2[a]	–2.5±0.3[b]	–2.0±0.4[b]
Quantum yield of photosynthesis	0.039±0.012[a]	0.024±0.003[b]	0.036±0.006[a]	0.046±0.016[c]
Light intensity at saturation of the light curve of photosynthesis, μmol photons $\cdot m^{-2} \cdot s^{-1}$	144±11[a]	363±17[b]	278±17[c]	423±19[d]
Light compensation point, μmol photons $\cdot m^{-2} \cdot s^{-1}$	32±7[a]	36±5[a]	69±6[b]	153±8[c]

N o t e. The percentages of the initial energy power of the irradiators (1200 W) and the actual temperatures that were set in the phytotron compartment, depending on the type and used power of the irradiator are indicated. Characteristics of phytolamps by variants of the experiment, see Figure, Table 1.

[a, b, c, d] The mean values in a row, marked with the same letter, do not differ statistically significantly at $p \le 0.05$.

In variants 2 and 4, the light intensity at saturation of the light curves of photosynthesis was higher (363 and 423 μmol photons $\cdot m^{-2} \cdot s^{-1}$, see Table 2) compared to the irradiation intensity during cultivation (305 and 309 μmol photons $\cdot m^{-2} \cdot s^{-1}$, see Table 1). In these variants, Chl a works more efficiently; therefore, the rate of dark reactions of photosynthesis in the leaves of the upper tiers did not become a limiting factor.

The maximum rate of photosynthesis at the plateau of the light curve in plants grown under LEDs in variant 1 was the lowest in comparison with other variants. The saturation of the photosynthesis rate curve began at a light intensity of 144±11 μmol photons $\cdot m^{-2}$ s^{-1} (see Table 2) which is lower than the light intensity during plant growth (193.00 μmol photons $\cdot m^{-2} \cdot s^{-1}$, see Table 1) and is apparently associated with the limitation of the rate of dark reactions of photosynthesis.

Analysis of the activity of light reactions of the photosynthetic apparatus by the parameters of variable fluorescence shows (Table 3) that the maximum quantum yield of the photochemical reaction in PS II (F_v/F_m) was comparable in plants in variants 2, 3, and 4 and turned out to be slightly higher in variant 1 with a reduced light intensity. In plants grown under LEDs, the real quantum yield Y(II) of the primary photochemical reaction of PS II in all variants was higher in comparison with plants under DNaT-600, especially variants 1 and 4.

3. Variable leaf fluorescence parameters of cucumber (*Cucumis sativus* L., hybrid Tristan F_1) plants in growing by the aeroponic technology in a phytotron facility depending on the light and temperature conditions as per test variants ($n = 8$ with 4-fold analytical repeatability of measurements, $M \pm SEM$)

Parameter	Variant 1, 75 %, 23 °C	Variant 2, 100 %, 25 °C	Variant 3, 100 %, 26 °C	Variant 4, 100 %, 24 °C
Fv/Fm	0.782±0.007[a]	0.744±0.005[b]	0.741±0.003[b]	0.743±0.005[b]
ETR	32.6±2.0[a]	40.9±3.1[b]	51.0±3.4[b]	31.8±2.3[a]
Y(II)	0.409±0.010[a]	0.342±0.006[b]	0.260±0.007[c]	0.467±0.010[d]
NPQ	1.020±0.100[a]	0.812±0.080[b]	1.019±0.110[a]	0.533±0.060[c]

N o t e . Fv/Fm — potential quantum yield of the photosystem II (PS II), ETR — relative electron transport rate through PS II, Y(II) — actual quantum yield of PS II, NPQ — coefficient of non-photochemical quenching of PSII chlorophyll a fluorescence. The percentages of the initial energy power of the irradiators (1200 W) and the actual temperatures that were set in the phytotron compartment, depending on the type and used power of the irradiator are indicated. Characteristics of phytolamps by variants of the experiment, see Figure, Table 1.

[a, b, c, d] The mean values in a row, marked with the same letter, do not differ statistically significantly at $p \le 0.05$.

The electronic transport rate (ETR) was slightly lower under LEDs compared to DNaT-600 lamps. A higher rate of electron transport in plants under DNaT-600 lamps could lead to an increase in the synthesis of high-energy equivalents (in particular, ATP NADP-H_2), providing high rates of CO_2 absorption both

at a radiation intensity in the PAR region of 412.98 μmol photons · m^{-2} · s^{-1} (see Table 1) and at high irradiation intensity (1200 μmol photons · m^{-2} · s^{-1}). In the variant 4 (under LEDs), a decrease in non-photochemical quenching (NPQ) was observed, which characterizes a decrease in thermal dissipation when light energy is used not for photosynthetic processes, but for maintaining the proper rate of other biochemical reactions.

4. Growth parameters of cucumber (*Cucumis sativus* L., hybrid Tristan F1) plants in growing by the aeroponic technology in a phytotron facility depending on the light and temperature conditions as per test variants ($N = 3$, $M \pm$SEM)

Parameter	Variant 1, 75 %, 23 °C	Variant 2, 100 %, 25 °C	Variant 3, 100 %, 26 °C	Variant 4, 100 %, 24 °C
Plant height, cm/number of leaf tiers	88/18	119/21	148/27	101/19
Yield, kg/m^2:				
on day 43 of growth	16.6±1.0[a]	21.5±1.1[b]	24.7±1.1[c]	17.6±1.2[a]
on day 55 of growth	19.4±1.1[a]	27.3±1.3[b]	0	20.5±1.1[a]
Average yield increase in additional (compared to control) growing season, %	14.4[a]	26.9[b]		16.5[a]
Duration of productivity period, days	55	55	43	55
Temperature difference vs control	–3	–1		–2
Lagging behind control in terms of the first harvest of marketable harvest, days	13	5		7

N o t e. In each variant, the yield for three cultivation cycles was assessed for 36 plants. Variant 3 (DNaT-600 lamps) was a control. The percentages of the initial energy power of the irradiators (1200 W) and the actual temperatures that were set in the phytotron compartment, depending on the type and used power of the irradiator are indicated. Characteristics of phytolamps by variants of the experiment, see Figure, Table 1

[a, b, c] The mean values in a row, marked with the same letter, do not differ statistically significantly at $p \leq 0.05$.

With high activity of the photosynthetic apparatus of leaves under DNaT-600 lamps, one could expect an increase in the rate of growth and development of plants. Indeed, when growing plants in aeroponic installations with limited height (according to the "city farm" model with a trellis height of 1.2 m), plants under DNaT-600 lamps significantly outpaced plants in other variants both in growth rate and in the onset of the fruiting phase (Table 4). Conventionally, the fruiting process in the experiment under DNaT-600 lamps can be divided into three stages. The first stage is rapid growth, development, and advancement in the formation of fruits in comparison with other variants where LEDs were used; the second stage is active uniform fruiting; the third - a slowdown in fruiting and a stop of the growing season due to the fact that, in the given spatial conditions, the plants have reached their maximum height.

The rapid growth and development of plants under DNaT-600 resulted in an earlier first harvest (by 13, 5 and 7 days, respectively) (see Table 4) than with LED irradiation, and also to an advance of the first maximum of harvest for variant 3 compared to variants 1 and 4 by 10 and 7 days, respectively.

The vegetation and harvesting periods with DNaT-600 irradiation was 43 days (the growth stopped, since the plants reached the maximum stipulated height of the trellis). By this time, under DNaT-600 irradiation, the total yield (see Table 4) exceeded that in all variants with LED irradiators, i.e., by 32.8% compared to variant 1, by 13.0% to variant 2, and by 28.7% to variant 4. To obtain additional data, we extended the observation of plants that retained their viability under LED irradiator (variants 1, 2 and 4) by 12 days (until the onset of a pronounced slow-down of the production process on day 55). The yield increase was 14.4% (variant 1), 26.9% (variant 2), and 16.5% (variant 4) compared to the yields for these variants at the date of completion of plant vegetation under DNaT-600 (variant 3). Only then (that is, in terms of the total output for 55 days), the yield in variant 2 exceeded that in variant 3 (DNaT-600) by 10.5%; in other variants (1 and 4), the total yield of marketable products for 55 days was still less than that of plants under DNaT-600 for 43 days. When comparing the cost of electricity and the

resulting increase, the advantage of a faster plant growth with DNaT-600 irradiation is obvious The data obtained are comparable with the total productivity of this hybrid in industrial greenhouses, where the growing season, according to the originator, usually lasts 90-110 days, and the yield is 35.7-50.0 kg/m^2.

The reason for the shortened growing season and the corresponding decrease in yield under DNaT-600 lamps is associated with the physiological aging of plants [32], as well as with an excess of IR radiation. This factor must be regarded when growing plants under DNaT-600 lamps in "city farms" (that is, with height restrictions). Note that the aeroponic installation and technology developed by us, in this case, have an advantage over growing on substrates. The design of the aeroponic installation allows the stems to be lowered, to place them inside the aeroponic trough, which contributes to an increase in the rooting volume and rejuvenation of vegetative plants. However, in our experiment, when using this technique, the DNaT-600 lamps would have an additional advantage, which would affect the objectivity of the comparison of the DNaT-600 and LED irradiators.

It is worth noting that with the general automatic regulation of the temperature in the phytotron, its own temperature gradient was established in each compartment, depending on the type and power of the irradiator. This property of the luminaires affects the entire production process, so it was decided not to level the actual temperature difference. The ratio of thermal and light energy for LED and sodium lamps was different. According to data of MechaTronix Co., Ltd. (Netherlands), in LED phyto-lighting 35% of the energy is converted into convection heat, 15% is emitted in the form of radiation heat, and only 50% falls on the light energy in the PAR region within the given spectrum boundaries. In phyto-luminaires with DNaT-600 lamps, the efficiency in the PAR region is 34%, 55% of the energy is emitted in the form of radiation heat, and 11% in the form of convection heat [33, 34]. This factor must also be regarded when designing phytotrons for "city farms" to create the necessary temperature conditions.

Comparison of data on the yield and wavelength distribution in the spectra of LED irradiators (see Fig.) shows that an increase in the proportion of RL in the wavelength range 600-700 nm led to an increase in plant productivity. In terms of radiation intensity in this area, the LED irradiators in the 2nd variant were superior to DNaT-600 lamps by 40 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. In addition, the maximum intensity of the luminous flux inside the RL of the spectral region for the irradiators in variant 2 is at λ_{max} = 660 nm in contrast to the DNaT-600 lamps which have a maximum at 610 nm. That is, LEDs were optimized for the red spectrum at wavelengths λ = 630-680 nm (in the region of maximum absorption of chlorophyll).

The differences that we observed between the variants of the experiment can be associated with a longer high activity of the photosynthetic apparatus with a slow increase in the leaf area and plant growth in general. A similar pattern was described earlier in the work of Chermnykh et al. [35] who showed that in cucumber plants, leaves with a relatively low growth rate are able to carry out active photosynthesis for a long time (in contrast to leaves which quickly reach their maximum size). Our data indicate that plants grown at a higher PAR (variants 2 and 3) and a temperature optimal for the growth of the Tristan F_1 hybrid more efficiently use high-intensity light than plants in variants 1 and 4 (see Table 2) at temperatures below the optimum for the hybrid under study. These results make it possible to assess the possibility of using LEDs in the formation of irradiation regimes for plants in cultivation facilities of various types. With relatively comparable energy costs of DNaT-600 and LEDs, the latter benefit from the duration

of use in the process of growing plants [36]. However, we note that the payback of the LED in comparison with the DNaT-600 is achieved much later and there is generally no correct justification of the economic efficiency of the LEDs. It is reported [37] that the long term use of LED irradiators led to a decrease in energy costs (up to 70%) compared to traditional light sources, in particular, when growing cucumber seedlings [38] and seedlings of ornamental crops [39]. According to other authors, due to high capital costs, five-year energy costs per mole of photons produced are 2.3 times higher for LED irradiators. For both technologies, long-term costs are low as compared to the cost of electricity consumed [40].

Comparison of variant 2 with variants 1 and 4 shows that even small changes in the LED spectrum lead to significant changes in growth and production processes (see Table 4). LEDs are characterized by a smaller portion (by 12%) blue (λ= 450 nm) and a larger (by 12%) far red (λ = 730 nm) light (see Table 1). Also, in variant 2, the red part of the spectrum with λ = 660 nm is also 12% higher than that of the red one with λ = 630 nm (see Fig.). The LEDs we used in variant 2 could be effectively used as the only light source when growing cucumber plants in closed premises.

However, further research is needed to better understand how plants respond to changes in the spectral composition of light in vegetation structures (greenhouse, phytotron, "city farm") and how to adjust the intensity and spectrum of light in order not only to speed up production processes with a quality crop, but also to optimize energy consumption. The energy costs make up to 35-40% in the cost of the product, sometimes even more, depending on the region. Although today LEDs for growing plants are not inferior in efficiency to traditional lamps DNaT-600, it is still far from the creation of "ideal" phytolamps. Further research is needed to determine the regulatory role of all PAR sites, the effect of near infrared light (in addition to a single LED light source) on growth, morphology, fruit quality and, in general, on the production process in cucumber plants.

Thus, in cucumber plants, at 305 and 413 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (variants 2 and 3, the LED and DNaT-600 lamps, respectively) and temperatures of 25 and 26 °C, a photosynthetic apparatus is formed that can work effectively when the light intensity is increased to 1200 μmol photons $\cdot m^{-2} \cdot s^{-1}$. In variants 2 and 3 as compared to variants 1 and 4 (LEDs), high values of the CO_2 balance (photosynthesis—respiration) were revealed. When using LEDs, the real quantum yield Y(II) of photosystem II in the light is higher, and the ETR is slightly lower than for DNaT-600 lamps. In variants 2 and 4, the coefficient of non-photochemical quenching of fluorescence (NPQ) decreased. This elucidates the effects of different parts of the light spectrum on cucumber plants, the slowed growth of plants under LEDs and explains the greater yield during active fruiting period under DNaT-600 lamps. When plants are irradiated with DNaT-600 (variant 3), a leaf surface is formed faster and plant growth in height is accelerated. DNaT-600 lamps also provides earlier yielding of the Tristan F_1 hybrid plants. In an aeroponic phytotron simulating a "city farm" conditions with a 1.5 m height limitation of the lamp position, the use of DNaT-600 lamps leads to a shorter growing period, by 12 days (without plant lowering), than under the LEDs with a higher total yield than under the LEDs. Plants grew more slowly under LEDs, which made it possible to extend the growing season by another 12 days (until a visible decrease in productivity). During this period, the optimal distance between the top of the plants and the lamp was established (about 0.3 m) which contributed to high fruiting. Ultimately (taking into account the additional vegetation time), the LEDs in variant 2 provided a higher yield than the DNaT-600, but the energy consumption was also higher. Continuation of

these studies is necessary to determine the role of different regions of photosynthetically active radiation and the influence of other spectral regions (for example, near infrared and UV-A radiation) on the production process and the quality of marketable products in cucumber plants. This is necessary to use the LED as the only light source, especially when designing and creating "city farms".

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 948-957
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Spectroscopic techniques

UDC 633.15:581.192:58.088

doi: 10.15389/agrobiology.2021.5.948eng
doi: 10.15389/agrobiology.2021.5.948rus

USING INFRARED SPECTROSCOPY AND RAMAN SPECTROSCOPY TO EVALUATE THE CONFORMATION OF BIOMOLECULES IN MAIZE (*Zea mays* L.) LINES

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The authors declare no conflict of interests
Acknowledgements:
Supported by GVM from Russian Science Foundation (grant No: 19-79-30062) as well as the Interdisciplinary Scientific and Educational School of Moscow University "Molecular Technologies of Living Systems and Synthetic Biology"
Received July 15, 2020

Abstract

Currently, there are few non-invasive methods that allow you to control the content and conformation of molecules in plant cells and tissues not only in the laboratory but also in the field. Infrared microscopy and Raman spectroscopy (IR and Raman spectroscopy) are actively used to analyze the role of molecules of certain substances in crop breeding. Using methods of vibronic spectroscopy, we investigated changes in the content and conformation of chloroplast molecules of various maize lines. Using infrared (IR) spectroscopy (3500-3000 cm^{-1}) it was found that in the chloroplasts, the proportion of vibrations of OH-groups and intramolecular and intermolecular H bonds is maximum for the maize line ZPPL 186, and vibrations of NH-groups of amides (proteins) is minimal for ZPPL 225. It has been proven that ZPPL 186 chloroplasts are characterized by the maximum proportion of stretching vibrations from alkane molecules, carboxylic acids (region 2920-2860 cm^{-1}) and deformation vibrations of aromatic structures (band at 1000 cm^{-1}), and for the line M1-3-3-sdms, the fraction of stretching vibrations of O=C=O bonds (band at 2300 cm^{-1}) is characteristic. Using Raman spectroscopy (ranges of 1250-500 cm^{-1} and 1535-1400 cm^{-1}), it was found that differences in the chloroplasts of different maize lines are associated with changes in the conformation of chloroplast carotenoid molecules (rather than cellulose molecules). It was found that in two samples (except ZPPL 225), carotenoid molecules are in the 15-trans form with different conformation of the polyene chain. We note that the conformation of carotenoids of the ZPPL 186 line is characterized by a minimum amount of rotation outside the plane of the polyene chain and has more pronounced vibrations of the lateral CH_3-group. It was assumed that carotenoids of leaf chloroplasts of various maize lines lack interactions with aromatic amino acids of proteins. According to the authors, the combined use of IR and Raman spectroscopy of the leaf chloroplast fraction can be recommended for monitoring the content and conformation of biomolecules in maize breeding.

Keywords: *Zea mays* L., inbred line, leaf, chloroplast, Raman spectra, infrared spectra, conformation changes, carbohydrates, carotenoids, proteins, maize breeding

The growth of the world's population increases the requirements for yields

and profitability of agricultural products, which can be achieved through fundamental research that allows the development of effective analytical methods for use in plant breeding [1-3]. Currently, there are few non-invasive methods that allow you to control the content and change in the conformation of molecules of bioactive organic compounds in plant cells and tissues not only in laboratory but also in the field. Infrared microscopy and Raman spectroscopy (IR and Raman spectroscopy) are actively used to analyze the role of molecules of certain substances in crop breeding [3-6]. In most cases, measurements using these methods can be carried out in the field directly on plant tissues, as well as on fractions isolated from the plant material. Both methods of vibronic spectroscopy make it possible not only to record spectra, the bands of which characterize the content and conformational changes of certain molecules [7-9], but also to distinguish plant genotypes based on these data [1, 2]. The ability to quickly monitor the amount and physicochemical state of bioactive organic compounds and cellular compartments (for example, carotenoids and chloroplasts) in plant leaf homogenates and to identify the correlation of these parameters with economically significant traits would make it possible to use the parameters recorded by spectroscopy as markers for assessing the agronomic efficiency of the studied forms in plant breeding. In addition, these methods can be used in the manufacturing industry to quickly check the quality of incoming raw materials, as well as for control in continuous production [10-12]. Based on the analysis of IR spectra in plants, additional characteristics were obtained not only of the structure of various molecules, but also modifications of their conformation (by changing the proportion of characteristic vibrations of chemical bonds in molecules) [13-15].

Unlike IR spectroscopy, Raman spectroscopy makes it possible to monitor changes in the structure of molecules in cells and tissues without drying the preparations [3, 12, 16-18]. Using this method, the features and differences in the distribution of protein and pigment molecules in plant cells in different hybrids and lines of maize are described. It is worth noting that this approach makes it possible to reveal the presence of a positive correlation between the change in chlorophyll fluorescence in chloroplasts and the content and structure of carotenoid molecules in whole leaves of maize lines and hybrids [3, 18].

It is obvious that the formation of a methodology for studying the functional state of a plant using spectral methods will reveal new molecular mechanisms that can be used in the breeding of maize.

In this work, it has been proved that the combined use of IR and Raman spectroscopy of a plant leaf can be recommended as a minimally invasive method for monitoring the content and possible differences in the conformation of biological molecules when testing lines and hybrids of maize.

The aim of this work was to comprehensively analyze changes in the content and conformation of protein, carbohydrate and pigment molecules in leaf chloroplasts in maize inbred lines by IR and Raman spectroscopy.

Materials and methods. Maize (*Zea mays* L.) lines ZPPL 186, ZPPL 225, and M1-3-3-sdms (originated by Maize Research Institute, Zemun Polje, Belgrade, Serbia) were used in the study. The lines possess high grain quality, yields and are adapted to cultivation technologies [1, 3, 12]. Thirty seeds of each line (weighing 313±9, 382±17 and 196±5 mg, respectively), after treatment with hydrogen peroxide for 30 min and washing with water, were incubated in a Petri dish at 22 °C with constant non-contact wetting until the appearance of roots with a length of at least 5 mm. The germinated seeds were placed in the ground and grown at 16-hour day length until the three-leaf stage (laboratory test). The quality of the seeds was assessed by germination rate (the ability of seeds to germinate and give normally developed seedlings under certain conditions within the accepted time frame; GOST 13056.6-75) and

germination energy (the ability of seeds to germinate in a shorter period of time, from 1 to 15 days; GOST 13056.6-75).

To isolate chloroplasts, cooled (0-4 °C) leaves (5 g portions, samples were kept in a polyethylene bag or wet filter paper) were separated from the veins, chopped with scissors and homogenized at 0-4 °C three times (10 s each) in a buffer (0.04 M sucrose, 20 mM Tris-HCl, pH 7.8, 35 mM NaCl, 1 mM EDTA) chilled to 4 °C. The homogenate was filtered through four layers of nylon and centrifuged (1500 rpm, 5 min, 0-4 °C). The supernatant was poured into precooled tubes and centrifuged again (5000 rpm, 44 °C for 10 min). The supernatant was discarded; the resulting pellet was resuspended in 5 ml of isolation medium. Glycerin (30% of the volume obtained) was added and frozen at –73 °C. Before recording the Raman and IR spectra, the suspension was thawed and diluted with a buffer (15 mM NaCl, 400 mM sucrose, 50 mM Mes-NaOH, pH 6.5) at a ratio of 10 μl of suspension per 5 ml of buffer [19, 20].

Cellulose was isolated from maize leaves according to the description [21] using 3% sodium hypochlorite solution, 5% hydrogen peroxide solution and a mixture of 3% sodium hypochlorite solution and methanol.

Raman scattering of carotenoids in leaf chloroplasts was recorded using a DFS 24 Raman spectrometer (JSC LOMO, Russia) with a laser (Ciel, Eurolaser GmbH, Germany) (wavelength 473 nm), registration system MORS 1/3648 (LLC MORS, Troitsk, Russia) based on a linear CCD matrix TCD1304DG (Toshiba, Japan) with an LPO2-473RS-50 filter (Semrock, USA). The laser power on the sample was 3 mW, the signal registration time was 10 s [23].

Fourier-transform infrared spectroscopy (FTIR spectrometry) in the range of 400-4000 cm^{-1} was carried out using an IR-Prestig 21 IR spectrometer (Shimadzu Corp., Japan) with a measurement step of 4 cm^{-1}. Before the experiment, the leaf homogenate suspension (see above) was thawed and diluted with buffer (15 mM NaCl, 400 mM sucrose, and 50 mM Mes-NaOH, pH 6.5; 10 μl suspension per 5 ml buffer), dried and rolled into a tablet with bromide potassium (KBr) (mixing ratio 1:50) [12, 23].

The results were statistically processed using Microsoft Excel 2013 (Microsoft Corp., USA) and Statistica v.10 (StatSoft, Inc., USA). The primary processing of the Raman and IR spectra was carried out using the Origin Pro 2017 package (OriginLab Corp., USA). Statistical hypotheses were tested using a nonparametric Kruskal-Wallis H-test for a set of independent variables at the significance level of $p = 0.05$ ($n = 10$).

Results. The objectives of our study included recording the IR spectra of chloroplasts in three maize lines and analyzing the differences between them for protein and carbohydrate molecules, as well as performing additional Raman spectroscopy to detect changes in carotenoids contained in chloroplasts. In the IR spectra of chloroplasts in the range 4000-400 cm^{-1}, bands were found due to vibrations of bonds in the molecules of a number of organic compounds, including cellulose, proteins, carbohydrates, ethers, phenols (Fig. 1).

It was found that in the IR spectrum of chloroplasts in the region of 3700-3100 cm^{-1} there were bands of vibrations of hydroxyl groups, and in the region of 1500-900 cm^{-1} to vibrations of C–H, C–O and O–H groups of glycosidic bonds and glucopyranose ring of cellulose molecules [13-15]. Thus, the 3340 cm^{-1} band of the IR spectrum characterizes the O–H and CH_2–OH vibrations of cellulose bonds, the 2900 and 1374 cm^{-1} bands are characteristic of the C–H group bending vibrations, and the 1170 and 1059 cm^{-1} bands of C–O–C and C–OH groups, respectively. In the IR spectrum of the leaf chloroplasts, there was a band at 3414 cm^{-1} caused by stretching vibrations of hydroxyl groups and a band at 2904 cm^{-1} which characterizes the C–H vibrations of methylene and methine

groups of molecules. The IR spectrum also revealed a band at 1654 cm^{-1}, due to H−O−H vibrations of crystallized water, a band at 1375 cm^{-1} caused by deformation vibrations of the C−H bond, and a band at 1317 cm^{-1} of bending vibrations of CH_2-groups. The IR band at 1165 cm^{-1} is the stretching vibrations of the C−O −C bond, but it is also referred to as the bending vibrations of C−O or O−H in the C−OH groups. The IR band at 1085 cm^{-1} is the vibrations of the C−O−C bond in the glucopyranose ring. The band at 1058 cm^{-1} is the stretching vibration of the C−O bond in the C_3H−OH group, and the bands at 796 and 777 cm^{-1} correspond to vibrations of the glucopyranose ring associated with vibrations of CH- and CH_2-groups. The bands identified in the IR spectra in the range of 1500-1650 cm^{-1} correspond to proteins.

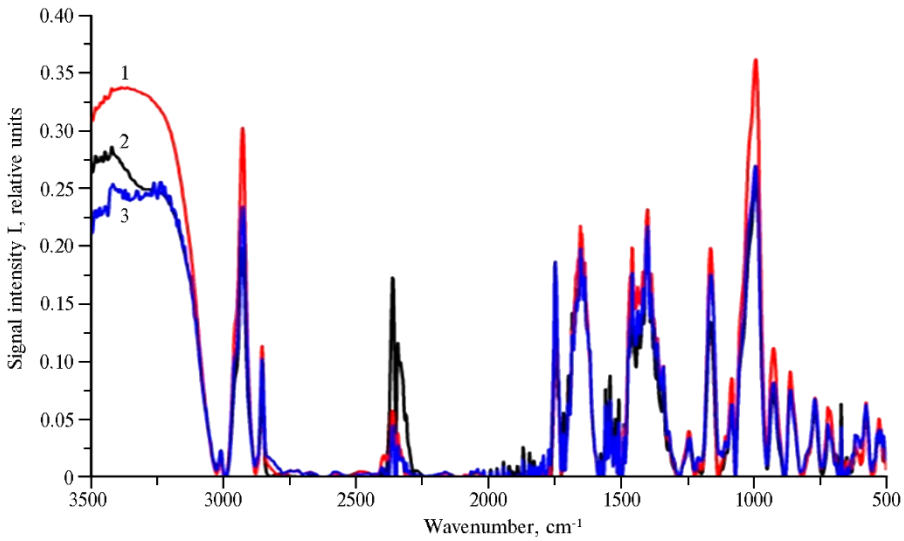

Fig. 1. **IR spectrum** (Fourier transform infrared spectroscopy) **of maize (*Zea mays* L.) leaf chloroplasts at the three-leaf stage, the lines ZPPL 186** (1)**, M1-3-3-sdms** (2), **and ZPPL 225** (3) (originator is Maize Research Institute, Zemun Polje, Belgrade, Serbia) (laboratory test). Fig. 1 shows typical normalized IR spectra.

Vibrations of free and bound OH-groups, intra- and intermolecular H-bonds, stretching vibrations of N−H bonds in primary and secondary protein amides, vibrations of hydroxyl OH-group (water, carbohydrates, amino acids), and stretching vibrations of NH-group (proteins, amino acids and their derivatives) are maximum for the leaf chloroplasts of ZPPL 186 line and minimum for the ZPPL 225 line (3500-3000 cm^{-1} region). Leaf chloroplasts of the ZPPL 186 line are also characterized by the maximum contribution of stretching vibrations of alkanes, carboxylic acids (range 2920-2860 cm^{-1}) and bending vibrations of bonds in aromatic compounds (band at 1000 cm^{-1}), the line M1-3-3-sdms shows vibrations of O−C−O bonds (band 2300 cm^{-1}). Thus, we have revealed differences in the composition of protein and carbohydrate molecules in chloroplasts of leaves of different maize lines. These differences may be due to the synthesis of new molecules of cellulose, proteins, carbohydrates, ethers, phenols. For example, a higher content of molecules of alkanes, carboxylic acids, and aromatic compounds in chloroplasts of a leaf was characteristic of ZPPL 186.

However, by using the IR spectroscopy, we were unable to reveal differences in the region from 1100 to 1600 cm^{-1} (see Fig. 1). It is known that this region is characteristic of symmetric and antisymmetric stretching vibrations of atoms, as well as in-plane and out-of-plane bending vibrations of molecules [14, 15]. Possible candidates for such differences could be cellulose and carotenoid

molecules from leaf chloroplasts. The content of starch, fiber and carotenoids in the tissues of the leaves of the studied maize lines is high [22]. Therefore, in the next series of experiments, we obtained IR spectra of cellulose, which had bands in the region of 4000-2500 cm^{-1} (stretching vibrations of O−H and C−H bonds) and bands in the region of 1500-500 cm^{-1} (stretching vibrations of C−C, C−O bonds and deformation vibrations of C−H and O−H bonds). The range of vibrations of OH-groups included three bands corresponding to free hydroxyl groups (at carbon atoms C2 and C6) and an OH-group, which are involved in hydrogen bonds (Fig. 2).

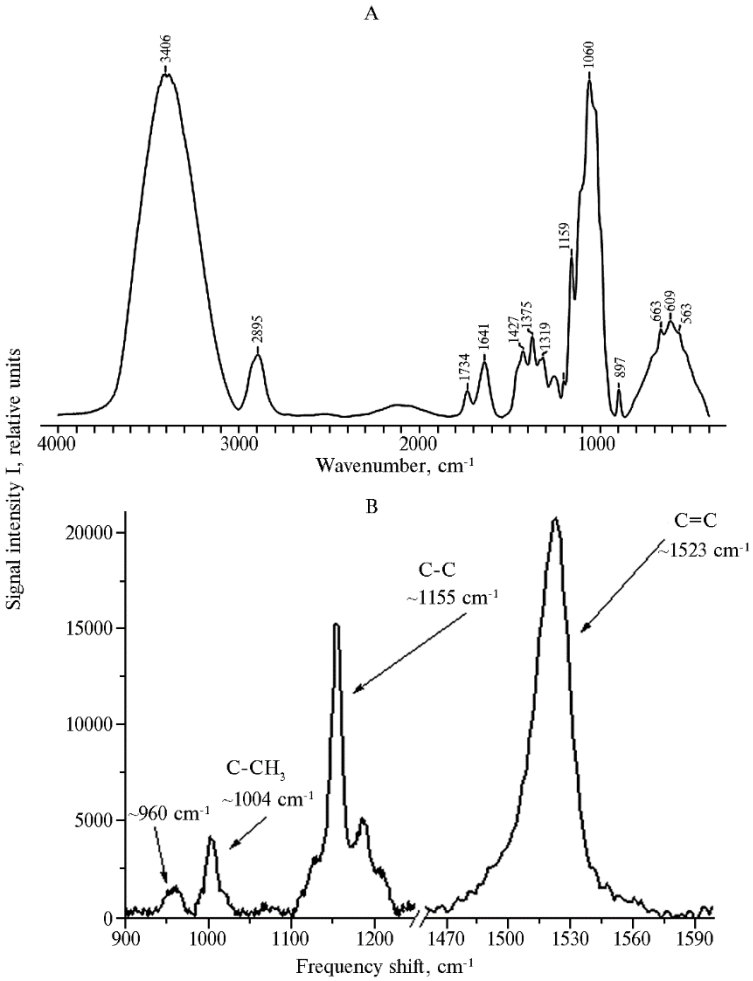

Fig. 2. IR spectra (Fourier transform infrared spectroscopy, A) **of cellulose from leaf tissue and Raman spectra (RS) of carotenoids in chloroplasts of leaves** (B) **in maize (*Zea mays* L.) line ZPPL 186** (originator is Maize Research Institute, Zemun Polje, Belgrade, Serbia) **at the three-leaf stage** (laboratory test)**.** Fig. 2 shows typical normalized IR and RS spectra.

Intramolecular hydrogen bonds between hydroxyl groups at the C2 and C6 positions (as well as at C3) are formed even in the presence of small number of OH-groups. In the IR spectrum in the range of 1500-900 cm^{-1}, we recorded the total intensity of vibrations of three hydroxyl groups of each glucopyranose unit of a cellulose molecule (C−H, C−O. and O−H vibrations). The bands at 1375 and 1319 cm^{-1} characterized the presence and manifestation of bending vibrationsof C−H- and CH_2-groups, respectively, in the cellulose molecule [8, 14]. However, using the IR spectroscopy, we were unable to detect differences

in the investigated region of wavenumbers for cellulose molecules.

In the next series of experiments, we used Raman spectroscopy to refine the results of IR spectroscopy in the 800-1800 cm^{-1} region. It is important that it is the maize leaf chloroplasts' Raman spectra in this region that are characterized by high-amplitude bands of carotenoids and low-amplitude or combined bands of cellulose molecules (the band at 1095 cm^{-1} corresponds to C–O–C vibrations, at 1477 cm^{-1} to H–C–H groups of atoms of the cellulose molecule). Thus, using Raman spectroscopy in the 800-1800 cm^{-1} range, one can additionally investigate the conformation of carotenoid molecules in chloroplasts (see Fig. 2, Table) [24-27]. In the Raman spectra, the 1523 cm^{-1} band originates from vibrations of the C=C bonds in the carotenoid molecule, the 1155 cm^{-1} band from the C–C bonds. In the case of the trans-conformation of double bonds in the carotenoid molecule, the band at 1155 cm^{-1} changes and acquires two pronounced arms, at 1190-1193 and 1210 cm^{-1}. The band at 1004 cm^{-1} corresponds to vibrations of the side methyl group C–CH_3. The 960 cm^{-1} band arises from out-of-plane C–H vibrations in the C–C bond. An increase in the intensity of this band is observed when the planar configuration of the molecule is disturbed: the greater the amount of pigment bound to the protein, the less pronounced out-of-plane twists between the C11 and C12 and, as a consequence, the intensity of the indicated band in the Raman spectrum.

The intensity (I) and band intensity ratios in Raman spectra of carotenoids in chloroplasts of leaves in the studied maize (*Zea mays* L.) lines at the three-leaf stage (n = 10)

Frequency shift, cm^{-1} (M±SD)	Mean I value (M)	Standard deviation (±SD)	Characteristic bands	
			positions	intensity ratios
Line M1-3-3-sdms				
1523.6±0.1	132.5	9.6	962/1006	0.45
1155.6±0.9	148.9	10.9	1006/1157	0.34
1191.3±0.4	46.7	3.7	1157/1190	3.18
1004.9±0.9	51.9	4.2	1006/1525	0.39
960.5±1.3	23.5	1.9	1526/1157	0.88
Line ZPPL 186				
1523.0±0.2	213.6	26.9	962/1007	0.38
1155.8±0.7	195.7	23.5	1007/1158	0.28
1190.4±0.4	55.4	7.3	1158/1190	3.53
1004.0±0.6	55.3	4.5	1007/1526	0.25
960.1±0.5	21.5	2.2	1526/1158	1.09
Line ZPPL 225				
1523.8±0.3	118.2	13.6	960/1005	0.48
1155.2±0.6	132.1	21.2	1005/1157	0.36
1189.7±0.9	43.4	6.7	1157/1189	3.04
1004.6±0.4	47.7	5.6	1005/1526	0.40
960.7±0.3	23.0	2.4	1526/1157	0.89

To normalize the contribution of each type of bonds to the Raman spectra of carotenoids, the ratio of the peak values is used, choosing, as a rule, a constant amplitude Raman band, the changes in which are minimal (intramolecular marker) [23]. Our results indicate that carotenoids in chloroplasts of different maize lines can be in different conformation. Thus, the conformation due to the length of the polyene chain of carotenoid molecules (the value is proportional to I_{1523}/I_{1155}) in leaf chloroplasts of maize lines can vary from 0.88 (M1-3-3-sdms) to 1.09 (ZPPL 186), and in two maize lines (except for ZPPL 225) carotenoid molecules are in the 15-trans conformation. With this conformation, the 1155 cm^{-1} band of the Raman spectrum has one pronounced shoulder at 1190 cm^{-1}. Note that the carotenoids in the leaf chloroplasts of the ZPPL 186 line had the minimum I_{960}/I_{1006} ratio which indicates an insignificant change in conformation caused by rotation of the carotenoid molecule outside the plane of the polyene chain, or the absence of such a change. In the ZPPL 186 line, the carotenoid molecule was characterized by more pronounced vibrations of the side CH_3-group.

It is known that the carotenoid bound to proteins of the light-harvesting complex of photosystem II has a characteristic intense band at 960 cm^{-1} in the Raman spectrum, which practically does not differ in amplitude from the bands at 1156 and 1004 cm^{-1} [24-27]. Note that in our experiments the amplitude of the band at 960 cm^{-1} of the Raman spectrum of carotenoids was significantly less than the amplitude of the bands at 1156 and 1004 cm^{-1} and did not differ in the Raman spectrum of chloroplasts in different lines, which probably indicates the absence of protein-lipid interactions.

In our opinion, the combined use of IR and Raman spectroscopy of the leaf chloroplast fraction can be recommended as a minimally invasive method to control the content and possible differences in the conformation of biological molecules when testing maize lines and hybrids [28-30].

So, using the methods of vibronic spectroscopy, we studied the changes in the content and conformation of chloroplast molecules in different lines of maize. Infrared (IR) spectroscopy (range 3500-3000 cm^{-1}) showed that in the molecules contained in the chloroplasts of the leaf (water, carbohydrates, proteins), the proportion of vibrations of OH-groups and intra- and intermolecular H-bonds is maximal in the maize line ZPPL 186, and the vibrations of NH-groups of amides (proteins) are minimal in the ZPPL 225 line. It was proved that the ZPPL 186 line is characterized by the maximum fraction of vibrations from alkanes, carboxylic acids (region 2920-2860 cm^{-1}) and deformation vibrations of aromatic structures (band at 1000 cm^{-1}) of chloroplasts, and for the M1-3-3-sdms line, the fraction of O=C=O bond vibration (band 2300 cm^{-1}) are typical. Using Raman spectroscopy (ranges 1250-500 cm^{-1} and 1535-1400 cm^{-1}), it was found that the differences in the studied maize lines are associated with changes in the conformation of carotenoid molecules in chloroplasts, but not cellulose molecules. It was found that in two samples (except for ZPPL 225), carotenoid molecules are in the 15-trans form with different conformation of the polyene chain. Note that the conformation of carotenoids of the ZPPL 186 line is characterized by the minimum rotations outside the plane of the polyene chain, while more pronounced vibrations of the side CH_3-group appear. It was suggested that in the studied lines, the carotenoids of leaf chloroplasts do not interact with the aromatic amino acids of proteins.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 958-967
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Mycotoxins

UDC 633.1:632.4:636.085.19

doi: 10.15389/agrobiology.2021.5.958eng
doi: 10.15389/agrobiology.2021.5.958rus

ADVANCES IN MYCOTOXICOLOGICAL RESEARCH OF FORAGE GRAIN CROPS

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The authors declare no conflict of interests
Received May 25, 2021

Abstract

Recently, production of forage from the vegetative mass of grain crops has been steadily growing in Russia (Z.L. Fedorova, L.V. Romanenko, 2016; V.V. Popov, 2017; E.A. Volkova et al., 2018). For the successful and safe use of these products, it is extremely important not only to strictly observe the recommended terms, mowing height, drying conditions and technology of silaging grain-stem mass, but also to have the most complete information about the sanitary quality of raw materials. The study of the peculiarities of contamination by toxigenic microscopic fungi and mycotoxins of wild and cultivated cereals has already begun (G.Yu. Laptev et al., 2014; A.A. Burkin, G.P. Kononenko, 2015; G.P. Kononenko et al., 2015; E.A. Yildirim et al., 2019). However, this aspect has not been studied with a focus on forage crops. This work, for the first time, presents data on contamination of vegetative grain crops with toxic metabolites of microscopic fungi and on changes in the content of mycotoxins over phases of plant development and in ears at the beginning of grain maturation. The aim of this work was a mycotoxicological study of common barley (*Hordeum vulgare* L.), soft wheat (*Triticum aestivum* L.), and oats (*Avena sativa* L.) during in the periods optimal for hay harvesting and in unripe ears of wheat and barley. The samples (spring barley *H. vulgare* cv. Vladimir, spring soft wheat *T. aestivum* cv. Ivolga, and oats *A. sativa* cv. Skakun) were collected from April 24 to August 11, 2019 (the fields of the Russian State Agrarian University — Moscow Timiryazev Agricultural Academy and the Williams Federal Scientific Center for Feed Production and Agroecology, Moscow Province). Beginning of tillering—ligule formation was noted as period 1, opening of the flag leaf envelope and appearance of the awns above the ligule—early milk ripeness — as period 2. At the stage of grain maturation, from the aboveground parts cut 3-5 cm from the soil surface the ears were separated. The concentrations of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), 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), and ergot alkaloids (EA) were measured by indirect competitive enzyme immunoassay (ELISA) test. The detected load of mycotoxins was generally low. AOL, EMO were present in small and comparable amounts of 15-32 μg/kg and 14-29 μg/kg, as well as CPA and EA with wider ranges of variation, from 34 to 180 μg/kg and from 2 to 115 μg/kg. Fusariotoxins T-2, DON, and ZEN appeared in single samples, and FUM was not detected. ROA was also absent, and PR was extremely rare and detected only in one sample of wheat. In all crops, tens of μg/kg MPA and STE were found, and AB_1 amounted to 1-3 μg/kg. Combined contamination of OA and CIT occurred only in barley (more often at tillering and ligule formation), while OA contamination occurred, though rare, in wheat and oats at the levels close to the detection limit. Lower contamination by mycotoxins was characteristic of vegetative oat plants compared to barley and wheat, which is practically important since fodder oat is popular as a green fodder for preservation, both separately and in crop mixtures. Wheat and barley ears at the beginning of grain maturation were noticeably different from the aboveground parts of the plants and showed a uniform tendency to reduce the frequency of mycotoxin detection to single cases or complete absence while maintaining the occurrence of EMO.

Keywords: wheat, barley, oat, plant biomass, mycotoxins, ELISA

Recently, production of forage from the vegetative mass of grain crops

(wheat, barley, oat, and triticale) has been growing in a number of regions of the Russian Federation mainly due to high nutritional value of conservation products thereof and due to the ability to successfully overcome critical situations threatening the reaping of a full grain harvest [1-3]. For the successful and safe use of hay and haylage from the grain crops, it is extremely important not only to strictlyobserve the recommended terms, mowing height, drying conditions and technology of silaging the grain-stem mass, but also to have the most complete information about the sanitary quality of raw materials. Of particular relevance is information about the contamination of crops by toxigenic microscopic fungi and their metabolites resulting in animal mycotoxicoses [4, 5]. For a large community of cereals, the accumulation of such information is just beginning. The abundance of *Fusarium* fungi in pastures of Manitoba province of Canada [6] and Croatia [7] has been studied. The abundance of such fungi and their toxins in 70 species of meadow plants in one of the ecosystems suitable for cattle grazing in Chaco province in northeastern Argentina has been assessed [8, 9]. Systematic affiliation and toxicogenic potential of *Fusarium* fungi from mycobiota of nine species of herbs in five agro-ecological zones of western Iran has been determined [10].

Russian researchers, described the contamination of meadow leguminous grasses with fungi *Fusarium*, *Alternaria*, *Cladosporium* and mycotoxins [11, 12], the features of colonization of wild and cultivated grasses by these fungi [13], the general mycotoxicological situation for the community of meadow plants [14] and industrial mixed sowing of ryegrass, timothy grass, fescue grass, festul lolium and cocksfoot grass [15]. The content of five mycotoxins in samples of ryegrass and timothy grass monocrops has been analyzed [16, 17]. Grain crops harvested without threshing for forage purposes have not been previously studied.

This work, for the first time, presents data on contamination of vegetative grain crops with toxic metabolites of microscopic fungi and on changes in the content of mycotoxins over phases of plant development and in ears at the beginning of grain maturation.

The aim of this work was a mycotoxicological study of vegetative barley, soft wheat, and oats during in the periods optimal for hay harvesting and in unripe ears of wheat and barley.

Materials and methods. Specimens of spring barley (*Hordeum vulgare* L.) cv. Vladimir, spring soft wheat (*Triticum aestivum* L.) cv. Ivolga, and oats (*Avena sativa* L.) cv. Skakun were collected from April 24 to August 11, 2019 (the fields of the Russian State Agrarian University — Moscow Timiryazev Agricultural Academy and the Williams Federal Scientific Center for Feed Production and Agroecology, Moscow Province).

Plant phenophases were described according to BBCH classification [18]. Beginning of tillering-ligule (tongue) formation was noted as period I (BBCH 21-39), opening of the flag leaf envelope and appearance of the awns above the ligule-early milk ripeness — as period II (BBCH 49-73). At the stage of grain maturation, from the aboveground parts cut 3-5 cm from the soil surface the ears were separated (waxy ripeness, BBCH 83-87).

The specimens were kept to an air-dry state in a ventilated room and were ground in a laboratory mill M20 (IKA, Germany). For extraction, a mixture of acetonitrile and water in a volume ratio of 84:16 was used at a flow rate of 10 ml per 1 g of the sample. The extracts after 10-fold dilution with buffer solution were used for indirect competitive enzyme-linked immunosorbent assay. The concentrations of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), 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), and ergot alkaloids (EA) were measured by indirect competitive enzyme immunoassay (ELISA) test (GOST 31653-2012 "Feedstuffs. Method of immunoenzyme mycotoxin determination", M., 2012). The lower limits of quantitative measurements corresponded to 85% level of antibody binding.

The data obtained were processed using the descriptive statistics in Microsoft Excel 2013 program, the proportion of positive samples (n^+) from the number of examined samples (n), the minimum, maximum content of mycotoxin (μg/kg) and the arithmetic mean value (M) for positive specmens was calculated.

Results. The studied cereals lacked mycotoxins FUM and ROA. PR was found only in one sample of wheat in quantity of 320 μg/kg. Recently, the similar situation with isolated cases of PR detection and lack of FUM and ROA was described for agrestic annual plants of Cruciferae family [19], as well as for sowing white mustard [20] and sunflower [21]. Unfortunately, information on the contamination of the green mass of corn is still extremely limited. In four studied specimens from the Rostov region, at the stage of three leaves, completion of flowering and physiological ripeness, there were no FUM and ROA (unpublished data of the authors). Nevertheless, it is known that at the beginning of growth, corn and rice are characterized by contamination with fungi *Myrothecium* spp. producing ROA [22].

1. The occurrence and concentration of mycotoxins in vegetative plants of spring barley (*Hordeum vulgare* L.) cv. Vladimir, spring soft wheat (*Triticum aestivum* L.) cv. Ivolga and oat (*Avena sativa* L.) cv. Skakun (Moscow Province, 2019)

Mycotoxin	Barley (n = 32)		Wheat (n = 11)		Oat (n = 11)	
	I (n = 14)	II (n = 18)	I (n = 2)	II (n = 9)	I (n = 3)	II (n = 8)
T-2	–	–	–	2 3	2 2	–
DON	1 95	–	–	–	–	1 160
ZEN	–	–	2 32	–	–	–
EA	14 7-16-37	17 2-8-35	1 3	7 6-115-425	2 6	4 1-2-2
AOL	14 18-30-49	18 13-29-40	2 27	7 16-26-32	2 32	4 15-21-25
AB1	6 2-3-3	15 2-2-3	–	4 1-1-2	–	2 1
STE	7 13-24-25	9 13-16-22	–	4 16-20-25	1 13	–
CPA	13 79-180-320	16 76-145-265	2 81	9 66-140-280	2 89	4 27-48-70
EMO	6 11-14-18	7 11-17-25	–	5 10-14-19	2 18	6 16-29-40
OA	5 6-8-10	8 4-5-6	–	2 5	2 7	–
CIT	7 19-23-33	1 30	–	–	–	–
MPA	5 19-23-30	11 13-20-38	1 21	8 11-24-40	–	3 14-18-21

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, EA — ergot alkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid. Period I comprises beginning of tillering—ligule (tongue) formation (BBCH 21-39), period II comprises opening of the flag leaf envelope and appearance of the awns above the ligule—early milk ripeness (BBCH 49-73); n is the number of studied specimens. The upper value in lines is the number of positive specimens (n^+) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

The most frequently detected mycotoxins in our study were AOL, CPA, and EA, therewith such list was added with MPA and EMO for wheat, EMO for oats, AB1, STE, and MPA. Besides, OA and CIT were regularly found in barley

as opposed to wheat and oats. Fusariotoxins T-2, DON, and ZEN appeared in single specimens (Table 1).

Neither toxin was found in vegetative oat plants, possibly, due to low concentrations close to determination limits. In practical terms, the fact of mild contamination of this culture with mycotoxins is very important, since oats are more often cultivated for green fodder both in pure form and in mixed crops with vetch, tare, and vetchling.

The boundaries of surveillance over the mycotoxicological status of grain fodder crops were selected accounting for the recommended terms of their harvesting for hay (period I, the end of the development of flag leaf at the latest) and for grain silage (period II, completion at the beginning of the milky-wax ripeness of grain).

Based on comparable sets of barley specimens for periods I and II, it is possible to generally assess the direction of changes in mycotoxin contamination (see Table 1). In both periods, the computed average concentration of mycotoxins in positive samples remained comparable. Therefore, concentration of AOL and EMO were equally low (from 14 to 30 μg/kg), contamination by OA was at the baseline, concentration of EA was low, from units to tens μg/kg. Nevertheless, period I differed from period II in a number of CIT, MPA, and AB_1 detection instances. At the initial phase, plants were characterized by an increased accumulation of CIT and a decreased accumulation of MPA and AB_1. It should be noted that the same tendency were observed for MPA and AB_1 in wheat and oats (see Table 1). A variation in the content of mycotoxins by growth periods denotes possible involvement of toxigenic micromycetes in the processes accompanying the change in the ontogenetic states of these plants. However, it is not yet possible to search for any correspondences with the composition of their mycobiota due to the lack of basic information. As is known, mycotoxins are produced by many species of *Alternaria*, *Fusarium*, *Aspergillus*, *Penicillium* genus, as well as other fungi capable of autonomous existence [23-26]. Possibly, some of them are adapted to living inside plants [27-29] and are able to provide the biosynthesis of these metabolites independently or with the participation of the host organism [30].

During period II, concentration of AOL, EMO, and MPA was equally low and stable in barley, wheat, and oats (from 14 to 29 μg/kg), contamination by OA and CIT was baseline or lacked. EA was usually found in small concentrations from units to tens μg/kg, with the exception of a few cases of exceeding the value of 100 μg/kg in wheat (see Table 1). Possibly, it was due to infection of a part of the ears with actively producing epiphytes of the *Claviceps* genus [31]. No differences in occurrence and degree of contamination AB_1 (1 and 2 μg/kg, respectively), STE (20 and 24 μg/kg), and CPA (140 and 145 μg/kg) were found in wheat and barley. In barley, AB_1 was found even less often, STE was not found, and CPA was by one order less (48 μg/kg). In general, the grain-stem mass in all three crops was characterized by moderate contamination and, according to this indicator, can be recognized as equally promising for laying on grain-silage. Oat cuttings at the late vegetation stages are also suitable for drying, since oat hay retains a high nutritional value at all stages of plant maturity.

In general, in terms of mycotoxin load, the vegetative mass of grain fodder crops differs little from weakly contaminated cruciferous plants and sunflowers [19-21]. The fact that we regularly detected AOL, EA, and CPA in both monocotyledonous and dicotyledonous plants suggests the presence of similarities in the composition of their mycobiota. Indeed, among the endophytes of many plants, fungi with a confirmed potential for AOL and EA biosynthesis have been described [32]; however, no information on the identification of active producers of CPA

[33] in the composition of the internal mycoflora of herbs was found in the available literature.

The differences in the profile of mycotoxins in some plant species, as well as in communities with an annual and interrupted development cycle, indicate the peculiarities in the composition of associated fungi.

In recent years, information on the species diversity of endophytes using molecular methods, in particular, among representatives of *Triticeae* tribe [34], common reed grass *Phragmites australis* (Cav.) Trin. ex Steud. [35], bamboo *Phyllostachys* spp., *Sasa* spp. [36], Siberian cheegrass *Achnatherum sibiricum* (L.) Keng ex Tzvelev [37, 38], and cocksfoot grass *Dactylis glomerata* L. [39] has been actively accumulated. It was established that in cultivated cereals (ryegrass, timothy grass, and wheat) the DNA content of *Alternaria* and *Cladosporium* fungi was 6 times lower and of *Fusarium* 14 times lower than in wild-growing cereals (cocksfoot, wheat grass, timothy grass) [13]. In furtherance, such methods will make it possible to start searching for specific micromycetes responsible for the formation of a natural level of mycotoxin contamination of forage plants, including grain fodder crops.

Ripening ears of wheat and barley significantly differed in the content of mycotoxins from the vegetative biomass of period II (Table 2). T-2, DON, and AOL were more often found in barley, which could be due to fungal infection, since increased contamination with these toxins is observed at intense infection with *Fusarium* and *Alternaria* [40, 41]. Omelchenko et al. [42] noted the relationship between DON accumulation and the maturation of wheat ears affected by the micromycete *Fusarium*.

2. The occurrence and concentration of mycotoxins in immature ears of spring barley (*Hordeum vulgare* L.) cv. Vladimir and spring soft wheat (*Triticum aestivum* L.) cv. Ivolga (Moscow Province, 2019)

Микотоксин	Barley (n = 13)	Wheat (n = 6)
T-2	4	–
	2-7-10	
DON	4	–
	79-150-260	
ZEN	1	–
	15	
EA	2	4
	2	3-9-11
AOL	7	2
	17-74-355	16
AB_1	1	2
	1	1
STE	1	2
	14	15
CPA	1	3
	97	32- 44-54
EMO	4	1
	8- 47-150	12
OA	–	–
CIT	1	–
	24	
MPA	1/18	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, EA — ergot alkaloids, AOL — alternariol, AB_1 — aflatoxin B_1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid. Period I comprises beginning of tillering—ligule (tongue) formation (BBCH 21-39), period II comprises opening of the flag leaf envelope and appearance of the awns above the ligule—early milk ripeness (BBCH 49-73); n is the number of studied specimens. Upper value in lines is the number of positive specimens (n^+) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

In the ears, the occurrence of AB_1, STE, CIT, and MPA decreased in several cases or was completely absent (See Table 2). for mature grains of wheat and barley, the situation was similar. Particularly, AB_1 was not detected, and the

cases of STE, CIT and MPA detection did not exceed 5% [43]. Contamination of wheat ears by CPA was also significantly lower as compared to biomass (see Table 2), which corresponded to the previously obtained data on its low occurrence (1%) in mature grain [43]. In barley ears, this toxin was found only in one case, and in the grain, it was not found in any of the 92 samples [43]. On the contrary, the frequency of EMO detection remained the same in ripening ears and vegetative mass (30-40%) (see Tables 1, 2), and in threshed grain it reached 72.3% [43]. Since mycotoxin distribution between vegetative parts and ears changes multidirectionally, it can be assumed that the processes of maturation are accompanied by blocking or activation of mycotoxin biosynthesis by associated producers, reformation of the mycobiota composition with their replacement by other fungi or a change in the localization of toxigenic fungi in the plant. Interestingly, in mature corn plants with multiple combined contamination of leaves, stems and flowers, mycotoxin contamination of cobs was not found (unpublished data).

Our characteristics of the main grain fodder crops on the accumulation of fungal metabolites toxic to animals are important for the development of a well-grounded approach to the formation of poly-species herbage, the economic feasibility of which is beyond doubt. Similar projects have yet to be implemented for corn and triticale, as well as for sorghum, which are increasingly being introduced into field fodder production. In the future, we can count on a more detailed description of the mycotoxicological status of crops using a combined high-performance liquid chromatography and mass spectrometry assays [44, 45]. These methods were used to analyze wild-growing grasses in Norway [46] and were successfully applied to assess mycotoxin contamination of the 2017-2018 wheat and barley grain harvest from the Ural region [47].

Thus, mycotoxins slightly contaminated the green mass of wheat, barley and oats, when harvesting early for hay and later for grain silage. It have been established that alternariol, cyclopiazonic acid, emodin and ergoalkaloids regularly occur at a low level; mycophenolic acid and sterigmatocystin contaminate biomass of all cultures; the aflatoxin B_1 concentration corresponds to a basal level; fumonisins and roridin A are absent. Wheat and barley ears at the beginning of grain maturation were noticeably different from the aboveground parts of the plants and showed a uniform tendency to reduce the frequency of mycotoxin detection to several cases or complete absence while maintaining the occurrence of emodin. The revealed features of the mycotoxin accumulation during the initial and final plant growth and in ripening ears testify to the active role of toxigenic microscopic fungi in the course of plant development and serve as a convincing argument in favor of the need for complex research projects aimed at interpretation of mechanisms of their interaction with associated fungi.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 968-978
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 582.736:632.4:636.085.19

doi: 10.15389/agrobiology.2021.5.968eng
doi: 10.15389/agrobiology.2021.5.968rus

TOXINS OF MICROMYCETES IN GENERATIVE ORGANS OF PLANTS OF THE FAMILY *Fabaceae*

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The authors declare no conflict of interests
Received July 18, 2021

Abstract

During the study of the role of associated microscopic fungi in the adaptation of plants to external influences, researchers focus mainly on such key aspects as i) the shift in the composition of the internal mycobiota during growth, ii) the direction of fungal colonization of vegetative and generative organs, and iii) concomitant changes in the metabolic status of the plant organism (J.A., Wearn et al., 2012; V. Arbona et al., 2013; J. Hong et al., 2016). The dynamics of DNA accumulation of *Alternaria*, *Cladosporium* and *Fusarium* fungi in different months of plant growth was revealed in meadow grasses of the Fabaceae family (O.P. Gavrilova et al., 2017; A.S. Orina et al., 2018) and seasonal fluctuations in the content of toxic metabolites characteristic of these groups of micromycetes were found (A.A. Burkin, G.P. Kononenko, 2018, 2019). The predominant localization of mycotoxins in leaves was established for meadow clover, white clover, Caucasian goat's rue, Washington lupine and melilot (G.P. Kononenko et al., 2019). In this study, we describe for the first time the complexes of toxic fungi metabolites in the generative organs of legumes. The aim of the work was to study the component composition and content of mycotoxins in the whole plants, flowers and beans of perennial legumes of 6 genera of the *Fabaceae* family. Meadow grasses of the genera *Trifolium* L. — meadow clover (*T. pratense* L.), alsike clover (*T. hybridum* L.), zigzag clover (*T. medium* L.), white clover (*T. repens* L.); of *Lathyrus* L. — meadow peavine (*L. pratensis* L.), spring peavine (*L. vernus* (L.) Bernh.); of *Vicia* L. — bush vetch (*V. sepium* L.), cow vetch (*V. cracca* L.); of *Lotus* L. — deer vetch (*L. corniculatus* L. s.l.); of *Lupinus* L. —Washington lupine (*L. polyphyllus* Lindl.), and of *Galega* L. — Caucasian goat's rue (*G. orientalis* Lam.) were collected from natural grass stands of the Moscow region in May—the first half of August 2019, wood vetch (*V. sylvatica* L.) and Japanese peavine (*L. japonicus* Willd. subsp. pubescens Korobkov) — in the second half of August of the same year on of the Kandalaksha Gulf of the White Sea (Republic of Karelia). The aboveground parts of plants, as well as flowers and beans, were kept at room temperature to an air-dry state and crushed in a laboratory mill. For extraction, a mixture of acetonitrile and water was used (84:16 v/v) at the ratio of 10 ml per 1 g of the sample. Extracts after 10-fold dilution with a buffer solution were assessed using indirect competitive enzyme immunoassay. The content of the mycotoxins — T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (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) was determined using commercial and research certified enzyme immunoassay systems (GOST 31653-2012). For the generative organs of most of the examined plants, both common features (preservation of the mycotoxin profile typical of the whole plant, with the absence or decrease in the content of a number of fungal metabolites) and peculiarities were revealed. In particular, in the flowers of three species of the genus *Trifolium* L., in general, the mycotoxin complex characteristic of the vegetative part was preserved, but the occurrence and accumulation of fusariotoxins were higher. The flowers of two species — alsike clover and zigzag clover were characterized by combined contamination of OA and CIT in comparable quantities, rare for plants. With a general low contamination, fusariotoxins T-2, DON and ZEN were present only in generative organs in the deer vetch. In all representatives of the genera *Vicia*, *Lathyrus*, *Lupinus*, and *Galega* the metabolic background in flowers as a whole was found weakened, in beans it turned out to be similar to the aboveground part without a sharp variation in the content of mycotoxins.

Keywords: legumes, flowers, beans, mycotoxins, ELISA

In recent years, when studying the mechanisms of adaptation to external influences, plants are increasingly considered as complex systems, which include micromycetes [1, 2]. Various connections can be established between fungi and a plant: from complete isolation to joint participation in biochemical processes [3-5]. The main plant responses to a change in habitat conditions include a shift in the composition of the internal mycobiota and the direction of colonization by fungi of vegetative and generative organs [6, 7], as well as the accompanying changes in the metabolic status of the organism [8-10].

In meadow grasses of the *Fabaceae* family, the dynamics of DNA accumulation of the fungi *Alternaria*, *Cladosporium*, and *Fusarium* was studied in different months of plant collection [11, 12], and seasonal fluctuations in the content of toxic metabolites characteristic of these groups of micromycetes were revealed [13-15]. With regard to the organotropism of endophytic fungi in these plants, preliminary data were obtained [16], but for meadow clover, white clover, Caucasian goat's rue, Washington lupine, and melilot, the predominant localization of mycotoxins in leaf blades was established; the same complex of metabolites with an increased content of individual components is retained in flowers of *Melilotus* sp. [17]. Mycotoxicological examination of generative organs in other members of this family was not carried out.

In this work, we were the first to describe complexes of toxic metabolites of imperfect fungi in the generative organs of leguminous plants.

Our purpose was to study the component composition and content of mycotoxins in flowers and beans in perennial legumes of the genera *Trifolium*, *Lathyrus*, *Vicia*, *Lotus*, *Lupinus,* and *Galega*.

Materials and methods. Meadow grasses of the *Fabaceae* family from natural grass stands of the Moscow Province were collected regularly, at weekly intervals in May—the first half of August 2019. According to the guides [18, 19], grasses were assigned to six genera: *Trifolium* L. — meadow clover *T. pratense* L., alsike clover *T. hybridum* L., zigzag clover *T. medium* L., white clover *T. repens* L.; *Lathyrus* L. — meadow peavine *L. pratensis* L., spring peavine *L. vernus* (L.) Bernh.; *Vicia* L. — bush vetch *V. sepium* L., cow vetch *V. cracca* L.; *Lotus* L. — deer vetch *L. corniculatus* L. s.l., *Lupinus* L. — Washington lupine (*L. polyphyllus* Lindl.); and *Galega* L. — Caucasian goat's rue *G. orientalis* Lam. Wood vetch (*V. sylvatica* L.) and Japanese peavine (*L. japonicus* Willd. subsp. *pubescens* Korobkov) were collected in the second half of August of the same year on the coast of the Kandalaksha Bay of the White Sea (Republic of Karelia).

The aboveground parts of plants, cut at a height of 3-5 cm from the soil surface, as well as flowers and beans separated from them, were immediately after collection placed in a ventilated room, kept at room temperature to an air-dry state, and ground in a laboratory mill. For extraction, a mixture of acetonitrile and water in a volume ratio of 84:16 was used at a flow rate of 10 ml per 1 g of the sample. Extracts after 10-fold dilution with phosphate buffered saline (pH 7.4) with Tween 20 were used for indirect competitive enzyme immunoassay.

The content of mycotoxins T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (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 analyzed using commercial and research certified enzyme-linked immunosorbent assay tests (GOST 31653-2012. Feedstuffs. Method of immunoenzyme mycotoxin determination. Moscow, 2012). The lower limits of quantitative measurements were 1 (EA, AB_1), 2 (T-2), 4 (OA, STE, ROA), 15 (AOL, ZEN, EMO), 20 (CIT, MPA), and 50 μg/kg (DON, FUM, CPA, PR) and corresponded to 85% of antibody binding.

The data were processed using descriptive statistics in Microsoft Excel 2013. We calculated the proportion of positive specimens (n^+) from the total number of those tested (n), the minimum, maximum content (μg/kg) of mycotoxins and the arithmetic mean value of the indicator (M) for positive samples.

Results. In the vegetative part of meadow clover, as noted earlier [9], the most common fusariotoxin was T-2, EA, AOL, CPA, EMO, OA, AB1, MPA, and PR were regularly detected; CIT, STE were rarely detected, and ROA was absent. In flowers, the component composition as a whole remained the same, while the frequency of occurrence and content increased for fusariotoxins (T-2, DON, ZEN, FUM), remained unchanged for AOL, OA, and AB1, and decreased for the rest of the components (Table 1).

1. The occurrence and concentration of mycotoxins in aboveground parts (1) **and flowers** (2) **of *Trifolium* L. plants** (Moscow Province, 2019)

Mycotoxin	*T. pratense* L.		*T. hybridum* L.		*T. medium* L.		*T. repens* L.	
	1 (n = 13)	2 (n = 13)	1 (n = 8)	2 (n = 8)	1 (n = 4)	2 (n = 3)	1 (n = 9)	2 (n = 9)
T-2	13	13	6	8	4	3	9	8
	2-8-55	2-69-795	3-4-7	2-10-16	2-6-9	8-65-180	2-5-15	2-83-470
DON	3	8	2	7	–	2	–	–
	68-115-160	63-110-160	95, 95	120-210-400		100, 170		
ZEN	3	6	2	7	4	3	3	3
	19-22-25	16-21-32	17, 17	20-45-84	15-19-25	25-45-61	19-20-21	19-22-26
FUM	1	1	–	5	–	2	–	–
	90	250		85–195–300		200, 355		
AOL	13	13	8	8	4	3	9	9
	64-130-180	63-125-315	30-70-105	44-300-630	120-155-210	240-290-370	21-38-54	40-76-160
OA	13	13	5	8	4	3	–	4
	6-20-31	6-17-54	5-7-10	10-55-110	8-9-10	9-31-56		5-7-8
CIT	3	2	5	7	–	2	–	2
	28-36-51	38, 40	21-39-59	30-79-180		32, 49		21, 25
STE	5	1	3	5	1	3	3	2
	12-17-21	30	12-17-22	12-41-75	16	24-29-37	12-13-13	21, 38
AB1	11	12	2	6	3	2	2	5
	1-2-4	1-3-8	1, 2	2-5-9	1-1-1	4, 8	2, 2	1-2-2
CPA	13	13	8	8	4	3	9	9
	39-230-590	31-95-245	50-135-240	63-160-315	62-86-105	47-77-135	40-72-105	32-76-115
MPA	12	8	8	7	4	3	9	9
	26-44-100	22-40-94	25-36-52	45-90-125	26-39-60	58-61-64	16-28-40	24-33-50
EA	10	6	6	7	4	3	7	5
	1-10-47	1-6-27	1-7-15	3-18-50	1-5-10	3-4-4	2-3-12	1-2-4
EMO	13	13	7	8	4	3	9	9
	315-960-2820	115-300-850	10-355-2240	33-130-240	32-82-125	50-195-405	50-150-250	35-185-645
PR	12	7	–	7	3	3	–	–
	335-530-795	305-405-575		400-585-795	425-530-670	670-790-945		
ROA	–	–	–	1	–	2	–	–
				18		10, 14		

N o t e. Mycotoxins are T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), ergot alkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B1 (AB1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), and PR-toxin (PR); n is the number of studied specimens. The upper value in lines is the number of positive specimens (n^+) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

For white clover plants, we observed the ubiquitous or similar occurrence of the same mycotoxins, but in amounts lower than in meadow clover [13]. The flowers were characterized by an increased concentration of T-2, a tendency to an increase in the content of AOL, OA, CIT, EMO while maintaining the same amounts of CPA and MPA. In aslike clover and zigzag clover, which occupy an intermediate position in the content of mycotoxins [13], increased contamination with fusariotoxins and AOL was observed in flowers with an increase in indicators for other components. Consequently, in the flowers and vegetative part of plants of the genus *Trifolium*, both common features and differences in the composition and content of mycotoxins occurred (see Table 1).

All plants showed a tendency towards greater accumulation of fusariotoxins in flowers. Apparently, this phenomenon is associated with the similarity of their physiological reactions, leading to a general direction of shifts in the distribution of micromycetes. The detection of all analyzed fusariotoxins suggests a combined contamination of the flowers of meadow clover, aslike clover, and zigzag clover with a medium complex of *Fusarium* fungi, the identification of which has not yet been carried out. It is worth noting, that in recent years the presence of toxigenic species, in particular *F. graminearum* Schw., *F. sporotrichioides* Sherb., *F. culmorum* (W.C. Sm.) Sacc. was noted in endophytes in a phylogenetically diverse group of wild-growing cereal grasses, and the study of their distribution in leaves, inflorescences, and seeds has begun [20-23].

An increase in the accumulation of AOL in flowers is also among the interspecific features, which is especially pronounced in aslike clover, less clearly in zigzag clover and white clover, and is not characteristic of the meadow clover. It is important to note that in cereal grasses the active producer of this toxin, the small-spore species *Alternaria alternata*, is referred to as typical endophytes [7].

Besides, OA and CIT were detected in the flowers of aslike and zigzag clover in comparable amounts, which was quite unusual (see Table 1). This situation is extremely rare. It was observed only in one of the 22 examined legume species - licorice (*Glycyrrhiza glabra* L.) [15] and was not observed in crucifers [17]. The simultaneous occurrence of these toxins has been described in both higher plants and lichens [24], but, as a rule, the amount of CIT is an order of magnitude higher than that established for OA. The joint presence of these mycotoxins with a frequency of 38% was also detected in grain, but not in equal amounts [25]. There is still no clarity about the sources of OA and CIT in grasses; however, it is known that habitat conditions have a noticeable effect on biosynthetic processes in the micromycete *Penicillium verrucosum* [26-28], which is capable of producing both metabolites [29]. In flowers of meadow clover and white clover, CIT was found only in some of the samples in quantities close to the limit of its determination (see Table 1), and in the seeds of these plants only OA was detected and there was no CIT (unpublished data of the authors).

The mycotoxicological assessment of one of the representatives of the genus *Lotus*, the horned flower, performed in this work for the first time, showed that in the aboveground part of the plant, the complex of fungal metabolites included AOL, CPA, and EMO, as well as OA and EA in smaller amounts (Table 2). It was not possible to detect OA and EA in the same amounts in flowers, while retaining the three main contaminants, but IFC and PR were detected. The composition of the beans turned out to be similar to the vegetative mass and differed only in the increased content of EMO. Fusariotoxins were found only in flowers and beans: T-2 in all samples, ZEN and DON in some cases. This feature is of undoubted scientific interest and indicates the need for a more detailed examination of *L. corniculatus* on expanded samples of material.

In two *Vicia* species (*V. sepium* and *V. cracca*), known to be weakly contaminated with mycotoxins [15], there was a tendency to a decrease in their number in flowers as compared to the vegetative part, while the occurrence and concentration of AOL remained the same, and both indicators for EA decreased (see Table 2). In forest vetch (*V. sylvatica*), which we sampled in Karelia, in the terrestrial part and flowers, the content of AOL was also comparable (25 and 32 μg/kg), and the differences in EA (30 and 1 μg/kg) were quite significant.

In two species of the genus *Lathyrus*, characterized by different levels of contamination [15], there was a uniform decrease in the content of mycotoxins in flowers compared to the vegetative parts. Nevertheless, in *L. vernus*, the AOL and

CPA contamination remained unchanged (Table 3), as in the *L. japonicus* specimens from the geographically remote area (Karelia) with 23 and 43 μg/kg AOL and 50 and 42 μg/kg CPA in the aerial parts and flowers, respectively.

2. The occurrence and concentration of mycotoxins in aboveground parts (1), **flowers** (2) **and beans** (3) **of deer vetch and *Vicia* L. plants** (Moscow Province, 2019)

Mycotoxin	*V. sepium* L.		*V. cracca* L.		*Lotus corniculatus* L. s.l.		
	1 (n = 4)	2 (n = 4)	1 (n = 5)	2 (n = 5)	1 (n = 4)	2 (n = 3)	3 (n = 3)
T-2	1	2	2	2	–	3	3
	3	2, 3	2, 3	2, 2		2-3-3	3-6-10
DON	–	–	1	1	–	–	1
			100	95			71
ZEN	–	–	1	-	–	1	2
			16			16	16, 18
FUM	–	–	2	-	–	–	–
			380, 420				
AOL	4	4	4	5	4	3	3
	20-26-34	20-29-35	14-29-38	23-33-47	22-36-50	31-42-48	40-41-44
OA	1	–	1	1	4	-	3
	5		8	9	5-7-8		5-10-18
CIT	1	–	1	–	–	–	1
	47		30				23
STE	1	–	2	–	–	–	–
	14		12, 17				
AB_1	–	–	–	–	–	–	–
CPA	4	4	5	5	3	3	2
	160-200-275	79-140-195	50-130-200	48-100-160	39-65-97	52-70-105	61, 105
MPA	4	4	–	–	–	2	2
	16-23-38	14-16-18				13, 20	25, 32
EA	4	3	5	3	3	–	2
	10-19-26	2-3-5	3-14-35	2-9-24	2-2-3		3, 11
EMO	2	1	–	1	4	3	3
	13, 25	16		25	40-75-140	49-75-120	165-205-250
PR	–	–	–	–	–	1	–
						260	
ROA	–	–	–	–	–	–	–

N o t e. Mycotoxins are T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (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), and PR-toxin (PR); n is the number of studied specimens. The upper value in lines is the number of positive specimens (n^+) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

Specific features extended to beans. In the spring vetch, the composition of mycotoxins was replenished with T-2, DON and PR, the frequency of detecting MPA was reduced with the same regular detection and constant concentration of AOL and CPA, as in the aboveground part, and in the meadow, there were clear differences in practically no contamination of beans and vegetative plants was observed (see Table 3).

For plants of the genera *Lupinus* and *Galega*, despite the contrasting content of mycotoxins [14], the differences between the generative organs and vegetative ones had common features (Table 4). In many-leaved lupine of 14 components characteristic of the aerial part, all were found in flowers except FUM, CIT, and PR, and in beans 8 were found, excluding DON, STE, and AB_1. The occurrence and content of all other mycotoxins, except for AOL, in flowers and beans decreased. For the eastern goa's rue, a generally similar trend was observed: in flowers, of 12 mycotoxins 8 were detected, except for FUM, OA, CIT, STE, in beans 7, except for ZEN and AB_1, with one case of OA detection. The frequency of detection of T-2, CPA, MPA and EMO decreased while their average content remained unchanged (see Table 4).

Thus, despite the interspecific features, perennial leguminous plants as a whole are characterized by the preservation of mycotoxin complexes typical for the vegetative part in flowers, with a tendency to a decrease in their content. It is

possible that in plants with a long interrupted growth cycle, only a part of producers is involved in specific processes accompanying the transition to the generative phase of development. It should be noted that in two species of annual cruciferous plants (white mustard and field cabbage), the set of mycotoxins in flowers turned out to be much wider due to the group of fusariotoxins DON, DAS, ZEN, FUM, as well as MPA, EMO, and PR [17, 30]. In this regard, in the future, it is of interest to examine annual leguminous grasses that are rarely found in biocenoses of Central Russia, such as *Vicia angustifolia* Reichard, *V. hirsula* (L.) S.F. Gray, *V. tetraspermum* (L.) Schreb. and *V. pannonica* Crantz.

3. The occurrence and concentration of mycotoxins in aboveground parts (1), **flowers** (2) **and beans** (3) **of *Lathyrus* L. plants** (Moscow Province, 2019)

Mycotoxin	*L. vernus* (L.) Bernh.			*L. pratensis* L.		
	1 (n = 20)	2 (n = 4)	3 (n = 8)	1 (n = 9)	2 (n = 4)	3 (n = 6)
T-2	–	–	3 2-2-3	9 2-6-10	4 2-3-6	5 3-4-5
DON	–	–	4 64-74-83	7 105-220-455	1 97	5 125-175-240
ZEN	–	–	–	9 19-36-52	4 16-27-42	6 24-29-33
FUM	–	–	–	4 95-450-1095	–	4 89-205-355
AOL	20 21-59-100	4 39-54-63	8 33-54-86	9 60-350-960	4 10-160-580	6 98-150-190
OA	4 4-5-6	–	6 5-12-23	9 16-36-59	1 32	6 43-66-105
CIT	4 23-30-40	–	3 24-27-33	8 40-65-125	1 32	6 32-42-56
STE	6 11-15-19	–	2 12, 15	9 19-63-120	1 10	6 22-30-38
AB1	2 2, 2	–	3 2-3-4	9 3-10-19	1 2	6 2-3-4
CPA	18 72-160-255	4 79-130-180	8 74-115-225	9 63-145-315	2 66, 79	4 53-65-78
MPA	10 15-19-25	–	1 16	9 29-46-79	2 13, 22	5 26-41-60
EA	20 3-21-100	4 4-6-8	5 2-8-20	9 4-36-155	1 3	6 3-7-12
EMO	9 12-21-38	2 19, 19	6 12-24-60	9 17-71-130	1 18	6 17-25-37
PR	–	–	1 232	4 400-720-1585	–	4 425-610-815
ROA	–	–	–	4 14-185-255	–	6 3-44-110

N o t e. Mycotoxins are T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), ergot alkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B1 (AB1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), and PR-toxin (PR); n is the number of studied specimens. The upper value in lines is the number of positive specimens (n^+) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

The taxonomic assignment of micromycetes responsible for the formation of a complex of mycotoxins in herbs is still unclear. Nevertheless, in the mycobiota of higher plants of 17 families, including *Fabaceae*, the dominance of representatives of the genera *Curvularia, Acremonium, Alternaria, Penicillium, Fusarium, Stemphylium*, and *Cladosporium* [16] has already been shown, among which potentially toxigenic species are known [31]. In addition, associated fungi of other systematic groups may also be present in it, since modern science receives more and more evidence of the transfer of genome sites in the process of evolution from one organism to another [32, 33]. Participation in the toxinogenesis of endophytic fungi proper, involved in mutually beneficial symbiosis and do not exist autonomously, cannot be ruled out.

The role of endophytic fungi in plant responses to biotic and abiotic influences remains the focus of attention of researchers [34-38]. However, information on the distribution of endophytes in the host organisms is still extremely limited and contradictory. On plants of 17 families, including *Fabaceae*, it was shown that the fungi associated with them are localized mainly in the stems and leaves, to a lesser extent populate flowers, fruits, ears and inflorescences, and the root system is the most favorable organs for their existence [16]. In cereals (cocksfoot, wheatgrass, and timothy grass), the organotropic confinement of fungi *Fusarium, Alternaria*, and *Cladosporium* was established by quantitative PCR: the amount of DNA in generative organs (ears/panicles) was significantly higher than in stems and leaves [39].

An analysis of the results obtained in this work shows that toxin-forming micro-mycetes can be involved in the formation of generative organs in leguminous plants and in the response to changes in the environment. It can be assumed that they are involved in metabolic shifts that serve to transmit transformed signals from receptors that perceive changes in external factors through the "disturbance" of the hormonal status, the system of secondary messengers, genome activity, or the behavior of the cytoskeleton [40]. Given this nature of the interaction with the plant, associated toxin-forming micromycetes should rather be attributed to symbions than to endocommensals, although the data obtained in this work do not give any grounds to judge the typification of micromycetes that provide the production of mycotoxins.

4. The occurrence and concentration of mycotoxins in aboveground parts (1), **flowers** (2) **and beans** (3) **of *Lupinus* L. and *Galega* L. plants** (Moscow Province, 2019)

Mycotoxin	*L. polyphyllus* Lindl.			*G. orientalis* Lam.		
	1 (n = 17)	2 (n = 7)	3 (n = 7)	1 (n = 15)	2 (n = 5)	3 (n = 8)
T-2	17	7	6	9	5	3
	3-5-11	4-6-9	2-3-4	2-3-4	3-3-4	2-2-3
DON	16	1	–	–	–	–
	63-89-125	63				
ZEN	17	7	4	7	5	–
	16-29-47	16-34-50	12-17-21	16-25-33	19-27-34	
FUM	1	–	–	1	–	–
	56			95		
AOL	17	7	7	15	5	8
	65-155-295	63-105-130	19-145-785	11-31-66	25-47-81	14-27-63
OA	14	4	2	1	–	1
	5-8-12	5-6-6	5, 6	5		5
CIT	5	–	–	3	–	–
	20-36-74			23-24-25		
STE	15	1	–	5	–	–
	12-24-33	15		11-18-38		
AB1	17	7	–	3	4	–
	3-5-8	2-3-4		2-2-2	2-2-2	
CPA	17	7	4	14	5	4
	130-475-870	150-260-355	68-73-79	41-115-255	82-110-140	74-105-125
MPA	16	7	2	8	5	1
	13-29-47	18-23-29	14, 16	15-22-37	16-20-22	20
EA	16	7	4	12	4	4
	8-70-160	2-4-10	3-9-16	3-12-50	2-3-4	2-3-3
EMO	17	7	6	8	4	2
	31-155-355	25-49-85	26-84-130	12-14-15	13-17-25	11, 26
PR	7	–	–	–	–	–
	205-290-400					
ROA	–	–	–	–	–	–

N o t e. Mycotoxins are T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), ergot alkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B1 (AB1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), and PR-toxin (PR); n is the number of studied specimens. The upper value in lines is the number of positive specimens (n^+) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

Thus, in the generative organs of perennial legumes of the genera *Trifolium, Lathyrus, Vicia, Lotus, Lupinus*, and *Galega*, the mycotoxin complexes are generally similar to those found in the vegetative parts and in some species have differences in the content or ratio of individual components. A number of features have been established in the contamination of flowers of plants of the genus *Trifolium*: the combined occurrence of ochratoxin A and citrinin in comparable quantities (*T. hybridum* L., *T. medium* L.), a sharply increased content of alternariol (*T. hybridum*), increased accumulation of T -2 toxin, deoxynivalenol, zearalenone and fumonisins (*T. pratense*, *T. hybridum, T. medium*), and T-2 toxin (*T. repens*). In two species of the genus *Lathyrus*, a common feature was a tendency towards a decrease in the content of mycotoxins in flowers, while for beans there was a similarity with a whole plant (*L. pratensis*) and replenishment of the composition with T-2 toxin, deoxynivalenol, PR-toxin with a decrease in the detection rate of mycophenolic acid (*L. vernus*). In sheepfoot (*Lotus corniculatus*), fusariotoxins were found only in the generative organs. In the examined representatives of the genera *Vicia, Lupinus*, and *Galega*, the metabolic background in flowers was generally weakened, and in beans it did not have clear signs of a change in the component composition and a sharp variation in the content. Taxonomically related species and subspecies of the genera *Lotus, Vicia, Lupinus*, and *Galega* are promising for the successful continuation of work on assessing the contribution of toxigenic micromycetes to internal processes and plant responses to external factors. The comparative mycotoxicological analysis of the generative and vegetative organs, carried out by us, in the future it is advisable to extend to plants and other taxonomic groups, which will make it possible to form more definite ideas about the physiological role of associated microscopic fungi.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 979-989
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Southern and subtropical gardening

UDC 634.232:581.4:631.559

doi: 10.15389/agrobiology.2021.5.979eng
doi: 10.15389/agrobiology.2021.5.979rus

MORPHOLOGICAL AND BIOLOGICAL PECULIARITIES OF SWEET CHERRY PRODUCTIVITY DEVELOPMENT IN THE SOUTH OF THE NON-CHERNOZEM ZONE

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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 within the framework of the theme "Develop effective breeding technologies, create geographically and edaphically differentiated varieties of forage and fruit crops of a new generation using modern breeding methods based on the wide use of biodiversity of plant resources of cultural and natural flora" of the Programs for fundamental research RAS (AAAA-A19-119121090123-8)
Received July 8, 2021

Abstract

Sweet cherry (*Cerasus avium* L. Moench) is a valuable fruit crop; its industrial planting is concentrated mainly in the South of Russia because of insufficient winter hardiness. Nowadays, 16 varieties have been adapted in the Non-Chernozem zone, and 11 varieties bred in the Russian Lupin Research Institute are under the testing. A deep understanding of the patterns of development and formation of the yield components allows for a better use of genetic potential of the species. This study, for the first time, revealed the significant variability in morphobiological indices of new sweet cherry varieties in the Non-Chernozem zone conditions, which provides new possibilities for genotype selection and commercial planting. The yield components are shown to be related to each other but only some correlations are significantly valid. The cluster analysis grouped the varieties by growth and fruiting similarity, and the factors with the highest contribution were found. The work aimed to study morphobiological parameters determining productivity of sweet cherry plants and to highlight genotypes which are valuable for breeding and commercial use. The experiment estimated 23 sweet cherry varieties for 9 morphobiological traits, i.e., the number of annual shoots, the average length of annual shoots, the number of sprays ("May bouquets"), the number of flower buds per annual shoot, the number of flower buds per sprays, the number of flowers per flower bud, yield, crown width, trunk circle (the garden experimental plots, the All-Russian Lupine Research Institute, Bryansk Province, 1991-1996). Estimation of variation coefficients allowed us to classify the varieties into two groups. The first group consists of varieties with high variation degree (more than 10 %) of such correlated traits as the number of annual shoots, the average length of annual shoots, the number of sprays, the number of flower buds per annual shoots and the number of flower buds per sprays. In this group, the varieties Podarok Petelinu, Teremoshka, Bryanochka, 2-3-67, 2-6-36, 2-3-45, Odrinka, Krasnaya plotnaya, Yantarnaya, 2-5-2 and 2-3-35 formed correlation pleiades. The pleiades had different power and strength. The varieties of this group are appropriate for breeding for a complex of economic valuable traits. The all tested genotypes made the second group with the variation degree for the number of flowers per flower bud (Cv = 1.0-6.0 %), crown width (Cv = 2.0-5.0 %), and trunk circle (Cv = 0.3-0.4 %) less than 6 %. Only seven of 36 pair correlations are significant. The significant pair correlations are the average length of annual shoots—the number of annual shoots (r = –0.49, p = 0.016); the average length of annual shoots—the number of sprays (r = 0.73, p = 0.000); the average length of annual shoots—crown width (r = 0.74, p = 0.000); the average length of annual shoots—trunk circle (r = 0.42, p = 0.044); the number of annual shoots—the number of flower buds per annual shoots (r = 0.77, p = 0.000); the number of sprays—crown width (r = 0.59, p = 0.003); crown width—trunk circle (r = 0.54, p = 0.008). There is no link between the yield and its components. The cluster analysis resulted in four clusters grouping the varieties that are similar in terms of the generalized indicator of the studied traits. It makes easier to select initial lines for breeding. The lack of the significant valid

correlations between yield and morphological traits made us to apply the factor analysis which revealed four factors with eigenvalues of > 1. The contributions of these factors to the observed variability are 35.9, 18.6, 11.9, and 11.5 %. The other four factors can be regarded as scree ones.

Keywords: sweet cherry, varieties, variability, productivity, correlation, clustering, factor analysis, the Russian Non-Chernozem zone

Sweet cherry, as a fruit crop, recently appeared in the variety of the Non-Chernozem zone. The first zoned sweet cherry varieties were Bryanskaya rosovaya, Iput, and Revna (1983) bred at the Russian Lupin Research Institute (RLRI). Nowadays, the industrial range of such zone includes 16 varieties bred in the Russian Lupin Research Institute, for which copyright certificates have been obtained, 3 varieties bred in the Federal Horticultural Research Center for Breeding, Agrotechnology and Nursery (FHRCBAN), and one variety bred at the Institute for Fruit Growing [1].

The selection of fruit crops abroad is carried out in accordance with the European Breeding Program [2]. In Russia, programs for breeding and research on varieties of fruit, berry and nut crops have been developed for these purposes [3, 4]. The biological characteristics of the growth and fruiting of sweet cherries are insufficiently studied. The authors of previous studies on southern varieties characterize sweet cherries as vigorous with weak branching and predominant fruiting (70-75% of fruit buds) on sprays ("May bouquets") [5-7]. In central Russia, fruiting is concentrated mainly on annual shoots and sprays [8, 9]. Most of these works are of a general nature and do not contain sufficient information on the quantitative relationships of biological traits affecting the productivity of sweet cherries.

Yield is a complex trait, mainly due to its defining morphobiological parameters and their ability to withstand environmental stress factors [10]. In sweet cherries, the process of crop formation lasts from the differentiation of fruit buds in July to ripening of fruits in June-July of the next year. For this reason, there is a high risk of annual exposure to abiotic and biotic stressors at any stage of the annual plant development cycle. [11-13]. Reduced yield of fruit crops is caused by low cold resistance of plants [14-16], high temperature during flowering reducing fruit set [17, 18], disturbance of stages of morphogenesis [19], instability of fruiting [20-22], and abnormal temperatures during formation flower buds [23-25].

Many researchers emphasize the role of the variety, its yield ability, and stability [26-28] in the formation of crop in case of weather anomalies, which can lead to freezing of trees and the defeat of plantations by fungal diseases [29-31]. In recent years, considerable attention has been paid to the selection and use of vegetatively propagated rootstocks for fruit crops both abroad [32-34] and in Russia [35-37]. Their influence has been shown not only on growth and productivity [38, 39], but also on disease resistance [40] and product quality [41-43].

Recently, biplot analysis is used for in-depth study of genotypes in multivariate experiments. This is a two-level graph, where samples are displayed as dots and variables as vectors, which makes it possible to more fully interpret the interaction of the genotype and the environment [44]. The integral indicator of the breeding value of the genotype provides a comprehensive assessment of the yield and its stability, which is important when selecting the starting material [45, 46].

This study, for the first time, revealed the significant variability in morphobiological indices of new sweet cherry varieties in the Non-Chernozem southern zone conditions, which provides new possibilities for genotype selection and commercial planting. It was found that yield components are interconnected, but only some of them have reliable correlations. By means of cluster analysis, the varieties were grouped by growth and fruiting similarity. The factors with the highest to the formation of yield have been identified.

The work aimed to study morphobiological parameters determining productivity of sweet cherry plants and to highlight genotypes which are valuable for breeding and commercial use.

Materials and methods. The study was conducted in 1991-1996 on a garden experimental plot of the Russian Lupin Research Institute (Bryansk Province).

The experiment estimated 23 sweet cherry (Cerasus avium L. Moench) varieties for nine morphobiological traits. The length of parent branch and all branching of the 2nd and subsequent orders by age-specific zones has been measured. Structural elements were counted for each variety on four parent branches of the lower crown layer (based on technical capabilities). The number and length of annual shoots, the number of fruit buds on annual shoots, sprays, and flowers in a fruit bud were determined. The productivity per 1 running (linear) meter of the parent branch was assessed, the crown width and the trunk circle were measured, and the yield was determined [6].

The experimental data were statistically processed according to the recommendations [3, 4] using one-way ANOVA test [47], and multidimensional statistical methods (cluster and factor analyzes) [48, 49] in the STATISTICA 7.0 package (StatSoft, Inc., USA). The mean values for the traits (M), standard deviations ($\pm$SD), and coefficients of variation (Cv) were calculated.

Results. The use of a limited range of varieties and forms in breeding results in nuclear and cytoplasm uniformity of hybrids, reducing their adaptability and increasing genetic vulnerability. Involvement in breeding of varieties and hybrids of various economically valuable traits increases the variance of traits and creates the possibility of selecting valuable genotypes.

An extremely important characteristic of the starting material is the degree of its variability. In this study, the diversity of varieties was assessed using two indices — the limit (lim) and the coefficient of variation (Table). The minimum and maximum number of annual shoots in the specimens differed 7.4-fold, the number of sprays 21.3-fold, the average length of annual shoots, crown width, trunk circle, the number of fruit buds on annual shoots, the number of sprays, flowers in the bud and yield 1.6-4.0 times.

Coefficient of variation (Cv) allows comparing traits with different units of measurement and largely reflects the reaction of genotypes to external influences. According to the variability of traits, the studied genotypes were divided into two groups. The first group comprises varieties with a high degree of variation of the number of annual shoots (8 genotypes, Cv = 12-28%), the average length of annual shoots (9 genotypes, Cv = 11-23%), the number of sprays (13 genotypes, Cv = 11-24%), the number of flower buds per annual shoots (9 genotypes, Cv = 11-26 %), and the number of flower buds per sprays (17 genotypes, Cv = 7.0-49 %). In the rest genotypes, variability of these traits did not exceed 10 %. In this group, the varieties with high (> 10%) coefficient of variability of correlated traits forming the correlation Pleiades have been identified: Podarok Petelinu ($V_2V_3V_4$), Teremoshka ($V_1V_3V_4$), Bryanochka ($V_1V_2V_3$), 2-3-67 ($V_1V_2V_4$), 2-6-36 ($V_2V_3V_4$), 2-3-45 (V_1V_3), Odrinka (V_2V_4), Krasnaya plotnaya (V_2V_4), and Yantarnaya (V_3V_4), 2-5-21 (V_1V_3), 2-3-35 (V_1V_2).

All tested genotypes made the second group with the variation degree not exceeding 6% for the number of flowers per flower bud (Cv = 1.0-6.0 %), crown width (Cv = 2.0-5.0 %), and trunk circle (Cv = 0.3-0.4 %).

Comparison of the coefficients of variation denotes significant opportunities for selection of varieties for breeding according to the first group of traits and less - according to the second group of traits, which are probably under stricter genetic control.

Statistical characteristics of the structure of the yield of sweet cherry (*Cerasus avium* L. Moench) varieties in the south of the Non-Chernozem zone of the Russian Federation (Bryansk Province, settlement Michurinskii, 1991-1996)

Variety	Index for 1 lin.m. of parent branch										Number of flowers per bud, units		Yield, dt/ha		Crown width, m		Trunk circle, cm	
	annual shoots				Number of sprays		Number of fruit buds											
	number		average length, cm				on annual shoots		on sprays									
	M±SD	*Cv*, %	*M*±SD	*Cv*, %	*M*±SD	*Cv*, %	*M*±SD	*Cv*, %	*M*±SD	Cv, %	*M*±SD	*Cv*, %	*M*±SD	*Cv*, %	*M*±SD	*Cv*, %	*M*±SD	*Cv*, %
Podarok Pitelinu	27.5±0.99	3	18.3±2.34	13	40.3±5.60	14	102.6±26.78	26	133.3±9.31	7	3.0±0.48	5	39.5±1.13	0.4	4.6±0.14	3	38.2±0.85	0.4
Teremoshka	23.9±3.82	16	13.8±0.42	3	8.2±1.60	20	69.6±7.70	11	22.1±4.40	20	2.9±0.15	1	59.8±1.41	0.2	3.0±0.18	5	33.1±2.11	0.4
2-5-21	23.5±3.82	16	12.9±1.69	13	4.6±0.55	11	130.0±13.00	10	22.2±6.16	28	3.7±0.03	4	38.9±2.69	0.4	4.0±0.28	4	35.4±2.54	0.4
Veda	23.2±0.64	3	29.6±1.50	5	49.2±1.62	3	122.3±8.54	7	136.5±12.24	9	2.7±0.15	5	53.6±1.56	0.3	4.6±0.21	3	35.2±0.23	0.4
2-3-35	22.4±5.09	23	18.8±2.66	14	5.8±0.06	1	127.8±1.28	10	22.2±2.86	13	4.4±0.14	6	34.7±3.22	0.6	4.6±0.24	3	40.8±1.84	0.3
Ovstuzhenka	21.6±1.98	2	26.0±1.82	7	52.3±5.72	11	84.4±2.52	3	146.4±17.52	12	3.0±0.26	5	75.5±1.06	0.2	4.8±0.25	3	35.8±1.13	0.4
Zolotaya loshchitskaya	20.6±1.98	10	18.7±0.95	5	32.8±7.92	24	78.2±3.90	5	131.4±31.44	24	4.1±0.15	4	31.0±2.12	0.5	3.2±0.10	4	35.1±0.42	0.4
2-3-67	18.6±0.85	5	29.3±6.67	23	88.5±13.32	14	103.3±19.57	19	354.5±169.92	48	3.1±0.16	5	19.0±0.99	0.7	5.0±0.28	3	43.7±2.57	0.3
Raditsa	14.7±1.41	10	22.2±1.54	7	42.7±7.31	17	125.5±6.30	5	192.8±30.72	16	3.3±0.15	2	57.0±4.24	0.3	4.6±0.21	3	42.6±2.26	0.3
2-7-39	14.7±0.54	4	28.3±0.28	1	39.3±0.39	1	64.6±9.75	15	118.2±57.82	49	3.5±0.04	4	62.0±0.35	0.2	5.4±0.28	3	54.3±1.41	0.3
2-8-28	14.4±2.83	20	14.1±0.84	6	22.0±0.88	4	52.5±3.18	6	60.3±24.60	41	3.0±0.14	5	27.5±1.70	0.5	4.2±0.28	3	37.5±3.25	0.4
Revna	12.3±1.13	9	25.5±0.26	1	60.3±3.60	6	61.9±0.62	1	265.4±26.50	10	3.5±0.15	4	45.3±1.13	0.3	4.4±0.14	3	40.7±0.85	0.3
2-7-5	11.4±1.41	12	22.2±1.47	7	29.0±3.77	13	57.9±5.22	9	76.6±6.84	9	3.2±0.14	4	64.3±0.42	0.2	3.0±0.14	5	38.1±0.54	0.4
2-6-36	11.2±0.42	4	27.1±4.59	17	35.4±3.96	11	66.1±5.28	8	96.5±11.52	12	3.4±0.12	5	54.0±0.50	0.3	5.8±0.33	2	47.4±0.85	0.3
2-6-32	10.8±0.57	5	26.7±1.89	7	46.0±2.76	6	41.7±5.04	12	124.3±13.64	11	3.1±0.17	5	20.8±0.44	0.7	5.0±0.27	3	34.4±3.52	0.4
Odrinka	11.8±0.42	4	25.6±3.38	13	45.7±10.12	22	46.8±10.34	22	146.7±8.76	6	2.8±0.15	6	38.2±0.14	0.4	4.4±0.24	3	41.1±0.71	0.3
Rozovyi zakat	10.5±2.97	1	23.5±0.72	3	51.9±4.16	8	73.5±9.00	15	260.5±58.80	33	2.3±0.17	4	51.9±2.69	0.3	5.4±0.14	2	34.3±1.56	0.4
2-3-30	11.0±0.14	1	16.6±3.34	22	32.0±3.20	10	50.3±3.57	7	99.3±34.65	35	3.5±0.09	4	70.8±2.12	0.2	4.0±0.28	4	36.2±2.97	0.4
2-3-45	10.1±1.48	15	37.8±2.66	7	97.9±13.72	14	60.0±1.80	3	274.5±38.36	14	3.4±0.14	1	64.5±2.47	0.2	6.6±0.21	2	41.8±0.99	0.4
Tyutchenka	8.4±1.27	15	26.9±1.35	5	56.3±2.80	5	40.3±4.40	10	242.4±108.90	45	2.9±0.30	5	60.5±3.25	0.2	5.2±0.21	3	44.5±1.56	0.3
Krasnaya plotnaya	4.8±0.28	6	35.4±7.00	20	31.3±2.79	9	53.7±6.48	19	110.6±16.50	15	2.9±0.14	5	51.0±2.83	0.3	5.2±0.28	3	39.9±2.81	0.4
Yantarnaya	4.7±0.14	3	30.2±0.60	2	32.8±5.82	16	33.3±4.65	14	98.2±20.58	21	3.0±0.14	5	31.3±1.41	0.5	5.0±0.33	3	39.1±1.56	0.4
Bryanochka	3.7±0.71	20	27.0±2.97	11	25.7±3.25	13	50.8±5.10	10	83.3±5.81	7	3.0±0.15	5	62.7±2.83	0.2	4.6±0.28	3	38.6±3.67	0.4
lim	3.7-27.5		12.9-3.78		4.6-97.9		33.3-130.0		22.1-354.5		2.3-4.4		19.0-75.5		3.0-6.6		33.1-54.3	
min/max	7.4		2.9		21.3		3.9		16.0		1.9		4.0		2.2		1.6	

All studied traits that form the yield correlate although with the different degree of the relationship (Fig. 1).

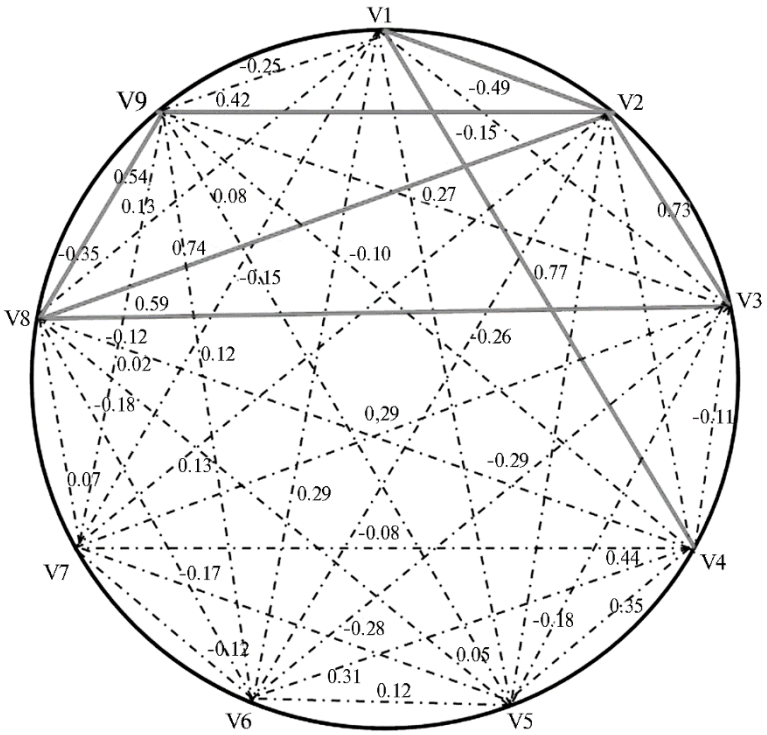


Fig. 1. Matrix of complete correlations of structural fruiting traits in 23 varieties of sweet cherry (*Cerasus avium* L. Moench) in the south of the Non-Chernozem zone of the Russian Federation: solid lines represent reliably significant relationships, dash-dotted lines unreliable relationships; V1 — the number of annual shoots, V2 — average length of annual shoots, V3 — the number of bouquet branches, V4 — the number of fruit buds on annual shoots, V5 — the number of fruit buds on bouquet branches, V6 — number of flowers in a fruit bud, V7 — yield, V8 — crown width, V9 — trunk circumference (trait values are given per 1 linear meter) (Bryansk Province, settlement Michurinskii, 1991-1996).

Only seven of 36 pair correlations were significant: the average length of annual shoots—the number of annual shoots (V_2—V_1) (r = –0.49, p = 0.016); the average length of annual shoots—the number of sprays (V2—V3) (r = 0.73, p = 0.000); the average length of annual shoots—crown width (V_2—V_8) (r = 0.74, p = 0.000); the average length of annual shoots—trunk circle (V_2—V_9) (r = 0.42, p = 0.044); the number of annual shoots—the number of flower buds per annual shoots (V_1—V_4) (r = 0.77, p = 0.000); the number of sprays—crown width (V_3—V_8) (r = 0.59, p = 0.003); crown width—trunk circle (V_8—V_9) (r = 0.54, p = 0.008). For most traits, correlations were weak and statistically insignificant. No link between the yield and its components is of special interest. Indeed, vegetative traits do not directly affect the crop, but they indirectly determine its formation. For instance, the length of annual shoots is so important that it is used to classify varieties. Such varieties as Teremoshka, Zolotaya loshchitskaya 2-8-28, 2-5-21, 2-3-30 were characterized by the shortest shoots (13-19 cm). This type of branching promotes restrained growth of the tree. The longest shoots (30-38 cm) were specific for varieties Veda, Krasnaya plotnaya, Yantarnaya, Ovstuzhenka, 2-3-45, 2-3-67. The amount of annual growth determines not only the habitus of the tree, but also serves as the basis for the formation of fruit buds.

It should be noted that there are groups of traits that were interconnected by high correlation coefficients, called correlation pleiades. The correlated traits

of the pleiades are characterized by power (G) and strength (D) indices. The most powerful and strong pleiade was $V_2V_3V_8$ (G = 3.0; D = 0.69), slightly inferior to it in strength $V_2V_8V_9$ (G = 3.0; D = 0.57), the weakest was $V_2V_1V_4$ (G = 3.0; D = 0.34). Probably, the close relationships between the traits of these pleiads were largely controlled genetically.

The data obtained through cluster analysis are very informative. The grouping of varieties by the nature of growth and fruiting is shown on the dendrogram obtained by the Ward's method of clustering, where the Euclidean distance was the measure of the relationship (Fig. 2). The resulting cluster included two groups.

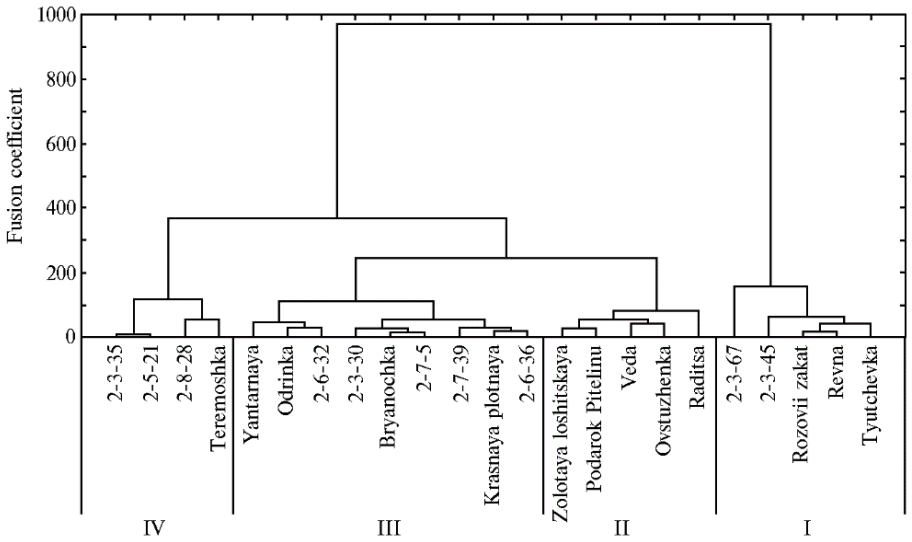
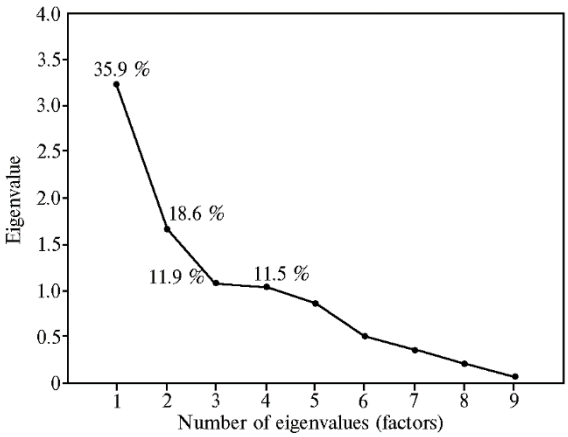


Fig. 2. The dendrogram of similarities and differences between sweet cherry (*Cerasus avium* L. Moench) varieties with regard to the generalized index of productivity elements (Ward's method): I-IV — groups of the same type of growth and yielding (Bryansk Province, settlement Michurinskii, 1991-1996).

The first group (I sub-group) comprised 5 varieties: Tyutchevka, Revna, Rozovyi zakat, 2-3-45, 2-3-67 with long shoots (24-38 cm) and 77-86 % flower buds on sprays. The second group included three sub-groups of varieties (II, III, and IV). Sub-groups II and III included 14 varieties heterogeneous in terms of growth and fruiting. They were highly branched, had medium-sized shoots, mixed fruiting (53-60% of flower buds were located on sprays, and 40-47% on annual growth). The subgroup IV included 4 varieties with predominant fruiting at one-year growth: 2-8-28 (71%), Teremoshka (76%), 2-3-35 (81%), 2-5-21 (86%). The emergence of varieties with this type of fruiting was associated with their inbred origin. The increased homozygosity contributes to the manifestation of hidden recessive genes controlling this trait.

Since there were varieties in each group that were similar in terms of the generalized index of the studied traits, varieties of different groups and subgroups, significantly different in terms of productivity, should be used as initial pairs for crosses. The difference in varieties by the nature of growth and fruiting must be taken into account when forming the crown, pruning trees and carrying out other agro-technical measures.

The lack of the significant valid correlations between yield and morphological traits made us to apply the factor analysis. This method allows identification of hidden, but objectively existing patterns affecting the formation of yield and characterizing factors in accordance with the contribution of each variable.



from Fig. 3. Influence of factor load on "yield" trait of sweet cherry (*Cerasus avium* L. Moench) varieties with regard to contribution of each variable (Bryansk Province, settlement Michurinskii, 1991-1996).

Fig. 3 shows the curve derived from the spectrum of eigenvalues. The number of chosen factors was determined by the break in the linear dependence curve on the graph of eigenvalues of the primary data matrix at point 4 (Y = 1.04; > 1). According to criteria, Kaiser and Cattell [50, 51] indicated factors with eigenvalues exceeding 1. The larger the eigenvalue, the greater the factor contributes to the total variability, which indicates its significance. In our work, the following first four factors with the most significant contribution to the observed variability have been revealed: factor 1 (35.9 % of total variability) mainly characterizing the length of shots, crown width, and the number of sprays; factor 2 (18.6%) — number of shots, number of fruit buds on shots; factor 3 (11.9 %) — trunk circle, number of flowers per bud; and factor 4 (11.5 %) — yield and number of buds on spray. The remaining factors (see Fig. 3) had an insignificant share of the total variance and belonged to the so-called factorial or "stony" scree [51].

By its genetic nature, sweet cherry belongs to crops of the southern regions. Thanks to the creation of winter-hardy varieties of sweet cherry, it became possible to promote this culture to the northern regions. It has recently appeared in the range of the Non-Chernozem zone (1). Sweet cherry has a high yield potential, which is realized in optimal weather conditions. According to the literature, the period of laying and differentiation of fruit buds lasts 117-130 days (from July of the current year to April of the next year); the studies identified five stages of organogenesis, or phenological phases, when plants are most sensitive to stress, which leads to a reduction in yield components [10, 11]. Unfavorable conditions during this period lead to the death of flower rudiments and loss of crop production [10].

Studies of Upadysheva [9] conducted for two years on three varieties of sweet cherry has shown that the active growth of shoots and fruit formations (sprays) serves the basis for increasing productivity. Works of other authors studying the fruiting nature of sweet cherry also emphasize the significant contribution of the load of trees with flower buds, their placement on annual branches and sprays of different ages [9, 10, 35], the number of flowers in fruit buds, trunk circle and crown size [37, 42] in the formation of productivity. At the Russian Lupin Research Institute, the work was carried out for 6 years on 23 sweet cherry varieties by 9 traits to give a deeper assessment of the link between morphobiological traits and the productivity of sweet cherry. It was established that varieties significantly differed in the nature of fruiting (see Fig. 2). Based on our data, the number of flower buds on annual shots varied from 33.3 to 130 and on the sprays from 22.1 to 354.5. In Tyutchevka, Revna, Rozovyi zakat, 2-3-45, 2-3-67 cultivars, sprays were mainly fruiting (77-86 %). Another 14 varieties had a mixed type of fruiting: 53-60 % of flower buds were located on sprays, 40-47 % on annual shots. Teremoshka, 2-8-28, 2-5-21, 2-3-35 varieties had flower buds on annual shots (71-86 %). The manifestation of this trait is associated with the inbred origin

of these varieties. In addition, these varieties are distinguished by moderate tree growth. The latter is considered as a significant economic characteristic in industrial plantings.

Therefore, we have revealed a significant morphological variability of 23 sweet cherry varieties under the conditions of the Non-Chernozem zone of the Russian Federation, which allow us to select valuable genotypes for breeding and production. High degree of variability (over 10%) by the number of annual shots, average length of annual shots, number of flower buds on annual shots, and the number of sprays was noted in the varieties Podarok Petelinu, Teremoshka, Bryanochka, Odrinka, Krasnaya plotnaya, Yantarnaya, and 2-3-35. Low degree of variability (less than 6%) by the number of flower buds, number of flowers on flower buds, crown width, and trunk circle was revealed in all studied varieties. Herewith, Ovstuzhenka, 2-3-45, Bryanochka, Tyutchevka, 2-7-5, 2-7-39, Teremoshka, and 2-3-45 were distinguished in terms of yielding. The link between the yield indices was different. The "yield" as a trait variable did not correlate with any of the studied yield structure elements. Factor analysis identifies hidden but objectively existing patterns that affect the yield formation. Our findings identified four factors with the most significant contribution to yield formation. Cluster analysis grouped the varieties with regard to their growth and fruiting features. Four clusters identified according to the generalized index of the studied characteristics will facilitate the selection of breeding forms. Varieties of different groups and clusters with significant differences in productivity elements should serve as pairs for crosses.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 990-998
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 634.25:632.4:632.9(470.62)

doi: 10.15389/agrobiology.2021.5.990eng
doi: 10.15389/agrobiology.2021.5.990rus

BIOLOGIZED CONTROL OF THE MAIN DISEASES OF CHERRY PLUM IN HUMID SUBTROPICS OF THE KRASNODAR REGION

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Received September 22, 2021

Abstract

Chemical fungicides are usually used to combat phytopathogens in stone fruit crops, in particular cherry plum (*Prunus cerasifera* Ehrh.). Biofungicides are an alternative to chemicals. Biofungicides preparations are based on saprotrophic bacteria and fungi. Most of these drugs suppress the reproduction of fungal plant pathogens thus reducing the infectious load. These drugs induce immune responses in plants, increasing their resistance to pathogens and unfavorable environmental factors. In this work, for the first time in humid subtropics, we have given a comparative assessment of the biological effectiveness of the biofungicides Baktofit, Vitaplan, Gamair, Fitosporin-M, used together with the chemical fungicides Skor and Horus at half the rate of application. Our goal was to develop systems of biologic protection of cherry plum from the main diseases (clusterosporium disease, moniliosis, and gray rot of fruits) based on biological products in combination with reduced doses of chemical fungicides in conditions humid subtropics of the Krasnodar Territory. The work also aimed to assess the effect of such systems on the yield and annual growth of axial shoots of cherry plum. The research was carried out in 2015-2017 in plantings of cherry plum variety Obilnaya at the production sites of the State Unitary Enterprise of the Krasnodar Territory "Oktyabrsky" (Sochi). We compared the effect of various *Bacillus subtilis* bacteria-based fungicides, namely Baktofit, SP (Sibbiopharm, Russia), Vitaplan (AgroBioTechnology, Russia), SP, Gamair, SP (AgroBioTechnology, Russia), and Fitosporin-M, Zh (BashInkom, Russia) in mixes with half the norms of Horus (Horus®, Syngenta AG, Switzerland) and Skor (Skor®, Syngenta AG, Switzerland), as well as the *Trichoderma harzianum*-based preparation Glyokladin, Zh (Agrobiotekhnologiya, Russia) without mixing with Horus and Skor. Trees were sprayed with fungicide solutions twice during the spring season, in the bud-swelling phase and in the phase of active shoot growth after flowering. The water was control during the treatment. The chemical fungicides Horus (1st treatment) and Skor (2nd treatment) were as a reference. The intensity of the development of clusterosporiosis, monilioz and gray rot of fruits and the biological effectiveness of the preparations used, the yield and the value of the annual growth of axial shoots were assessed. According to a three-year experiment, the bacterial biofungicide Fitosporin-M in combination with half the consumption rates of Horus and Skor and the fungal biofungicide Glyocladin without chemical fungicides showed the maximum statistically significant biological effectiveness in protecting cherry plum from clusterosporia, gray rot and brown monilial rot. The efficiency of Gamair turned out to be slightly lower, but it still exceeded the indicators of the reference treatment with chemical fungicides. The efficiency of Baktofit in most cases was lower than the reference. Vitaplan showed the lowest efficiency in all variants. The biological effectiveness of all tested preparations against monilial brown rot was lower than in the case of gray rot and clusterosporiosis. The yield of cherry plum when treated with Fitosporin-M, Glyokladin and Gamair was practically the same in all tests (9.8-11.5 t/ha), being approximately 1.8-1.9 times higher than the control values (5.4-5.7 t/ha) and 1.1-1.2 times higher than the reference (8.9-9.1 t/ha). When using Baktofit, the yield of cherry plum was almost equal to the reference (8.7-9.4 t/ha), while when treated with Vitaplan it was regularly below the reference (6-7 t/ha). The preserved yield upon treatment with biological preparations reached 9.7-11.5 t/ha. The increase in the growth of the axial shoots of cherry plum compared to the control turned out to be maximum (1.7 times) for Glyokladin and slightly less for Fitosporin-M and Gamair, while when using Baktofit and Vitaplan it was 15-25 % lower than the reference values and only 1.1-1.2 times higher than the control values. Thus, biological plant protection products based on *Bacillus subtilis* and T*richoderma harzianum* can be successful against the main diseases of cherry plum in the subtropics of Krasnodar Territory. It is

acceptable to use bacterial preparations in a mixture with lower rates of chemical fungicides. This approach is more environmentally friendly and reduces the cost of plant protection.

Keywords: biological plant protection, biofungicides, fungal plant pathogens, stone fruit crops, biological effectiveness, *Bacillus subtilis*, *Trichoderma harzianum*

The natural and climatic conditions of the subtropics of the Krasnodar Territory make it possible to obtain high yields of stone fruit crops, in particular cherry plum (*Prunus cerasifera* Ehrh.). However, they also favor the intensive development of a number of harmful phytopathogenic fungi [1]. The most significant causative agents of stone fruit diseases in Sochi are *Monilinia fructigena* (Pers.) Honey and *M. laxa* (Aderh. & Ruhland) Honey, as well as recently discovered invasive species *M. fructicola* (G. Winter) Honey [2], which cause moniliosis (i.e., specific fruit rot and burns of flowers and young shoots of cherry plum). *Botrytis cinerea* Pers., the causative agent of gray rot, and *Wilsonomyces carpophilus* (Lev.) Adask., J.M. Ogawa & E.E. Butler (=*Clasterosporium carpophilum* (Lev.) Aderh., =*Stigmina carpophila* (Lev.) M.B. Ellis), the causative agent of clusterosporium disease, a complex disease of stone fruits resulted in the death of shoots, damage to fruits, and perforated leaf spot are important [3].

Chemical fungicides are usually used [4, 5] to combat the listed plant pathogens of cherry plum, despite the negative consequences of their use, e.g., toxicity, indiscriminate action, and the risk of resistance of plant pathogens [6]. An alternative to chemical fungicides are biofungicides, the preparations based on saprotrophic bacteria and fungi [7, 8]. Currently, their use has already become one of the most promising areas in the protection of agricultural crops [9, 10]. Most of these drugs simultaneously suppress the reproduction of plant pathogenic fungi, reducing the infectious load, and induce immune responses of plants, increasing their resistance to plant pathogens and other unfavorable environmental factors [11, 12]. In recent years, biofungicides have been produced in Russia based on highly effective strains of the bacterium *Bacillus subtilis* and the fungus *Trichoderma harzianum*, known as natural antagonists of plant pathogenic fungi and inducers of the immune response of plants [13-15]. Such drugs are increasingly used for plant protection, since they are able to suppress various plant pathogens and are characterized by high environmental safety [16-20]. However, so far there are only a few studies devoted to their use for the protection of stone fruit crops in Russia [21, 22].

In this work, for the first time in humid subtropics of the Russian Federation, we have given a comparative assessment of the biological effectiveness of the biofungicides Baktofit, Vitaplan, Gamair, and Fitosporin-M used together with the chemical fungicides Skor and Horus at half the rate of application on plantings of Cherry plum Obilnaya.

Our goal was to develop systems for biologic protection of cherry plum from the main diseases (clusterosporium disease, moniliosis, and gray rot of fruits) based on biological products in combination with reduced doses of chemical fungicides, and to assess the effectiveness and effect of such systems on the yield and annual growth of the axial shoots of cherry plum in the context of humid subtropics of the Krasnodar Territory.

Materials and methods. The research was carried out in plantings of cherry plum variety Obilnaya (production sites of the Oktyabrsky State Unitary Enterprise, Sochi, a 5 km distance from the Black Sea coast, 2015-2017). Trees were treated with ready-made fungicide solutions.

The following chemical fungicides were used: Horus (Horus®, WDG, 750 g/l, 0.3 kg/ha, Syngenta AG, Switzerland) and Skor (Skor®, EC, 250 g/l, 0.2 l/ha, Syngenta AG, Switzerland); bacterial fungicides based on *Bacillus subtilis* — Baktofit, SP (strain IPM 215, BA-10000 IU/g, titer of at least 2 billion spores/g;

Production Association Sibbiopharm Ltd, Russia), Vitaplan, SP (strain VKM-B-2604D, titer no less than 10^{10} CFU/g; AgroBioTechnology, Russia), Gamair, SP (strain M-22 VIZR, titer no less than 10^{11} CFU/g; AgroBioTechnology, Russia), Fitosporin-M, Zh (strain 26 D, titer no less than 1 billion live cells and spores/m; BashInkom, Innovation & Research Enterprise, Ltd, Russia); fungal biofungicide based on *Trichoderma harzianum* fungus Glyokladin, Zh (strain 18 VIZR, titer no less than 10^9 CFU/ml; AgroBioTechnology, Russia).

The experimental design was as follows. Control plants were sprayed with water (2 applications); standard with Horus, 0.3 kg/ha (1st application), Skor, 0.2 l/ha (2nd application); treatment 1 with Baktofit, 2 kg/ha + Horus, 0.15 kg/ha (1st application), Baktofit, 2 kg/ha + Skor, 0.1 l/ha (2nd application); treatment 2 with Vitaplan, 0.12 kg/ha + Horus, 0.15 kg/ha (1st application), Vitaplan, 0.12 kg/ha + Skor, 0.1 l/ha (2nd application); treatment 3 with Gamair, 0.15 kg/ha + Horus, 0.15 kg/ha (1st application), Gamair, 0.15 kg/ha + Skor, 0.1 l/ha (2nd application); treatment 4 with Fitosporin-M, 2 l/ha + Horus, 0.15 l/ha (1st application), Fitosporin-M, 2 l/ha + Skor, EC, 0.1 l/ha (2nd application); treatment 5 with Glyokladin, Zh, 3 l/ha (2 applications).

Since bacterial preparations are quite well compatible with chemical fungicides [1, 21, 22], they were used in the form of tank mixtures with half the consumption rates of chemical fungicides (Horus for the 1st application, Skor for the 2nd application).

The control, the standard, and each treatment were performed in 4-fold replication with a randomized location of plots of a 20 m^2 area each [23]. The first spraying was carried out during the bud swelling phase, the second after flowering, in the phase of active growth and development of shoots and leaves. The dates of sprayings with biologicals were shifted for a period from 2 to 11 days, depending on annual weather conditions. Spraying with solutions was carried out in the morning at an air temperature not higher than +24 °C. Since the effect of biofungicides is long-term [24], the intensity of the development of clusterosporiosis in all variants of the experiment was assessed 10 days after each spraying, and gray and brown rot of fruits 1 month after the termination of the treatments.

The degree of development of clusterosporium disease, moniliosis, and gray rot in fruits was determined according to the generally accepted method [23]. The intensity of disease development was assessed on a 5-point scale with the following gradations: 0 means no damage, 1 stands for up to 10% the leaf surface affected, 2 for 11-25%, 3 for 26-50%, and 4 for over 50% of the surface is affected.

The disease intensity index R (%), reflecting the severity of damage to each plot (variant of the experiment), was calculated by the Abbott's formula [25]:

$$R = \frac{\Sigma(a \cdot b)}{N \cdot K},$$

where $\Sigma(a \cdot b)$ is the sum of the number of diseased leaves (a) multiplied by the corresponding lesion score (b), N is total number of leaves (healthy and diseased), K is the highest score of the scale.

Biological efficiency (BE, %) was calculated by the formula:

$$BE = \frac{K - b}{K},$$

where K is the disease intensity in the control, b is the disease intensity in the test variant.

For a more complete assessment of cherry plum protection schemes, the yield (measured at the time of harvest) and the growth of shoots during the growing season (measured at the end of the growing season) were determined.

The obtained data were processed using the descriptive statistics in Microsoft Excel and Statistica 10 programs (StatSoft, Inc., USA) (26). The arithmetic mean (*M*) and standard errors of the means (±SEM) were calculated. A linear univariate analysis was used to compare several independent groups of data (experimental variants) combined by one attribute (processing variants) [23]. When assessing the ratio of intergroup variability, the Fisher's test (*F*-test) was used to test the null hypothesis of equality of means for samples − experimental variants, for a significance level of $p < 0.05$ [23]. The least significant difference (LSD_{05}) was calculated, i.e., a value indicating the border of possible random deviations in the experiment, that is, the minimum difference between the mean values of the degree of disease development and yield for each variant of the experiment and control.

Results. Weather conditions in the spring and summer months of 2015-2017 in Sochi did not significantly differ from the climatic norm [27] and contributed to the intensive development of the main diseases of the cherry plum. The maximum degree of development of clusterosporiosis and fruit rot was noted in 2015 (Table 1), which was facilitated by high air humidity and early onset of spring. The results indicate a fairly high efficiency of biofungicides (59.84-96.8%) in the protection of cherry plum from clusterosporiosis (Table 1).

1. Intensity of clusterosporiosis (DI, %) **and biological effectiveness** (BE, %) **of protection schemes for cherry plum (*Prunus cerasifera* Ehrh.) cv. Obilnaya plants** ($N = 4$, M±SEM, production sites of the Oktyabrsky State Unitary Enterprise, Sochi)

Variant	2015		2016		2017	
	DI	BE	DI	BE	DI	BE
1	6.1±0.15	74.9±0.91	4.5±0.91	77.1±1.64	2.5±0.22	79.7±2.67
2	9.6±0.21	60.1±1.39	7.8±0.31	59.8±4.00	4.6±0.14	63.2±1.71
3	4.2±0.23	82.6±1.02	2.9±0.21	85.0±1.73	1.5±0.08	88.0±0.93
5	2.6±0.21	89.2±1.02	1.6±0.08	92.0±0.40	0.8±0.13	93.6±1.22
4	1.7±0.18	93.0±0.65	0.8±0.13	95.9±0.54	0.4±0.06	96.8±0.43
Standard	4.4±0.21	81.8±0.90	3.1±0.26	84.2±2.48	1.6±0.23	97.0±2.27
Control	24.1±0.49		20.0±1.98		12.6±0.77	
LSD_{05}	0.56	2.99	0.27	8.40	0.96	5.12
	$F_o = 1611.3 > F_t = 2.57$	$F_o = 134.82 > F_t = 2.77$	$F_o = 74.09 > F_t = 2.57$	$F_o = 2\ 0.92 > F_t = 2.77$	$F_o = 172.24 > F_t = 2.57$	$F_o = 49.04 > F_t = 2.77$

N o t e. For a description of the variants, see the section "Materials and methods". The differences are statistically significant ($F_o > F_t$) at the 95% level.

Over a 3-year period, the bacterial biofungicide Fitosporin-M showed the best result in protecting cherry plum from clusterosporiosis, the fungal biofungicide Glyokladin was in the second place (see Table 1), but it should be noted that it was used without a mixture with chemical fungicides. BE when treated with mixtures of bacterial biofungicides Fitosporin-M and Gamair with half the rates of chemical fungicides, as well as the use of Glyokladin in its pure form, gave a better effect than treatment with a full rate of chemical fungicides in the reference (Table 2). Even with the highest intensity of clusterosporiosis in June 2015, after the use of Fitosporin-M, Glyokladin, and Gamair, it was no higher than with the reference application of chemical fungicides, while Baktofit and Vitaplan were somewhat inferior to them in efficiency.

When using Glyocladin, even 1 month after the cessation of treatments, the intensity of clusterosporiosis was lower than when trees were treated only with chemical fungicides. This implies a prolonged protective effect of the fungal biofungicide, which is caused not only by the suppression of the causative agent of clusterosporiosis, but also by the activation of plant defense mechanisms [28].

For Stenley plum, the best protection against clusterosporiosis was also achieved when using Fitosporin-M and Glyokladin [1]. Similar results were obtained when testing biological products based on *B. subtilis* and *T. lignorum* in the

piedmont zone of the Krasnodar Territory to protect plums from clusterosporiosis, although in the latter case, the first treatment before bud blooming was carried out only with chemical fungicides, and after flowering, only biological products were used in pure form [22].

Biofungicides also showed good results against gray and monilial brown fruit rot: the degree of fruit rot development significantly ($p < 0.05$) decreased compared to the control (Table 2), although BE of all preparations against monilial brown rot was lower than for clusterosporiosis (see Table. 1) and gray rot (Table 3).

2. Biological effectiveness (BE) **of various protection schemes for cherry plum (*Prunus cerasifera* Ehrh.) cv. Obilnaya plants against monilial brown rot** ($N = 4$, $M \pm$SEM, production sites of the Oktyabrsky State Unitary Enterprise, Sochi)

Variant	2015	2016	2017
1	62.2±3.03	68.5±2.69	71.6±3.74
2	55.4±1.79	46.0±2.28	56.6±3.24
3	75.7±2.54	77.9±2.74	81.0±2.24
5	76.5±2.65	78.8±3.01	85.0±2.41
4	80.1±3.23	82.8±4.53	87.1±3.14
Standard	69.9±2.55	72.2±3.25	80.3±3.32
LSD_{05}	9.73	7.83	7.01
	$F_o = 11.06 > F_t = 2.77$	$F_o = 29.80 > F_t = 2.77$	$F_o = 23.04 > F_t = 2.77$

N o t e. For a description of the variants, see the section "Materials and methods". The differences are statistically significant ($F_o > F_t$) at the 95% level.

3. Biological effectiveness (BE) **of various protection schemes for cherry plum (*Prunus cerasifera* Ehrh.) cv. Obilnaya plants against gray rot** ($N = 4$, $M \pm$SEM, production sites of the Oktyabrsky State Unitary Enterprise, Sochi)

Variant	2015	2016	2017
1	67.6±2.60	74.5±3.23	77.8±2.35
2	58.4±2.74	51.1±1.64	61.5±2.55
3	82.3±3.85	84.7±1.97	87.1±2.37
5	83.1±4.19	85.7±1.78	88.5±3.11
4	87.1±13.4	90.0±2.56	92.4±1.71
Standard	76.0±2.77	78.5±3.86	87.3±1.78
LSD_{05}	9.73	7.83	7.01
	$F_o = 11.06 > F_t = 2.77$	$F_o = 29.80 > F_t = 2.77$	$F_o = 23.04 > F_t = 2.77$

N o t e. For a description of the variants, see the section "Materials and methods". The differences are statistically significant ($F_o > F_t$) at the 95% level.

As in the case of clusterosporiosis, all tested biological products showed a sufficiently high biological effectiveness across a three-year experiment. For Fitosporin-M, Glyokladin, and Gamair, it exceeded at least 1.1-1.2 times ($p < 0.05$) that observed in the standard (see Tables 2 and 3). The maximum effect was observed when Fitosporin-M was used in a tank mixture with chemical fungicides, while Glyokladin and Gamair were inferior in effectiveness. Bactofit and Vitaplan have shown the lowest efficiency in the protection of cherry plum from fruit rot in comparison with other biofungicides: for both biological products, BE was always lower than the standard by 20-40% (see Tables 2 and 3).

The best result in the fight against gray rot was obtained when using half rates of Horus and Skor in combination with Fitosporin-M (87.1-92.4%). Biofungicides Glyokladin and Gamair showed higher biological effectiveness than the reference, especially compared to Vitaplan, which also had the least effectiveness.

In general, the yield of cherry plum reflected the degree of the protective effect of each tested drug: it turned out to be the highest when using Fitosporin-M; however, Glyokladin and Gamair were inferior to it. So, in 2015, the highest yield was in trees treated with Gamair (Table 4).

Due to the intensive growth of plant pathogenic fungi, the yield of cherry plum in the control was significantly (1.3-2 times, $p < 0.05$) lower than in all

experimental variants (see Table 4), and the indicators of preserved yield in variants of application of biological preparations reached 9.7-11.5 t/ha. With Fitosporin-M, Gamair and Glyokladin, the yield exceeded 1.7-1.9-fold ($p < 0.05$) the standard, with Baktofit, the results were almost the same, and with Vitaplan, the yield was lower than in the stndard, but 1.1-1.3 times higher than in the control (see Table 4).

4. Yield (t/ha) **of cherry plum (*Prunus cerasifera* Ehrh.) cv. Obilnaya plants depending on treatment with biofungicides** ($N = 4$, M±SEM, production sites of the Oktyabrsky State Unitary Enterprise, Sochi)

Variant	2015	2016	2017
1	8.7±0.55	9.2±0.23	9.4±0.18
2	7.0±0.25	6.6±0.27	6.0±0.29
3	9.8±0.35	10.0±0.25	10.2±0.33
5	9.7±0.13	10.9±0.35	11.5±0.20
4	9.7±0.30	10.5±0.30	11.0±0.35
Standard	8.9±0.29	9.1±0.27	9.0±0.27
Control	5.4±0.26	5.7±0.27	5.6±0.24
LSD_{05}	0.96	0.82	0.81
	$F_o = 26.02 > F_t = 2.57$	$F_o = 49.82 > F_t = 2.57$	$F_o = 71.77 > F_t = 2.57$

N o t e. For a description of the variants, see the section "Materials and methods". The differences are statistically significant ($F_o > F_t$) at the 95% level.

It is known that the yield of cherry plum significantly depends on the intensity of development of annual shoots during the growing season, and the studied diseases, especially clusterosporiosis, inhibit the growth of shoots [1]. Consequently, the value of the annual growth of shoots can be an important indicator for assessing both the protective and growth-stimulating effects of the tested protection agents. Based on the results of measurements of the length of axial shoots of the current year in the autumn season after the completion of growth (Table 5), biofungicides Fitosporin-M, Gamair and especially Glyokladin in the experimental variants showed better results than chemical fungicides in the reference: the use of biological products led to a 1.2-1.7-forl ($p < 0.05$) increase in the average length shoots of the current year compared the control (see Table 5).

5. Shoot growth (cm/year) **of cherry plum (*Prunus cerasifera* Ehrh.) cv. Obilnaya plants depending on treatment with biofungicides** ($N = 4$, M±SEM, production sites of the Oktyabrsky State Unitary Enterprise, Sochi)

Variant	2015	2016	2017
1	62.2±3.00	67.6±2.60	74.9±0.90
2	55.4±1.80	58.4±2.70	60.1±1.40
3	75.7±2.50	82.3±3.90	82.6±1.00
5	76.5±2.70	83.1±4.20	89.2±1.00
4	80.1±3.20	87.1±13.40	93.0±0.70
Standard	69.9±2.60	76.0±2.80	81.7±0.90
Control	45.9±2.30	51.1±1.60	55.4±1.80
LSD_{05}	0.27	0.56	0.82
	$F_o = 74.09 > F_t = 2.57$	$F_o = 1611.3 > F_t = 2.57$	$F_o = 49.82 > F_t = 2.57$

N o t e. For a description of the variants, see the section "Materials and methods". The differences are statistically significant ($F_o > F_t$) at the 95% level.

Particularly noteworthy was the effect of Glyokladin which provided maximum growth of shoots over a three-year experiment. The length of annual shoots was approximately 1.7 times greater than in the control ($p < 0.05$). Fitosporin-M showed the best results, although the length of shoots were close to those for Glyokladin. In our opinion, it is due to not only a pronounced immunomodulatory, but growth-stimulating effect of this fungal biological product [29]. With Baktofit and Vitaplan, the increase exceeded the control values only 1.1-1.2 times and was 15-25% lower than the reference, while the treatment with chemical preparations alone in the reference gave an average increase of 1.5 times as

compared to the control.

Therefore, biofungicides Baktofit, Vitaplan, Gamair, and Fitosporin-M based on *Bacillus subtilis* and *Trichoderma harzianum* could be successfully used against the main cherry plum diseases in the subtropics of the Krasnodar Territory in mixtures with half the norms of chemical fungicides. Our findings comfier the high efficiency of biological preparations, in 2015-2017, the highest efficiency against clusterosporiosis was 93-97%, against moniliosis 80-87%, and against gray rot 87-92%. The highest yield of cherry plum in these years was 9.8-11.5 t/ha, the annual growth of the shoots reached 80-93 cm.

Particularly noteworthy was the effect of Glyokladin, with the use of which the growth of cherry plum shoots was maximal according to the results of three-year experiment: the length of annual shoots was approximately 1.7 times greater than the length of similar shoots in the control ($p < 0.05$). The best results were shown by Fitosporin-M, although the length of shoots were close to that with the use of Glyokladin. In our opinion, it is due to not only a pronounced immuno-modulatory but also growth-stimulating effect of this fungal biological product [29]. In the experimental variants with Baktofit and Vitaplan, the increase exceeded the control values only by 1.1-1.2 times and was 15-25% lower than the reference, while the treatment with chemical preparations alone in the reference gave an average increase of 1.5 times as compared to the control.

Therefore, biofungicides Baktofit, Vitaplan, Gamair, and Fitosporin-M based on *Bacillus subtilis* and *Trichoderma harzianum* could be successfully used against the main cherry plum diseases in the subtropics of the Krasnodar Territory in mixtures with half the norms of chemical fungicides. The studies carried out make it possible to judge the high efficiency of biological preparations. In 2015-2017, the highest efficiency against clusterosporiosis was 93-97%, against moniliosis80-87%, and against gray rot 87-92%. The highest yield of cherry plum in these years was 9.8-11.5 t/ha, the value of the annual growth of the shoots of cherry plum trees reached 80-93 cm.

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AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 5, pp. 999-1010
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 634.6+634.7:58.05(470+213.1)

doi: 10.15389/agrobiology.2021.5.999eng
doi: 10.15389/agrobiology.2021.5.999rus

ON SPECIFIC INFLUENCE OF THE AGROECOLOGICAL CONDITIONS OF HUMID SUBTROPICS OF RUSSIA ON PRODUCTIVE POTENTIAL OF *Actinida deliciosa* (kiwifruit)

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The authors declare no conflict of interests
Acknowledgements:
We express our gratitude to the DSci in agriculture, Director of the Adler Experimental Station VIR A.P. Boyko for his assistance in the successful implementation of this research work.
The work was carried out within the framework of the theme 0492-2021-0007 "To identify the base mechanisms of adaptation of agricultural crops, ornamental plants, and artificially created biocenoses to stress factors of various nature and to study the patterns of their manifestation to develop methods for stabilizing the production process and preserving decorativeness".
Received June 9, 2021

Abstract

Actinida deliciosa (kiwi), recognized for its delicious taste and health-promoting properties, is successfully cultivated in the subtropical regions of the planet. The peculiar climate of the humid subtropics of Russia influences the actinidia plants, in particular, the onset of phenological phases. During fruit formation (June-September), actinidia plants need irrigation depending on the amount of precipitation and temperature conditions. Here we show that in the humid subtropics of Russia, the periods of kiwi fruit formation differ from year to year for precipitations, which coincides with the moisture deficit in the soil. For the first time, we have developed a simple physiological indicator to promptly estimate water status of kiwi plants for timely irrigation based on the cell sap concentration in petioles which correlates with soil moisture in the 0-60 cm layer. Purpose of this work is to determine the complex of agroecological factors affecting the moisture supply and productivity of *A, deliciosa* in the changing weather conditions of the humid subtropics of Russia. The study was performed on late-ripening cv. Hayward planted in 1988 (a 5×4 m scheme, the total site area of 5.5 ha, three-tiered palmette shaping; the Adler experimental station of the Vavilov FRC All-Russian Institute of Plant Genetic Resources, Krasnodar Territory, 2016-2020). The average daily and maximum temperatures were recorded from May to October, the http://www.pogodaiklimat.ru data were used for precipitation. Moisture content of the soil, taken in 10-cm layers along the depth of the root layer (0-60 cm), was measured by the thermostat-weight method. The concentration of cell sap (CCS, %) in the leaves was measured refractometrically from May to September in ten plants (for 3 decades of each month). The yield of each bush (n = 30 in total, three replicates) was recorded. Annually, the average weight of a berry was determined using 100 berries. The weather conditions of the growing seasons significantly differed and showed abnormal precipitations and extreme temperatures (above 30 °C), affecting the water regime of plants and productivity. In May-June 2016-2020, the water content of leaf tissues was high (CCS 4.96-5.25 %), therefore, during the periods of budding, flowering, and fruit setting, the moisture supply of the plantings was optimal. In August, fruits began to grow actively and the initiation of the next-year generative organs occurred. However, high insolation and the temperature rise above 30 °C increased evaporations, while the atmospheric precipitations were minimal. The soil moisture reserves from May to October were optimal and ranged from 80 to 90 % of the least moisture capacity over the entire depth of the soil profile. However, the CCS increased from mid-September and exceeded 10 % at the end of the month despite the complete soil saturation with moisture. Tension in the environment and the peak of physiological processes caused water deficit in the leaves up to the CCS > 7 % which irrigation still could level. At the end of August or at the beginning of September (depending on weather conditions), the CCS steadily rose above 8 %, regardless of irrigation. During this period, a redistribution of water fractions from bound to free ecologically active form occurs. The earlier the transition of water and assimilation from the leaves

of kiwi plants to fruits begins, the higher their weight and yield. Thus, our findings show that in the humid subtropics of Russia, the studied agroecological factors have the greatest effect on the water regime and performance of *A. deliciosa* cv. Hayward plants during the period of fruit formation (in August-September).

Keywords: actinidia (kiwi), cv. Hayward, water regime, productivity, heat supply, moisture supply, humid subtropics

Actinidia deliciosa (A. Ghev.) C. Fliang & A.R. Ferguson (kiwi fruit) is naturalized in world production since 1970. In 2019, the number of kiwi fruits produced in the world reached 4,274,870 tons / year, and the planting area was over 268 thousand hectares [1]. The leading positions in this industry are held by China, Italy, New Zealand, Iran, and Chili [2]. The culture was successfully mastered in the subtropical regions of the planet, and its industrial cultivation became important due to its high nutritional and biological value [3-5]. The agrocenosis of *Actinidia deliciosa* is distinguished by the duration of fruiting (40 years or more). For instance, kiwi fruit plantations at the Adler experimental station of All-Russian Institute of Plant Genetic Resources were established in 1988 [6, 7].

This subtropical fruit and berry crop contains dry substances, sugars, acids, vitamins, polyphenols, amino acids, aromatic and mineral substances, and is used in the production of functional food that is of great importance in the prevention of diseases. Berries of *Actinidia deliciosa* serve as a natural concentrate of multivitamins and mineral elements and retain their valuable properties in their natural state for up to six months [8-10].

In New Zealand, where the kiwi crop was bred, three important climate determinants for its cultivation have been identified: optimal cooling (temperature 11 °C and below), 1100 degree-days above 10 °C during the growing season, and annual rainfall of 1250 mm or more [11].

The influence of temperature factors and precipitation on the yield of five *Actinidia argute* cultivars was studied in Poland, in the northeastern regions [12]. Along with climatic factors, soil conditions are important, including the physical state and biological properties of soils (content of enzymes, trace elements, etc.) [13]. Beginning of the growing season is due to the increased sap flow in the stems and roots of plants. Within days, the roots begin to extract water from the places of moisture [14]. In Italy, one of the leading countries in the production of kiwi in plantations, crops optimize irrigation regimes for the full development of plants and ripening of fruits [15]. Water supply to plants is one of the key factors determining the size and quality of the crop [16-18].

The peculiar climate of the humid subtropics of Russia influences the actinidia plants, in particular, the onset of phenological phases. Bud differentiation occurs from the 3rd decade of December until the 2nd decade of February, growing season begins in March, and budding and flowering occurs in April-May. Fruit formation lasts until September, ripening – from October until November, depending on variety. For actinidia, as well as for subtropical crops, the basic sum of active temperatures (more than 10 °C) up to 4000±100 °C is determined [8].

The reaction of actinidia plants to the timing of watering during the day, which affects the biological parameters of the culture, has been shown [19]. Researchers note a higher stem-to-fruit water gradient at half-day/early noon, which favors fruit growth, but not significantly [20]. In Spain, the effectiveness of drip irrigation was studied in fruit crops in comparison with irrigation (in terms of the effect on soil and plants) [21]. High temperatures, solar insolation, and increased transpiration activity aggravated the environmental conditions in the afternoon. In kiwi, watering at this time improves the moisture supply of the soil, increases the gas exchange of leaves and the flow of assimilation products to the fruits [22, 23].

Over the past 20 years, due to climate warming, the sum of temperatures

above 10 °C in the humid subtropics of Russia began to vary from 4559 to 4870 °C, while earlier it was 3735-4258 °C [24, 25]. During fruit formation (June-September), actinidia plants need irrigation depending on the amount of precipitation and temperature conditions. If the moisture supply of plants and, therefore, the water content of leaf tissues decreases, the concentration of cell sap in the leaves increases [26].

This work, for the first time, revealed an uneven distribution of precipitation in combination with a deficit of soil moisture during the period of actinidia fruit formation, which occurs in the humid subtropics of Russia over the years. For the first time, we suggest a physiological indicator (concentration of cell sap in actinidia petioles) for timely diagnosing and establishment of the optimal water regime, contributing to an increase in the productivity of actinidia.

Purpose of this work is to determine the complex of agroecological factors affecting the moisture supply and productivity of actinidia in the changing weather conditions of the humid subtropics of Russia.

Materials and methods. The studies on late-ripening *Actinidia deliciosa* cv. Hayward were performed during 2016-2020 at the Adler experimental station of the Vavilov FRC All-Russian Institute of Plant Genetic Resources (Krasnodar Territory). It was planted in 1988 according to a 5×4 m scheme, with three-tiered palmette shaping and the total site area of 5.5 ha. The soil under the culture is alluvial meadow low-humus. Content of humus in a layer is 0-20 cm was 5.67%, labile phosphorus 48.16 mg/100 g of soil, labile potassium compounds 18.3 mg/100 g of soil, pH_{wat}. 7.16-7.30. Drip irrigation was applied. The experimental design included three modes of prethreshold irrigation with soil moisture capacity (MC) of 90, 80 and 70%.

The average daily and maximum temperatures were recorded in dynamics each year from May to October, with using the precipitation data (according to http://www.pogodaiklimat.ru).

The moisture content in 10-cm soil layers along the depth of the root zone (0-60 cm), was measured by the thermostatic method (a 2V-151 drying chamber, Odessa Experimental Plant of Medical Equipment, Ukraine) and laboratory electronic scales (VSLT-300/3A Pioner, Ohaus Instruments (Shangha) Co., Ltd., PRC). The figures present a percentage of the soil moisture capacity (MC), which characterizes the greatest amount of water remaining after abundant moistening and draining of its excess in the absence of upward groundwater. The optimum moisture content in the soil for actinidia was 80% MC [26].

The concentration of cell sap (CCS, %) in the leaves was measured refratometrically from May to September in ten plants for three decades of each month [26, 27].

The yield was recorded for each bush (in three replicates, total number of bushes in a replicate $n = 10$). Annually, the average weight of a berry was determined using 100 berries.

Data were statistically processed according to Dospekhov [28] using Microsoft Excel and Statistica 10 (StatSoft, Inc., USA). Using *F*-test, a correlation analysis of the relationship between soil moisture in the root zone of soil and the concentration of cell sap was performed depending on the drip irrigation regime. Mean values (*M*), standard errors of means (±SEM) and coefficients of variation (*Cv*,%) were calculated [28].

Results. In the humid subtropics of Russia, three agroclimatic regions with regard to the heat and moisture supply have been identified [24, 29, 30]. Two of them (I and II) in terms of the sum of temperatures > 10 °C (3800-4300 and 3600-3800 °C) meet the needs of subtropical crops, but due to the recorded absolute

minimum temperature reaching –14...–15 °C (I) and –17...–18 °C (II), it is required to take into account the microlandscape when planting crops,. The amount of annual precipitation in the regions is 1600 and 2100 mm, respectively, the duration of the frost-free period is 250-300 and 200-250 days. With the introduction of GIS (Geographic Information System) in the Sochi National Park, it was established that the humid subtropical climate remains up to 300-400 m above sea level. The tropical and continental climates are characterized by significant fluctuations in weather factors, increasing to anomalous values.

The optimal placement of crops and a systematic analysis of the complex of factors affecting the productivity of agrophytocenosis contribute to an increase in the productive potential of the culture. One of the methods for optimizing conditions in agrophytocenosis is drip irrigation [31-33]. Optimization of abiotic conditions in agrocenoses leads to a significantly higher yield of economically valuable products per unit area compared to natural phytocenoses [33].

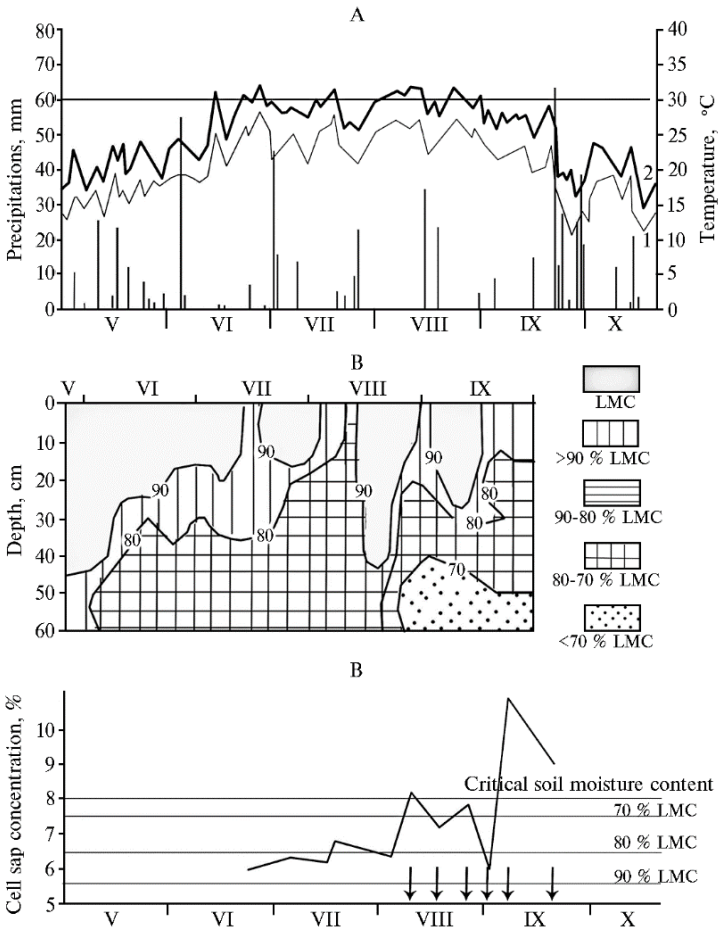

Fig. 1. Mean (1) **and maximum** (2) **ambient temperature** (A)**, soil moisture (%** MC) (B)**, and concentration of cell sap** (CCS) **in petioles** (B) **of *Actinidia deliciosa* (A. Ghev.) C. Fliang & A.R. Ferguson cv. Hayward in 2016, May-October**. MC is the minimum soil moisture capacity. Annual precipitation is 1682.0 mm; average annual temperature is 14.7 °C. Arrows indicate watering times (Adler Experimental Station VIR, Krasnodar Territory).

In this article, a detailed and comprehensive analysis of the influence of environmental factors (whether and soil) on the dynamics of water regime of Hayward kiwi plants and their productivity is given for 2016, 2019 and 2020, which differed in precipitation distribution and temperature regime.

Figure 1 shows data on the components of the weather-soil-water regime of plants in 2016. The observations of water regime of actinidia plants in 2016 starting from June showed that water content of leaf cells was high until August 8, but at the end of the 1st decade of August, the CCS exceeded 8%, and only subsequent watering in August contributed to its decrease (see. Fig. 1).

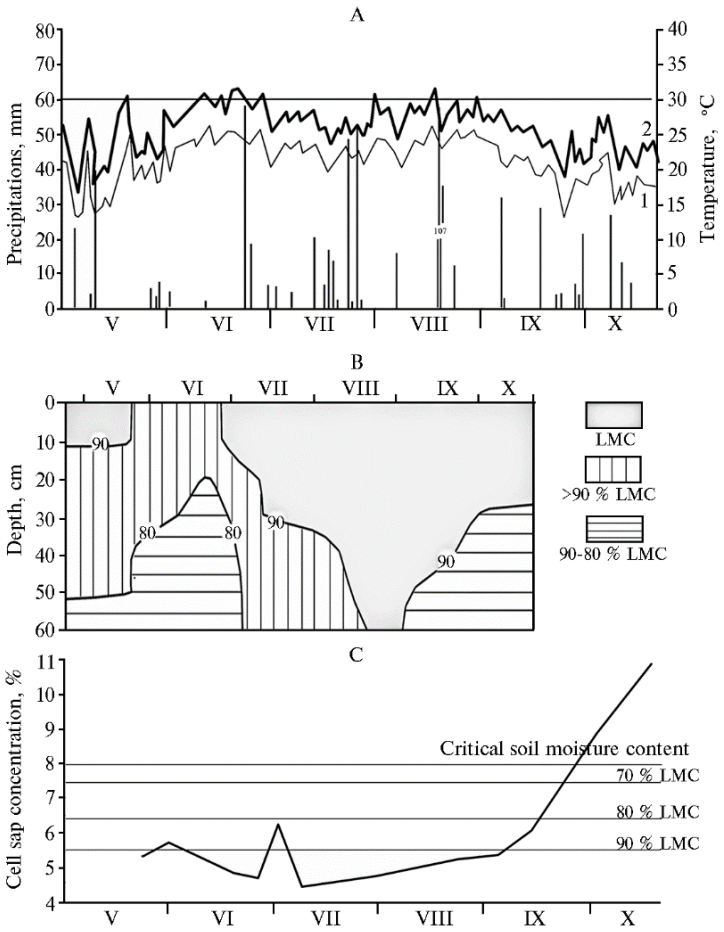

Fig. 2. Mean (1) **and maximum** (2) **ambient temperature** (A)**, soil moisture** (% MC) (B)**, and concentration of cell sap** (CCS) **in petioles** (B) **of *Actinidia deliciosa* (A. Ghev.) C. Fliang & A.R. Ferguson cv. Hayward in 2019, May-October**. MC is the minimum soil moisture capacity. Annual precipitation is 1420.4 mm; average annual temperature is 15.7 °C. Arrows indicate watering times (Adler Experimental Station VIR, Krasnodar Territory).

The thermal regime of 2019 was characterized by low temperatures and significant precipitation during the growing season (Fig. 2). The air temperature in July decreased by 0.8 °C compared to the long-term average values, while precipitation exceeded long-term data by 27%, and in August by 37%. The maximum temperatures (above 30 °C) were episodic in summer. Moisture reserves from May to October 2019 were optimum (80-90% MC) over the entire depth of the soil profile, while irrigation was not carried out. Nevertheless, the concentration of cell sap began to increase from mid-September, and at the end of the month, it was above 10% when the soil was full-saturated (see Fig. 2).

The sum of active temperatures in the driest 2020 reached 5164 °C. The average annual temperature was 15.8 °C, that is, it increased by 1.6 °C in comparison with the long-term average. The maximum air temperatures have been recorded since May. During the year, 1003 mm of precipitation fell, which comprised

59% of the norm (1703 mm). June, August and September were extremely dry. The drought worsened from March to the end of the year (Fig. 3). The intensity of the water regime in the soil profile was traced from the third decade of August in a layer of 30-60 cm. Irrigation in early September optimized the moisture regime in the layer of 50 cm. The water regime of actinidia plants was optimal until the first decade of August. Irrigation in early September could not improve the water regime of the crop (see Fig. 3).

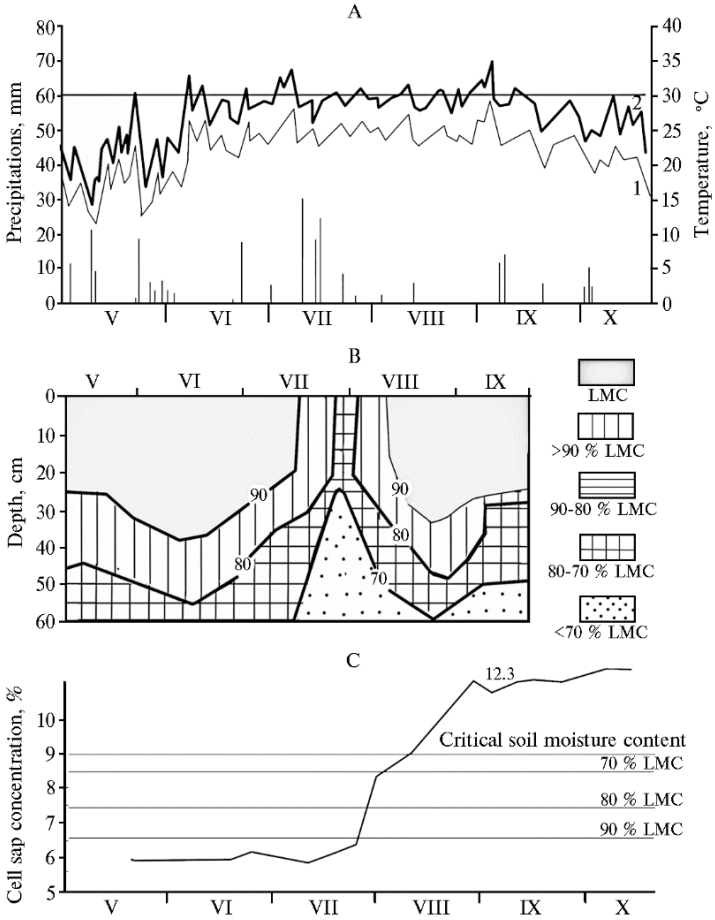

Fig. 3. Mean (1) **and maximum** (2) **ambient temperature** (A)**, soil moisture (%** MC) (B)**, and concentration of cell sap** (CCS) **in petioles** (B) **of *Actinidia deliciosa* (A. Ghev.) C. Fliang & A.R. Ferguson cv. Hayward in 2020, May-October**. MC is the minimum soil moisture capacity. Annual precipitation is 1003.0 mm; average annual temperature is 15.8 °C. Arrows indicate watering times (Adler Experimental Station VIR, Krasnodar Territory).

1. Concentration of cell sap (CCS, %) **in petioles of *Actinidia deliciosa* (A. Ghev.) C. Fliang & A.R. Ferguson cv. Hayward vs. soil moisture during vegetation period in the Russia humid subtropics** (n = 30, N = 3, M±SEM, Adler Experimental Station VIR, Krasnodar Territory, 2016-2020)

Parameter	Month					
	V	VI	VII	VIII	IX	X
CCS, %	4.96±0.43	5.25±0.09	5.73±0.11	7.14±0.17	8.80±0.30	9.87±0.35
Cv, %	8.8	11.3	16.5	17.8	23.6	15.5
Soil moisture, %	27.50	27.04	26.20	24.01	21.35	19.67

N o t e. For kiwi plants, the optimal soil moisture is 25% (80% MC), soil moisture level close to stress is 22%.

According to data obtained in August-September 2016-2020, concentration of the cell sap in actinidia leaf tissue increased significantly during these

months (Fig. 4, Table 1).

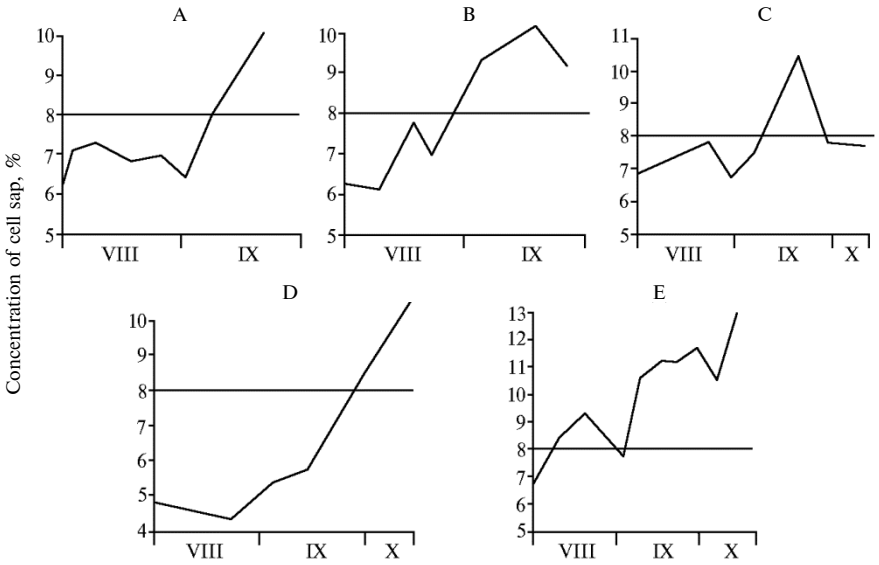

Fig. 4. Concentration of cell sap (CCS) **in petioles of *Actinidia deliciosa* (A. Ghev.) C. Fliang & A.R. Ferguson cv. Hayward at kiwi fruit maturity (August-October) in 2016** (A)**, 2017** (B)**, 2018** (C)**, 2019** (D)**, and 2020** (E)**.** The horizontal line marks the value of the CCS corresponding to the critical value of the water supply of plants **(**Adler Experimental Station VIR, Krasnodar Territory).

Correlation analysis of the relationship between soil moisture in the root layer of actinidia and the concentration of CCS in leaf petioles showed their close dependence. The correlation coefficient r was equal to 0.71885 at $F_{exp.} = 9.6236$, $F_{0.5} = 5.12$, which indicates a close statistically significant relationship between the analyzed factors. The mathematical model is as follows: $Y = 35.43 - 1.599x$, where Y is the soil moisture in the 0-60 cm layer, %; x is CCS, %.

High water content of leaf tissues in May-July was typical for the phases of budding, flowering, and setting. The increase in CCS in August over 7.14±0.17% coincided with the active growth of fruits. During this period, there is a redistribution of water fractions from bound to a free ecologically active form [34]. The content and ratio of water forms in the actinidia plant is in close correlation with agroclimatic factors in humid subtropics]35]. Goncharova [36] believes that the redistribution of water forms and products of assimilation from leaves to fruits can be considered self-regulation of transport flows by a plant organism, which has significant biological expediency under extreme conditions. Such self-regulation, in our opinion, based on long-term indicators of the change in the value of CCS in August-September, is also specific for actinidia.

The most unfavorable year in terms of yield and weight of actinidia cv. Hayward was 2019 due to high temperatures (> 30 °C) during the flowering phase (Table 2). Moisture provision during the ripening period was optimal without irrigation. However, the outflow of water with assimilates occurred at the end of September under moisture in the soil within 90% of the MC and along the entire depth of the root layer.

The minimum weight of the Hayward variety for 2016-2020 was 87 g, the maximum 135 g. On average, this fruit size corresponds to the characteristics of Hayward variety. The productivity of the Hayward variety in 2020, which was characterized by an acute lack of moisture and an early period (from the 2nd decade of August) of the outflow of water with metabolic products from leaves into fruits, was about 82% of the average productivity over 5 years. In a favorable

2018 (with drip irrigation) in the conditions of the Russian subtropics, a yield of 105.8 c/ha was obtained (see Table 2). The average yield of kiwi in the world in 2018 was 160 kg/ha (https://www.global-trademag.com/global-kiwi-fruit-market-2019-new-zealand-and-italy-are-the-leading-exporters-of-kiwi-fruits/).

2. Yield of *Actinidia deliciosa* (A. Ghev.) C. Fliang & A.R. Ferguson cv. Hayward in the Russia humid subtropics ($n = 30$, $N = 3$, $M \pm$SEM, Adler Experimental Station VIR, Krasnodar Territory)

Parameter	Year					
	2016	2017	2018	2019	2020	$M \pm$SEM
Yield, kg per bush	7.3	8.6	23.5	4.6	8.6	10.5±3.3
Yield, c/ha	32.8	38.7	105.8	20.7	38.7	47.3±6.4
Of mean yield, %	69	82	224	44	82	100

N o t e. The yield was assessed based on the average yield per bush according to the total number of female plants per hectare (450 plants).

The cultivation of the Hayward actinidia variety in the Republic of Abkhazia showed a similar influence of agroclimatic factors on the crop yield. Depending on the duration of the period of maximum temperatures and the amount of precipitation during the formation of fruits, the yield of the Hayward variety averaged from 50 to 130 c/ha [9].

In the countries of Western Europe, active selection work is being carried out on various types of actinidia in order to increase their productivity [37-39], fruit quality [40-42] and adaptability to local conditions [42, 43]. The greatest attention in the research is paid to the temperature factor [44, 45].

In the humid subtropics of Russia, as shown by multiple correlation analysis, the factors limiting the productivity of actinidia of Hayward variety are the amount of precipitation and air humidity during flowering and fruit ripening phases (excessive precipitation during flowering, its deficit during ripening). The amount of precipitation during the flowering phase is highly variable and uncontrollable, because of which this period becomes critical. The deficit of precipitation during the ripening period can be leveled by watering.

It should be noted that in the zones of traditional cultivation (New Zealand, Italy, China), much attention is paid to the issues of the influence of water scarcity and the methods and timing of irrigation on actinidia plants, the efficiency of the use of water resources by the culture [22, 46, 47]. It can be expected that under conditions of aridization of the climate and a shortage of water resources, the relevance of such studies will increase. The time of the onset of water stress (at the beginning of the season or at a later date) affects not only the yield, but also the content of biologically significant nutrients in fruits [47]. Therefore, it is believed that by choosing the timing of irrigation, it is possible to purposefully change some indicators of the quality of fruits in actinidia [47].

Since watering effectiveness of actinidia depends on its timing, it is important to have a simple and affordable way to assess the water status of plants. Many studies discuss the timing, methods, and effect of watering on the productivity and quality of actinidia fruits [23, 26, 43, 47]. However, in the available literature, we did not find data on how the optimal watering time for a plant was determined. The concentration of cell sap in the leaf petiole, which we proposed as such a criterion, has a significant advantage, since, firstly, it is a physiological indicator, and secondly, it is easy to determine it by a refractometric method. A mathematical model has been established for a close and significant relationship between CCS in leaf petioles and soil moisture in the root layer (0-60 cm). The optimal pre-threshold irrigation period for the crop is revealed (80% MC).

The mountain terrain and the variegation of the soil cover of the humid subtropics of Russia, as well as the varietal specificity of actinidia, determine the

possibilities of agroecological zoning of this young crop based on its adaptability. The study of the physiological status of actinidia when grown on the northern borders of the industrial cultivation area is of interest for understanding the genetic potential of the crop. The development of crop agroecosystem models can be of great importance for expanding the cultivation area and increasing the productivity of actinidia in the humid subtropics of Russia [48].

Therefore, a long-term study of the complex of agroecological factors of the humid subtropics of Russia during fruit formation (August-September) phase of *Actinidia deliciosa* cv. Hayward showed the effect of the amount of precipitation, soil moisture, and the concentration of cell sap in leaf petioles on plant productivity. High water content of leaf tissues during May-July was typical for budding, flowering, and setting phases. The increase in CCS in August over 7.14±0.17% coincided with the active growth of fruits. During this period, there was a redistribution of water fractions from bound to a free, ecologically active form. The earlier the period of the transition of the transport flow of water and assimilates in the actinidia plant from leaves to fruits begins, the higher their mass and yield. The increase in the CCS above 8% indicates the full technical ripening of the actinidia fruits. The parameters of the moisture content of crop were determined according to CCS values from 5 to 8%. An express method for refractometric measurement of the concentration of cell sap in actinidia leaves is proposed. The method provides rapid and reliable determination of the timing for watering actinidia in the field.

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