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## Reviews

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# MOLECULAR BASIS OF THE DWARFISM CHARACTER IN CULTIVATED PLANTS. II. DELLA-PROTEINS: STRUCTURE AND FUNCTIONS

(review)

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Abstract

The wheat gene *Rht* (*Reduced height*) which predeterminated the success of «Green revolution» and has been employed in creation of plant varieties with reduced stem elongation and resistant to stem lodging, encodes a protein containing highly conserved DELLA domain (J. Peng et al., 1999). Many other high-yield dwarf varieties also possess mutations in the genes coding DELLA proteins. Since the mutations did not affect viability and reproducibility of the plants the usage of the mutations for advanced plant breeding might be very promising (M. Ueguchi-Tanaka et al., 2007). Along with dwarfism, some mutations in the genes can lead to an opposite phenotype: to tall plants with spindly stems, the so called slender forms. What phenotype (dwarf or slender plant) would be developed depends on functionality of a protein region affected by the mutation. The paper considers in depth structure, posttranslational modifications, cellular localization and functions of DELLA proteins. The proteins being participants of complex protein-protein interactions play a role of repressors in gibberellin (GA) signal transduction. In the absence/or small concentration of GA DELLA proteins interact with specific transcriptional factor (TF) targets blocking their DNA binding activity and as a result restrain plant growth. In the presence of GA stem elongation is activated because this hormone promotes destabilization of DELLA proteins, releasing TF from their repression. Thus, restrained growth of the *rht* mutant as well as other naturally occurring dwarf plants is associated with accumulation of DELLA proteins in a result of their high stability. In turn, enhanced stability of the proteins can be caused by mutations in functionally important domains of either DELLA proteins or other players of GA signaling such as receptor GID1 and F-box proteins (GID2 in rice; SLY1 in *Arabidopsis*) of E3 ubiqutin protein ligase. They all participate in complex protein-protein interaction which is necessary for DELLA protein degradation via 26S-proteasome pathway (B.C. Willige et al., 2007; K. Hirano et al., 2010). The paper reviews a role of different functional motifs of DELLA protein in transduction of GA signal: DELLA, TVHYNP, polySTV on N-terminal part of a molecule and C-terminal GRAS-domain, containing the motifs LR, VHIID, PFYRE and SAW. DELLA-proteins possess no DNA-binding site. Most probably their repressive function is associated with GRAS-domain via protein-protein interaction of LR region with target TFs (R. Zentella et al., 2007; K. Hirano et al., 2010). The polySTV domain plays a regulator role. Post-transcriptional modifications in the region are potentially able to change partners in protein-protein interaction and/or cellular localization of the DELLA proteins (M. Ueguchi-Tanaka et al., 2005; K. Hirano et al., 2010). A function of DELLA protein as an integrator of hormone signal ways and external factors is also discussed. In the context the DELLA proteins are considered as «correctors» of plant growth reaction depending on growth conditions (X.-H. Gao et al., 2011). Thus, the activity of DELLA proteins might underlie the plant phenotypic flexibility and promotes restrained plant growth under unfavorable environmental conditions.

Keywords: dwarfism, retard growth, gibberellin signal transduction, DELLA protein, repression function, proteolysis

Most dwarf plants possess mutations in DELLA proteins which repress gibberellic signal. These mutations, not affecting plant viability and reproducibility, are helpful in crop breeding [1]. The "Green revolution" which ensured an

impressive rise in wheat yields in 1940-1970 was predetermined by the use of genes *Rht* (*Reduced height*) responsible for a decreased plant height and a thickened stem [2, 3]. However, some mutations in the genes encoding DELLA proteins can lead to strong elongation of the stem (slender forms) and a constitutive response of the plants even in the absence of gibberellin (GA). Eventually, the effect of mutation (dwarf or slender plant phenotype) depends on the region of DELLA protein molecule which is damaged or modified. Since DELLA proteins are essential for GA signal transduction cascade and involved in crosstalk between signaling pathways for some hormones and external cues [4], an integrative role of these proteins as central regulators in GA signaling is obviously due to their predisposition to protein-protein interactions. To date, many functionally active motifs of DELLA proteins and their interacting partners which can enhance or block DELLA-mediated repression are reported [5, 6].

This review is focused on the molecular mechanisms underlying growth changes in plants with mutant DELLA proteins and/or their interacting partners involved in the regulation of DELLA protein activity.

DELLA is known to inhibit plant growth while GA activates growth due to DELLA degradation that results in derepression of the genes involved in plant growth control [7, 8]. If a mutation leads to higher DEALLA stability, the plants are dwarf regardless of the GA presence or absence. An increased stability of DELLA protein may be caused by modifications in the domains necessary for binding to GA receptor GID1 (Gibberellin Insensitive Dwarf 1) and/or E3 ubiquitin-protein ligase which are involved in GA signaling. These induce 26S proteasome-dependent degradation of DELLA proteins. Changes in DELLA structure which resulted in dwarfism were reported in *D8* (*Dwarf plant 8*) and *D9* maize mutants [2, 9, 10], *Rht* (*Reduced height*) wheat mutant [2, 11], *DWF2* (*Dwarf 2*) turnip mutant [12] and *VvGAI* (*Vitis vinifera GAI*) grape mutant [13] (Table).

Mutants which growth is not suppressed by DELLA proteins are phenotypically slender. Such a phenotype could result from fully repressed DELLA gene expression (seeing GA-independent growth occurs in the absence of a repressor) or from changes in the domains involved in interaction with transcription factors. Such mutations are found in genes encoding proteins SLR1 (Slender rice 1) in rice [14] and SLN1 (Slender 1) in barley [15)], La and CRY in pea [16], StRGA (*Solanum tuberosum* repressor of *ga1-3*) in potatoes [17], PRO (Procera) in tomato [18] (see Table). Currently, genetic engineering makes it possible to modify the nucleotide sequences of DELLA genes in view to produce dwarf, semi-dwarf or slender plants.

*GAI* (*Gibberellic Acid Insensitive*), *RGA* (*Repressor of ga1-3*), *RGL1* (*RGA-like 1*), *RGL2* (*RGA-like 2*) and *RGL3* (*RGA-like 3*) genes are involved in DELLA control in *Arabidopsis* [14-16]. Only *GAI* and *RGA* seem to participate in growth inhibition. GAI and RGA proteins exhibit high homology (82 %). The roles of other genes in the repression of GA response differ but overlap (Fig. 1, A). All DELLA proteins (RGA mainly) are negative regulators of seed germination. RGA, RGL2 and RGL1 are also involved in flowering [1, 7, 22]. The role of RGL3 in plant development still remains unclear.

Dwarf *ga1-3* mutants with low GA level are often used to investigate the GA signaling in *Arabidopsis*. These mutants are a suitable model for studying plant growth with no effect of GA (see Fig. 1, B). The plant height of *ga1-3* dwarf mutants was not influenced by *gai* mutation but slightly changed by *rga* mutation. Double mutations *GAI* and *RGA* restored the height of *ga1-3* plants to the wild-type level. Mutations in 3-5 genes of DELLA proteins converted short *ga1-3* plants into giants [7, 20, 22, 24].

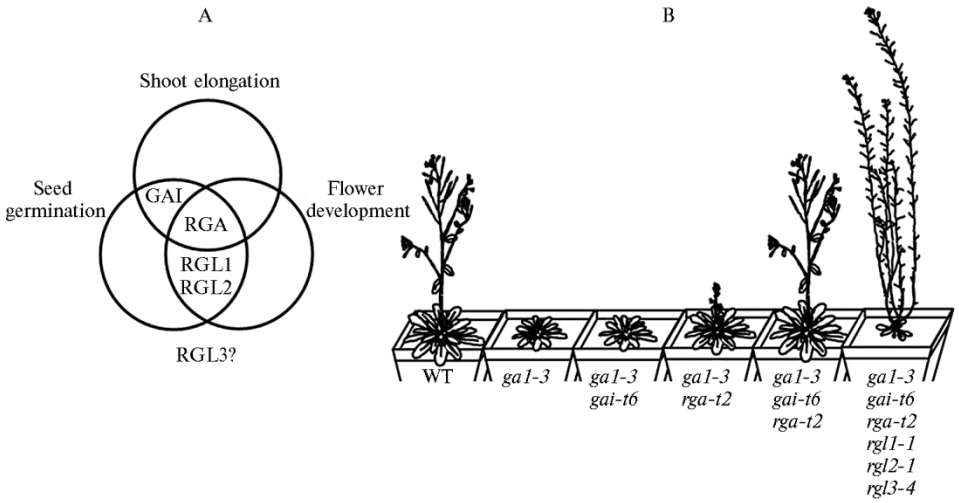


**Fig. 1. DELLA proteins involved in GA-mediated regulation of plant development in *Arabidopsis*:** A — partial overlapping functions of DELLA and GAI (Gibberellic Acid Insensitive), RGA (Repressor of *ga1-3*), RGL1 (RGA-like 1), RGL2, RGL3 (23), B — induction of GA-independent growth in dwarf *ga1-3* mutant with loos of repressing function of DELLA proteins [7, 22, 24]; WT — wild type; *gai-t6, rga-t2, rgl1-1, rgl2-1, rgl3-3* — mutations in DELLA proteins.

Special sites are responsible for the binding of DELLA proteins to specific interacting partners in GA signal transduction. The lesions in these sites can differently influence plant height. Highly conserved domains containing these sites are located at the N-end (DELLA, TVHYNP and polySTV) and the C-end (LR, VHIID, PFYRE and SAW) of DELLA molecule (рис. 2, A).

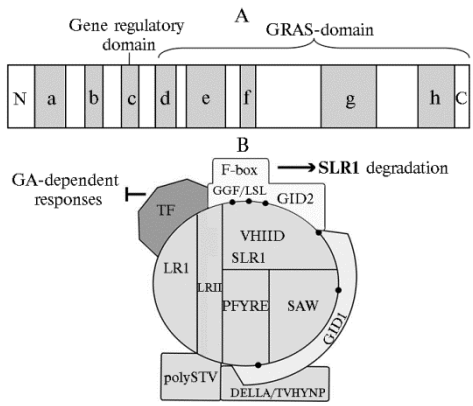


**Fig. 2. Conservative regions of DELLA-family proteins** (A, cited by [29] with additions) **and a model GID1 (Gibberellin Insensitive Dwarf 1)-SLR1 (Slender rice 1)-GID2 (Gibberellin Insensitive Dwarf 2) complex** (B, according to [5]).

A: DELLA (a), TVHYNP (b), VHIID (e), PFYRE (g) and SAW (h) named after characteristic amino acid sequences — DELLA for Asp-Glu-Leu-Leu-Ala (D-E-L-L-A), polySTV for polySer-Thr-Val (c). LRI (d) and LRII (f) are leucine rich repeats; GRAS domain (Gibberellic Acid Insensitive, GAI; Repressor of *ga1-3*, RGA; ScareCRow, SCR) is characteristic of GRAS family proteins and comprises several functional regions.

B: The regions of DELLA protein of rice (SLR1) which are involved in protein-protein interactions with GA receptor (GID1), GID2 protein (an F-box subunit of the E3 ubiquitin ligase complex) and a transcription factor (TF). Six sites containing 1 to 3 amino acids (the positions in the primary structure of the protein indicated) are pointed by dots downward clockwise (HFY315, PYL321, HFT3327, LQ361, G576, DRF490). A replacement of amino acids in these sites results in the loss of function of the domain. GGF and LSL are the GID2 domains necessary to interact with SLR1.

DELLA family proteins are nuclear-localized [10, 25, 26] and containing nuclear localization signal [10, 20]. DELLA means a characteristic conserved sequence of Asp-Glu-Leu-Leu-Ala ((D-E-L-L-A) at the N-end of the polypeptide. The main functions of DELLA and TVHYNP domains are to recognize the GA signal by binding GA to the receptor GID1 protein and its lid domain, and to stabilize GA-GID1 complex [27, 28] (see Fig. 2, B). The size but not primary structure of the region between these domains is functionally essential [29, 30]. DELLAs with deletions or mutations in DELLA and TVHYNP domains be-

come unable to form DELLA-GID1 complex. That is why they are not recognized by 26S proteasome and remain active negative regulator of the GA signal [31]. Mutations and deletions in DELLA and VHYNP motifs result in a dominant GA-insensitive dwarfing phenotype in which the plant height is not restored by exogenous GA [15, 30, 32]. For example, the dwarfing *gai* mutants in *Arabidopsis* and *slr1-d1*, *slr1-d2*, *slr1-d3* in rice with a lesion in DELLA/TVHYNP domain are unable to binding to GID1 [19, 20]. Interestingly, two *SLRL* genes (*SLR1-like1*, *SLR1-like2*) encoding proteins lacking DELLA domain are found in rice. These SLRLs are GA-insensitive and repress GA signal in the presence of GA [33] (see Table). The SLRL is believed to mediate growth inhibition in rice shoots at flooding [34].

**Mutations involved in the GA signal regulation**

| Mutant name | Protein | Loss/lesion of the protein function | Phenotype | Plant | References |
|---|---|---|---|---|---|
| | | DELLAs-mediated repression | | | |
| *gai-1* | GAI (Gibberellic Acid Insensitive) | GA-insensitive; failure to form GA-GID1-GAI complex; GAI stability (a 17-amino acid deletion in the conserved DELLA domain) | GA-insensitive dwarf plants | *Arabidopsis* | [19, 22] |
| *gai-t6* | GAI | GA-insensitive; failure to form GA-GID1-RGA complex; RGA stability; loss of repressor function (a 17-amino acid deletion in the conserved DELLA domain and a transposable element insertion in the C-end region of GRAS domain) | Normal height, a decreased GA-sensitivity | *Arabidopsis* | [35] |
| *rga-Δ17* | RGA (Repressor of *ga1-3*) | GA-insensitive; failure to form GA-GID1-RGA complex; RGA stability (lesion of DELLA domain due to deletion) | GA-insensitive dwarf plants | *Arabidopsis* | [22] |
| *rga-1* | RGA | GA-insensitive; loss of repressor function (67 amino acids deleted from the C-terminus) | Slight growth response to GA in dwarf mutants *ga1-3* | *Arabidopsis* | [35] |
| *rga-2* | RGA | Loss of repressor function (lesion of PFYRE domain due to $Asp_{478} \rightarrow Asn_{478}$ substitution) | Slight growth response to GA in dwarf mutants *ga1-3* | *Arabidopsis* | [20] |
| *rga-28* (*rga-t2*) | RGA | Loss of repressor function (lesion of LRI domain due to T-DNA insertion into coding region of *RGA*) | Slight growth response to GA in dwarf mutants *ga1-3* | *Arabidopsis* | [22] |
| *Brrga1-d* | BrRGA (*Brassica napus* repressor of *ga1-3*) | Failure of F-box of ubiquitin ligase complex to recognize DELLA proteins (lesion of VHIID region in the GRAS domain) | GA-insensitive dwarf plants | Rape | [12] |
| *d8-1* | D8 (Dwarf plant 8) | GA-insensitive; failure to form GA-GID1-D8 complex; D8 stability (a 4 amino acid deletion in the DELLA domain and single amino acid replacements in the DELLA and GRAS domains due to a deletion) | GA-insensitive dwarf plants | Maize | [2, 9, 10] |
| *d8(+)* | D8 | Increased D8 stability in the presence of GA ($Val_{103}$ insertion into the TVHYNP domain) | Deceased growth response to GA | Maize | [32] |
| *d9* | D9 (Dwarf plant 9) | GA-insensitive; failure to form GA-GID1-D9 complex, D9 stability (lesions in the DELLA and GRAS motifs due to single amino acid replacements) | GA-insensitive dwarf plants | Maize | [9, 10] |
| *rht-1* | Rht (Reduced height) | GA-insensitive; failure to form GA-GID1-Rht complex, Rht stability (DELLA lesion caused by a deletion) | GA-insensitive dwarf plants | Wheat | [2, 11] |
| *rht* | DELLA-subfamily protein | Decreased GA-sensitivity of DELLA protein (Leu→Pro substitution in the conserved DELLA domain) | Decreased growth response to GA | Sunflower | [36] |
| *cry-c* | CRY (Crypto-dwarf) | Loss of the repressor function ($Gly_{163} \rightarrow Gln_{163}$ substitution) | GA-insensitive growth (slender plants) | Pea | [16] |
| *cry-s* | CRY | Loss of the repressor function (a frameshift deletion of the conserved amino acid located at position 152) | GA-insensitive growth (slender plants) | Pea | [16] |
| *la* | LA | Loss of the repressor function of DELLA protein (a conserved $Gln_{85}$ excision due to an insertion) | GA-insensitive growth (slender plants) | Pea | [16] |

*Table continued*

| | | | | | |
|---|---|---|---|---|---|
| *pro* | PRO (Procera) | Loss of the repressor function of DELLA protein (the VHIID lesion resulting in failure of F-box of ubiquitin ligase complex to recognize DELLA proteins) | GA-insensitive growth (slender plants) | Tomato | [18] |
| *dwf2* | DWF2 (Dwarf 2) | GA-insensitive; failure to form GA-GID1-DWF2 complex, DWF2 stability (lesion of structure of the conserved DELLA domain) | GA-insensitive growth | Turnip | [12] |
| *Strga* | St RGA (*Solanum tuberosum* repressor of *gal-3*) | Loss of the repressor function of DELLA protein (lesion in the structure of the LRI domain) | GA-insensitive growth (slender plants) | Potato | [17] |
| *slr1-1* | SLR1 (Slender rice 1) | DELLA protein is not synthesized (a nonsense mutation) | GA-insensitive growth (slender plants) | Rice | [14] |
| *slr1-d1*, *slr1-d2*, *slr1-d3* | SLR1 | GA-insensitive; failure to interact with GID1, SLR stability ($Val_{49}\rightarrow Met_{49}$ substitution in the conserved DELLA domain in *slrl-d2* mutant or $Leu_{99}\rightarrow Phe_{99}$ and $Met_{106}\rightarrow Lys_{106}$ substitutions in the TVHYNP motif in *slr1-d3* mutant) | GA-insensitive dwarf plants | Rice | [37] |
| *slr1-d4* | SLR1 | GA-insensitive; failure to form a stable complex with GID1 and GID2, SLR stability (lesion in the SAW domain structure) | GA-insensitive dwarf plants | Rice | [5] |
| *sln1(b, c)* | SLN (Slender 1) | Loss of the repressor function of DELLA protein (the C-terminal end truncated) | GA-insensitive growth (slender plants) | Barley | [15] |
| *sln1d* | SLN | GA-insensitive; failure to form GA-GID1-SLN complex, SLN stability ($Gly_{46}\rightarrow Glu_{46}$ substitution in the conserved DELLA domain) | GA-insensitive dwarf plants | Barley | [15] |
| | | DELLA activator-mediated repression | | | |
| *spy* | SPY (Spindly) (N-acetylglucosamine transferase) | Not capable of DELLAs glycosylation | GA-insensitive growth (slender plants) | Rice, *Arabidopsis* | [38, 39] |
| | | Positive regulators (proteins of F-box containing E3 ubiquitin ligase complex) | | | |
| *sly* | SLY (Sleepy) | Loss of DELLA protein recognition (the C-terminal end truncated) | GA-insensitive dwarf plants | *Arabidopsis* | [35] |
| *sly1-d* | SLY | Gain of DELLA recognition regardless its interaction with GA-GID1 (E→K substitution in the C-end LSL) | GA-insensitive growth in dwarf mutant *gai* | *Arabidopsis* | [35] |
| *gid2* | GID2 (Gibberellin Insensitive Dwarf 2) | Loss of DELLA protein recognition (replacements of amino acids in the F-box domain) | GA-insensitive dwarf plants | Rice | [40] |
| | | DELLA-dependent negative regulator — a GATA transcription factor | | | |
| *gnl* | GNL/CGA1 (GNC-like/Cytokinin-Responsive GATA factor 1) | Loss of the negative regulator function | Slender plants | *Arabidopsis* | [41] |
| *gnc* | GNC | Loss of the negative regulator function | Slender plants | *Arabidopsis* | [41] |
| | | Positive regulator (repression of DELLAs activity) — a GRAS transcription factor | | | |
| *scl3* | SCL3 (ScareCRow-like 3) | Loss of the negative regulator function | Enhanced sensitivity to GA biosynthesis inhibitor | *Arabidopsis* | [42] |

The domain polySTV (polySer-Thr-Val) is a regulator. Transgenic rice plants with DELLAs deficient in polySTV were dwarfing [30]. Ser and Thr residues are found in polySTV which can be post-translationally modified (phosphorylated or N-acetylglucosaminated) [5, 29]. These modifications of DELLAs are believed to change their protein-protein interaction and (or) location. Phosphorylated DELLAs have been found in *Arabidopsis* and rice [43-45]. A role of DELLAs phosphorylation in GA signaling is discussed. SLR1 phosphorylation was shown to increase the protein stability in rice [45] though there was no reliable evidence for the effect of DELLA phosphorylation on the recognition of GA signal or degradation of the protein [7, 29, 43]. When seeking for negative regulators of GA signaling, the SPY (Spindly) protein was found in *Arabidopsis* and rice [38, 46]. The SPY is N-acetylglucosamine transferase which modifies proteins by Ser

and Thr glycosylation. The *spy* mutants of rice and *Arabidopsis* were phenotypically slender and characteristic of a decreased SPY expression [39]. It is believed that SPY-mediated DELLAs glycosylation blocks DELLAs phosphorylation. Nevertheless, it still remains unclear how does N-acetylglucosamination enhance the activity of DELLA-family proteins [7].

VHIID, LR (leucine repeats), PFYRE and SAW are the C-end located domains of the DELLAs (see Fig. 2, A), common to all members of a highly conserved GRAS family. The family comprises many transcription factors (TF). GRAS family is named after the first three members — GAI, RGA and SCR (ScareCRow) [47, 48]. The GRAS-containing TFs have been identified in *Arabidopsis* (33 TFs) and rice (60 TFs). These proteins are involved in plant growth and development and differ significantly by the presence of other functional domains [49].

Binding N-end DELLA and TVHYNP domains to the receptor protein GID1 is shown to be necessary but not sufficient to form a complex with E3 ubiqutin protein ligase and initiate proteolysis of DELLA proteins. The affinity to ubiqutin protein ligase increases only after an interaction between the GRAS domain of the DELLAs and GID1. A site-directed mutagenesis revealed the C-end region of VHIID together with PFYRE and SAW domains to be involved in association (see Fig. 2, B) [5]. In the VHIID mutants of rape (*Brrga1-d*) and SAW mutants of rice (*slr1-d4*) the DELLA proteins become more stable leading to dwarfism [5, 12] (see Table). The N-end-located VHIID and LRII of the DELLAs putatively bind to GID2 in rice and SLY1 (SLEEPY1) in *Arabidopsis* which are the F-box proteins of the ubiquitin-protein ligase complex degrading DELLAs (see Fig. 2, B). When GID2 (or SLY1) lesions, the DELLA-ubiquitin-protein ligase complex failed to form, and DELLAs stability increased leading to the GA-unresponsive dwarf plants [5].

Deletions in the GRAS domain often led to loss of the repressor function in DELLA proteins that was expressed in the GA-independent growth response and the appearance of slender phenotype [5, 20]. Thus, a regulatory role of DELLAs in transcription is GRAS-associated [34]. Using biochips, the DELLA-regulated GA-dependent genes (GA-activated but DELLA-repressed) have been found [7, 50]. Among these genes there were *GA20ox2* and *GA3ox1* involved in GA biosynthesis, and *GID1a* and *GID1b* responsible for GA reception. On the contrary, an expression of the genes encoding GA reactivation was DELLAs-inhibited and GA-stimulated [16, 42, 50]. *XERICO* is one more positive target for DELLAs. Еще одна позитивная мишень DELLA — ген *XERICO*. Overexpression of *XERICO* led to accumulation of abscisic acids, the phytohormone responsible for activating drought resistance. Thus, DELLAs contribute to GA biosynthesis and reception and, at the same time, induce biosynthesis of abscisic acids, an antagonist to gibberellin. The proteins encoded by the other target genes of DELLAs are transcription factors of bHLH (basic helix-loop-helix), MYB and GATA types.

Chromatin immunoprecipitation revealed DELLAs association with promoter regions of the regulated genes. However, a DNA-binding domain of DELLAs is not found [7, 42, 50]. A transcriptional repression in these associations seems to be due to DELLAs-dependent inactivation of TFs which contain a DNA-binding site [51] (see Fig. 2, B). It is believed that the sites responsible for repressive functions are distributed along the GRAS domain of DELLAs, making it difficult to identify. In addition, investigation of DELLA repressive role is complicated by indirect regulation when different mutations in a gene alter protein conformation that can inhibit the TFs inactivation. In this, DELLAs usually reserve the ability to bind GID1. In vitro interaction of the N-end located DELLA and TVHYNP domains with the GID1 receptor changed DELLA

molecule conformation leading to loss of repressor function in the C-end GRAS domain and gain of ability to bind the receptor [5]. Thus, the structure of the protein molecule as a whole seems to be essential in repressing. The role of LRI leucine repeats in DELLAs-mediated repression has been newly identified [5]. This region is suggested to be involved in protein-protein interaction with TFs. DELLAs can directly associate with certain transcriptional regulators of bHLH, JAZ (Jasmonic acid ZIM-domain; ZIM — Zinc-finger protein expressed in Inflorescence Meristem) and GRAS families, including other DELLAs. However, in these DELLAs-containing complexes the TFs were not associated with promoter regions and lost the ability to DNA binding. In the opinion of some researches, the hetero- or homodimer DEALLA formation is essential for GA-GID1-DELLA complex formation and further DELLA degradation [5, 7, 29, 52].

The scheme (Fig. 3) illustrates a role of DELLAs in GA signaling and indicates mutations which can affect DELLAs or other interacting partners in the signal transduction pathway resulting in dwarf or slender plant phenotype.

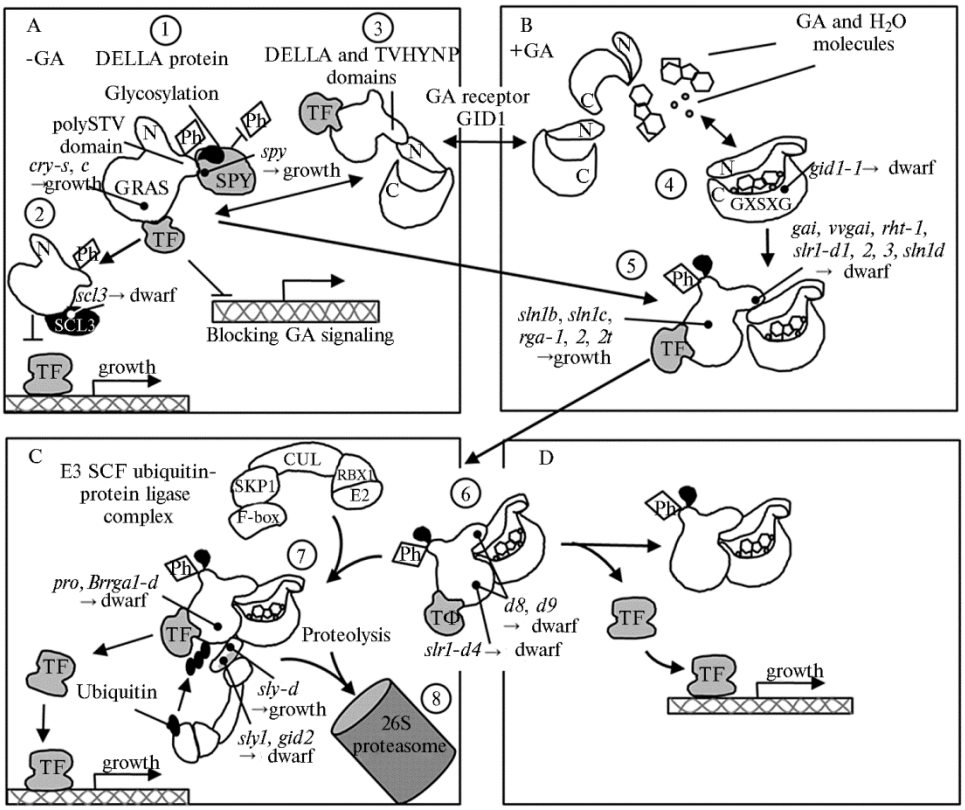


**Fig. 3. DELLA-family proteins involved in GA signaling.** Black and white dots indicate regions which are damaged due to mutations (indicated in italics) leading to a GA-unresponsive dwarf phenotype (→dwarf) or GA-independent growth (→growth).

A. At low level or in the absence of GA DELLA proteins and transcription factors (TFs) associate that blocks the signal transduction to the GA-dependent genes. Glycosylation by N-acetylglucosamine transferase (SPY, Spindly) within the polySVT (polySer-Val-Thr) domain blocks DELLA phosphorylation (P) and enhances DELLA repressor function (position 1). However, slow GA-independent growth can occur due to partial repression of DELLA activity by a transcriptional regulator SCL3 (ScareCRow-like 3) (position 2). DELLA protein, via specific N-terminal domains DELLA and TVHYNP, can bind to a gibberellin receptor GID1 (Gibberellin Insensitive Dwarf 1) but when GA is absent the GID1-DELLA complex is unstable and easily dissociates.

B. To initiate GA signal transduction, GA, with water molecules involved, is bound within the GA binding C-terminal pocket of the GID1 GA receptor (at GXSXG site). Then, due to folding of a lid structure connected to the N-terminal region of the DELLAs, a non-polar part of the GA molecule is trapped in the pocket to form a GA-GID1 complex (position 4). The GA-GID1 com-

plex is unstable but its stability increases due to a two-step association with DELLAs: the N-terminal DELLA domain of DELLA protein binds to the GID1 N-terminal lid domain (position 5), and the C-terminal GRAS (Gibberellic Acid Insensitive, GAI; Repressor of *ga1-3*, RGA; ScareCRow, SCR) domain of DELLA protein interacts with the C-terminal region of GID1 (position 6).

B. At DELLAs proteolysis-dependent inactivation a GA-GID1-DELLA complex has a high affinity to ubiquitin-proteasome multiprotein complex E3 SCF. E3 SCF comprises SKP-CUL-F-box (S phase Kinase-associated Protein 1 and Cullin) and RBX1 protein with Ring finger domain. F-box proteins recognize and bind the GRAS domain of DELLA protein (position 7). E3-associated ubiquitin-conjugated enzyme E2 tags ubiquitination of a target DELLA protein. The tagged DELLA protein undergoes proteolytic degradation by the 26S proteasome (position 8). Transcription factors, after a release from DELLA repression due to the degradation, activate the GA-dependent genes to launch growth and morphogenesis programs.

Г. In case of DELLAs proteolysis-independent inactivation which occurs under *GID1* overexpression the repressor function of DELLA protein is lost at a GA-GID1-DELLA complex formation (position 6).

It is believed that the DELLA-proteins as key components of GA signal transduction can integrate the signaling pathways of hormones and external stimuli [53]. The DELLAs activity may underlie the phenotypic plasticity and determine a low plant growth observed at strong light, drought, salinity, low temperature, an impoverishment of soil mineral composition and disease. An increased stability of DELLAs under the influence of stress factors (low temperature, salinity) evidences in support of the idea. DELLA proteins increased plant resistance to stress due to reducing growth rate and activating cellular antioxidant system [4, 7, 54]. In addition, the dwarf plants able to accumulate DELLA proteins possess an increased resistance to various stressors.

DELLA proteins are proved to be involved in light-dependent regulation (light signaling) of plant growth and de-etiolation of seedlings. They block PIFs (Phytochrome-Interacting bHLH Transcription Factors, the repressors of a phytochrome-dependent response). Additionally, DELLAs contribute to accumulation of the bZIP (basic region/leucine zipper) containing TF long-hypocotyl 5 (HY5), the main activator that promotes photomorphogenesis [17, 55, 56]. The mechanism of DELLAs-mediated PIFs repression is most well-studied. PIF3, PIF4 and PIF5 proteins are found in *Arabidopsis* and potato. When physically binding to promoters, they transcriptionally regulate expression of the genes encoding growth of etiolated seedlings [17]. DELLAs can form a complex with PIF by bHLH domain binding at low GA level in the light. When complexed with DELLAs, PIFs are unable to interact with promoters of the target genes. At dark and at high GA level the rate of DELLAs degradation rises interrupting repression of PIFs by the DELLAs. When released from DELLAs, PIFs bind to their target promoters to regulate transcription. Particularly, *GNC* (*GATA*, *Nitrate-Inducible*, *Carbon-Metabolism Involved*) and *GNL/CGA1* (*GNC-like/Cytokinin-Responsive GATA factor1*) are the genes transcriptionally controlled by the DELLAs and PIFs regulators. The encoded proteins (GATA TFs to bind GATA-containing DNA regions) play a repressor role in the GA signaling at seed germination, stem elongation and flowering. Their overexpressing mutants are dwarfs, whereas a loss-of-function GNC or GNL/CGA1 mutants result in slender plants [41]. It is shown that DELLA can also directly interact with another protein of the bHLH family — ALC (Alcatraz), responsible for the formation of separation layer and the dehiscence in *Arabidopsis*. In this, as in interaction with PIF, DELLAs prevent the ALC association with its target promoters thus blocking transcription [57]. It yet remains discussable whether the repression mechanism of DELLA-PIF/ALC models is applicable to other bHLH TFs. These studies will help to identify the DELLAs involvement in ontogenetic processes [7].

Like the bHLH regulators, DELLAs are able to directly interact with the TFs of GRAS and JAZ families [34]. A transcriptional regulator SCL3 (Sca-

reCRow-like3), like DELLAs, belongs to the GRAS-family proteins but lacks of DELLA domain. The effect of the interaction between SCL3 and DELLAs is antagonistic. SCL3 represses the DELLAs function, being, therefore, a positive regulator [42]. Mutation in *slc3* results in a wild-type growth rate under normal conditions which reduced in the plants treated with paclobutrazol, a GA-biosynthesis inhibitor. Interestingly, DELLAs activate *SCL3* expression. SCL3 protein, like DELLAs, has specific target genes, and does not contain DNA-binding domain that is why it regulates transcription due to the associations with other TFs. Interaction between SCL3 and DELLA oppositely influenced transcription of SCL3- and DELLA-dependent genes involved in GA response and GA metabolic gene expression [34]. A recent research reported on the role of SCL3 and DELLA in the root development. In this, other GRAS-family TFs, the SHR (Short-Root) and the SCR responsible for the endodermal cell specialization, were controlled by SCL3 and DELLA regulators [58]. It has been shown that DELLA proteins activated a jasmonic signal transduction. For that, DELLAs directly interact with a soluble protein JAZ1, the key repressor of jasmonic signaling, and block its ability to inhibit MYC2 protein, the bHLH/LZ (basic helix-loop-helix/leucine zipper) TF which is bound to its specific promoter. This TF family is named after gene *v-MYC* (V-mycavian mielocytomatosis viral oncogene) which is the main transcriptional activator of jasmonat-induced genes expression [59, 60].

Due to interaction with the members of various signaling pathways, DELLA proteins, additionally to the involvement in DEALLA-hinged GA signaling, are a central integrator of signal transduction pathways for many hormones and external stimuli. In the opinion of X.-H. Gao et al. [53], DELLA proteins can regulate plant growth and tolerance depending on environmental variables.

So, hereditary changes which determine the plant height are of interest for breeding. These can result from mutations in genes involved in regulation of cell metabolism at molecular, hormonal, epigenetic and transcriptional levels. Among the causes of shortened stems, breeders especially allocate changes in the regulation of GA activity common to various plant species. In breeding, the genotypes with mutations in DELLA proteins, the repressor of GA signal, seems to be the most viable, adaptive and expedient. These mutations often lead to a desired dwarf phenotype, but also slender forms can appear. Plant growth inversely correlates with DELLA resistance to degradation in the presence of GA. DELLA proteins are localized in the cell nucleus. Their molecules contain many high conserved regions involved in protein-protein interaction. DELLA proteins have no DNA-binding domains and indirectly regulate transcription of GA-dependent genes by blocking TFs that leads to growth retardation. GA, by inducing DELLAs proteolytic degradation via the ubiquitin-proteasome pathway, relieves DELLA-dependent repression of the TFs and restores growth. Mutants defective on ubiquitin-proteasome complex exhibit an increased stability of DELLA proteins leading to dwarfism. These mutations can affect either DELLA proteins, or their interacting partners in the signal transduction, i.e., proteins of soluble GA receptor (GID1) and F-box of ubiquitin ligase. Mutations leading to loss of DELLA repressor function seem to affect the GRAS domain. Whether a stability of DELLA proteins and their interactions with other proteins are influenced by posttranslational modifications (e.g., phosphorylation, glycosylation) remains an open question. The modifications affect the polySTV domain. Getting involved in diverse protein-protein interactions and being one of the key component in internal (hormones) and external (biotic/abiotic factors) signaling during plant growth, development and adaptation, DELLA-family proteins are not only a player of the GA signaling pathway, but act as a signaling integrator. Exactly activity of DELLA proteins is likely to underlie phenotypic plasticity and

enable a plant to restrain growth under adverse conditions.

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# ROLE OF PHYTOHORMONES IN THE CONTROL OF SYMBIOTIC NODULE DEVELOPMENT IN LEGUME PLANTS. II. AUXINS

(review)

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Abstract

This review refers to analysis of the modern data about the role of auxins in the regulation of nitrogen-fixing nodule development in the legume plants. The interaction of these hormones with cytokinins and components of signaling cascade activated by Nod factors during nodule organogenesis in legume plants with different types of nodules are discussed. Emphasis is being given to the participation of auxins in the control of initiation and early stages of nodule development. In early works the analysis of transgenic plants containing the fusions between promoters regulated by auxins and reporter genes, showed the accumulation of auxins in the places of nodule primordia development. It indicates a direct effect of these plant hormones on the process (U. Mathesius et al., 1998; C. Pacios-Bras et al., 2003). These studies became the basis for the suggestion that the auxin maximus in the cells giving later nodule primordia precedes these organs appearance. The creation of such peaks is dependent on Nod factors and cytokine response activation in plants, but it is differently regulated in legumes with determined (regulation of auxin biosynthesis) and non-determinated (control of auxin transport with flavonoids) type of nodules (A.P. Wasson et al., 2006; J. Plet et al., 2011; T. Suzaki et al., 2012). The effect of cytokinins on the auxin transport (auxin transporters PINs) during the formation of nodules has much in common with the participation of cytokinins in the control of lateral root development program, which may indicate an evolutionary relationship of two morphogenetic programs. In legume nodules and lateral roots development programs the initial stages have much in common, since both structures are formed as a result of the reactivation of differentiated root cells and have the features of structural similarity. However further divergence leads to the formation of structurally and functionally distinct organs that can be associated with unequal role that cytokinins and auxins play in controlling these processes. The review also examined the role of the balance of auxin and cytokinin hormones in determining the program of development of root cells. We discuss the experimental data using exogenous hormones, which showed that the founder-cells, giving rise to lateral roots, characterized by high plasticity of development processes, determined by the balance of auxins and cytokinins (L. Laplaze et al., 2007; Chatfield et al., 2013). However, an analysis of the data leads to the conclusion that the role of auxin in the control later stages nodulation in legumes is little investigated.

Keywords: legume-rhizobial symbiosis, auxins, phytohormonal balance, nodule organogenesis

Symbiotic interaction between the legumes and the *Rhizobiales* bacteria leads to appearance of nitrogen-fixing root nodules due to de novo organogenesis. This is initiated by lipo-chitooligosaccharides (Nod factors), the signals excreted by rhizobia [1]. Nodule development results from root cell reactivation, differentiation and transition to a division to produce a cluster of dividing cells which forms a symbiotic root nodule primordium. Nodulation in legumes is a unique model to study de novo body formation as influenced by an external regulation (in this particular case, by the signals produced by rhizobia).

There are numerous physiological and genetic evidences that the changes in phytohormone balance induced by Nod factors are necessary to successfully develop the root nodules [2-7]. These changes are mediated by regulation of the

genes encoding plant phytohormones' biosynthesis, activation and transport [5, 8]. Hormones produced by rhizobia are able to impact on the efficacy of symbiosis but not nodule formation [9].

This review is focused on the role of auxins in nodulation and also on the auxin-to-cytokinin balance as an essential component of root cell development program in legumes.

Auxins are involved in control of nodule initiation and initial stage of development. According to recent studies, the Nod factor-activated signaling cascade stimulates a cytokinin response in the cells of root cortex [10, 11]. In alfalfa *Medicago truncatula*, pea *Pisum sativum*, white clover *Trifolium repens* which produce an indeterminate type of nodules the cells of root pericycle, endodermis and inner cortical layer are cytokinin-responding and involved in the appearance of nodular primordia [5]. In contrast, in *Lotus japonicas* and soybean *Glycine max*, the legumes which have determinate nodules, a cytokinin response occurs in the cells of outer cortical layer [6].

However, experimental data indicate an important role of auxins in the regulation of initiation and development of nodules on the roots of legumes [4, 5, 7, 12, 13]. It has been known long enough that the nodule-like structures appeared in the legumes treated with inhibitors of polar transport of auxins, indicating these plant hormones to participate in root nodulation [4, 14]. Using genetic constructs containing auxin-regulated promoters *GH3:GUS*, DR5:GUS fused to the reporters, a local auxin accumulation was detected at the sites of primordia generation as an evidence for the direct effect of these plant hormones [17-19].

Rhizobial inoculation and treatment with Nod-factors led to disruption of local auxin accumulation and polar auxin transport (PAT) in the legumes producing indeterminate nodules [17, 18]. Two possible causes for PAT inhibition have been experimentally revealed. Firstly, the flavonoids which synthesis is activated by Nod-factors affect the PAT at the site of inoculation [17, 18, 20-22]. On the other hand, the PAT inhibition is possibly due to the effect of cytokinin on the distribution of PIN (PIN-FORMED) proteins, the transporters of auxins, in the root conducting system. Unlike wild type plants, the mutant cre1 deficient in cytokinin receptor exhibits no change in PIN protein localization in response to inoculation [5, 23]. No changes in the PIN distribution would result in no PAT inhibition.

An in-depth analysis of the mutant *cre1* allowed a molecular level understanding of Nod-factors, auxin and cytokinin interaction in the plants with indeterminate nodules. Mutant *cre1* does not respond to rhizobial inoculation by stimulated synthesis of flavonoids, and, as a result, no changes in PIN localization occur and the polar auxin transport is not disrupted [24]. However, the treatment of *cre1* mutant by exogenous flavonoids and PAT inhibitors restores nodulation [24]. Therefore, signaling pathway activation by Nod-factors stimulates the cytokinin receptor leading to expression of the genes encoding flavonoids. In turn, the flavonoids affect PIN proteins resulting in the inhibition of auxin transport. As a result, locally accumulated auxin triggers the initiation of the root nodule primordia formation and stimulates proliferation of pericycle, endodermis and inner cortical layer cells giving rise the nodules.

Ability of flavonoids to act as auxin transport regulators, in particular to affect PIN synthesis [25] and localization [26], has been shown for other plant species. Thus, in plants with indeterminate nodules the activity of Nod factors and cytokinins precedes the changes in auxin level and localization during root nodule development. Cytokinins influence the flavonoid synthesis and distribution of PIN proteins that is essential to form a local auxin gradient and to develop root nodule, a lateral organ generated de novo.

In the legumes with determinate nodules (*L. japonicus*) an auxin transport inhibition response to rhizobial inoculation was not observed, however, an increased auxin level at the site of nodule generation was shown experimentally with the use of auxin-regulated constructs [27, 28]. Moreover, the increased auxin correlated with the induced expression of gene *TRYPTOPHAN AMINOTRANSFERASE RELATED 1* (*TAR1*) involved in auxin biosynthesis control [29, 30]. Additionally, at initiation of organogenesis in *L. japonicus* the cytokinin action preceded auxin accumulation which, according to the authors' view, stimulated root cell proliferation affecting cell cycle regulators [29, 30].

Indeed, the role of cytokinins and auxins in the control of organogenesis may be due to their mutual effects on one another. Numerous publications show that cytokinins can regulate morphogenesis influencing the transport and metabolism of auxins. Hormone transport is crucial in the formation of auxin gradient that causes cell specialization and regulation of their proliferation and differentiation [31]. A case study of auxin transport in plants has been conducted for indole-3-acetic acid (IAA). In plant cells the IAA influx is mediated by proteins AUX1/LAX (AUXIN RESISTANT 1/LIKE AUX 1) and PGP (P-glycoprotein of the family MDR — multidrug resistance) [32]. An efflux of auxins from cell is regulated by PIN and PGP proteins embedded in the cell membrane diametrically to transporters AUX1/LAX [32]. In legumes the changes in auxin transport and biosynthesis occur after cytokinin involvement in the control of root nodulation. These data suggest that cytokinins can affect auxins in the early development of root nodules [4].

The mutual effect of cytokinin and auxin during organogenesis is quite well studied [33-36]. For example, cytokinins similarly regulate synthesis, localization and selective degradation of PIN proteins at formation of lateral roots in *Arabidopsis*, a model plant [33-36]. It is these mechanisms are found for the cytokinins to probably regulate auxin gradient formation during the development of lateral roots [36, 37]. Cytokinins and auxins are known to differently affect shoot and root development: shoot formation is cytokinin-stimulated while root formation is auxins-stimulated. In shoot apical meristem of Arabidopsis cytokinins stimulate cell proliferation and auxins, in contrast, affect cell differentiation. The root meristem auxins are necessary to maintain its activity, while cytokinins inhibit the development of root meristem [38, 39]. In this, the cytokinins affect root cell differentiation by inhibition of the stimulatory effect of auxins on cell division, i.e. the hormones act in close concert with each other. The balance between cell division and differentiation and, as a result, the control of meristem size are due to cytokinin-dependent stimulation of the auxin response repressors Aux/IAA. That suppresses expression of PINs (the auxin transporters) and leads to a change in the cytokinin-to-auxin balance in the root zone where the transition from cell division to differentiation occurs [38, 39]. These findings are in line with auxin-stimulated proliferation of the root cells producing nodule primordia in legumes.

Role of the auxin-to-cytokinin balance in the program of root cell development. In recent years, a lot of works report on a mutual influence of cytokinin and auxin, indicative of the role of their balance in determining cell development program. Exactly the balance may determine the choice of a development program. For example, it is known that in *Arabidopsis* lateral roots are derived from specific founder cells of pericycle as a result of high auxin level [40]. The exogenous auxin IAA led to an increase in the number of lateral roots [41]. On the contrary, the exogenous cytokinin 6-benzylaminopurine (6-BAP) blocks development of lateral root primordia (the regularity of cell division in primordium is impaired, leading to its flattening)

[37]. Moreover, under certain conditions the exogenous cytokinin, when used at the early lateral root primordia stage, is capable of causing a complete change of the development program, resulting in lateral root primordia transformation into active shoot meristem [42]. Thus, the experiments with exogenous hormones suggest that founder cells, giving rise to lateral roots, are characterized by high plasticity, and the vector of their development is determined by the balance of auxins and cytokinins.

In legume the development programs for nodules and lateral roots have much in common in the initial stages, since both organs derive from differentiated root cells after their reactivation and have the features of structural similarity [43-47]. However further divergence results in structurally and functionally distinct bodies that can be associated with different role the cytokinins and auxins, as regulators, play to control these processes. This has led some authors to the hypothesis that the nodules program has evolved on the basis of the program of lateral root formation [48]. This assumption is evidenced by the data that the Nod factors can stimulate not only the nodule, but the lateral root [49, 50]. Additionaly, the lateral root growth stimulation by Nod factors depends on the major genes (*NFP*, *DMI1*, *DMI2*, *DMI3* and *NSP2*) which control Nod factor-signaling cascade after the reception of Nod factors. The same genes regulate the activation of early nodulins and initial root cortex cell divisions at nodulation.

A comparative analysis of the root transcriptome in *M. truncatula* wild type plants and mutant *sickle* treated with Nod factors confirm this assumption. The mutant *sickle* is characterized by an increased susceptibility to inoculation and more intense response to Nod factors due to ethylene resistance [51, 52]. In wild type plants, and especially in mutant the exogenous Nod factors greatly increases the expression of genes that control root development (mostly these are the homologs of the *Arabidopsis* model plant genes) [53]. In *Arabidopsis* many of these genes are involved in control of cell division and formation of lateral root primordia, as well as the maintenance of stem cell pool and meristem structure of formed root center. The authors provide evidence that the increase in the expression of these genes is associated with the root nodule development. A significant increase in the number of root nodule primordia, but not lateral roots in mutant *sickle* indicate that induction of expression of the gene set is associated mainly with nodulation [53]. This supports the hypothesis that in legumes the same genes may control the initial stages of development in lateral roots and root nodules.

Thus, the appearance of nodules is preceded by maximum auxin accumulation in the cells which will produce root nodule primordia. Reaching auxin peaks depends on Nod factors and activation of cytokinin response, but differs in legumes with determinant nodules (auxin level control due to regulation of its biosynthesis) and indeterminant nodules (auxin transport regulation by flavonoids). The effect of cytokinins on PINs (the auxin transporters) and auxin transport at root nodule formation has much in common with the role of cytokinins in programing lateral root development that may indicate an evolutionary relationship of two these morphogenetic programs.

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# GENETIC MAPPING OF PEA (*Pisum sativum* L.) GENES INVOLVED IN SYMBIOSIS

(review)

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## Abstract

This article presents a review of current data on genetic mapping of pea (*Pisum sativum* L.) genes participating in development and regulation of arbuscular-mycorrhizal and legume-*Rhizobial* symbioses. By means of mutational analysis several regulatory symbiotic genes (*Sym*-genes) were identified in model and crop legumes, particularly, among them more than 40 pea *Sym*-genes. Some of them are already cloned and sequenced, and structural and functional similarity was demonstrated for orthologous *Sym*-genes in different legume species. The functions of these genes are diverse and include the control of perception of the microsymbiont's signal molecules, activation of the signal cascade (which is common for both legume-rhizobial and arbuscular-mycorrhizal symbioses), and consequent transcriptional changes in root cortex. To identify the sequence of mutated pea genes, an approach is used that is based on comparative genetic mapping and search for candidate gene in the genome of closely related legume plant barrel medic (*Medicago truncatula* Gaertn.). The web site www.phytozome.net (D.M. Goodstein et al., 2012) presents the current state of the barrel medic's genome sequencing in the form of genome browser, which facilitates the search for homologous genes and the sequence analysis of candidate genes. Significant similarity of pea and barrel medic genomes allows development of gene-based molecular markers, comparison of obtained pea genetic map with *M. truncatula* genome, and pea gene cloning after finding mutations with similar phenotypic manifestation. Currently, most of pea *Sym*-genes are mapped in genome; that resulted in identification of the sequences of 14 symbiotic genes. In particular, authors of the present review were able to sequence the pea genes *Sym35* — the homologue of *NIN* of *Lotus japonicus* (Regel.) K. Larsen (A.Y. Borisov et al., 2003), *Sym37* — the homologue of *NFR1* of *L. japonicus* (V.A. Zhukov et al., 2008), *Sym33* — the homologue of *IPD3* of barrel medic (E. Ovchinnikova et al., 2011), *Cochleata* — the homologue of *NOOT* of barrel medic (J.M. Couzigou et al., 2012). In recent years, considering the development of modern technologies of Next Generation Sequencing and massive genotyping, an avalanche of data on mapping pea gene-based data is being accumulated. Saturation of pea genetic map with markers, undoubtedly, will facilitate mapping of symbiotic genes and identification of their sequences; this will help to broaden the understanding of how the system of genes, which control interactions with beneficial soil microorganisms, functions in pea.

Keywords: legumes, legume-rhizobial symbiosis, arbuscular mycorrhiza, symbiotic plant genes, genetic mapping, synteny, gene-based molecular markers

Mutualistic interactions with microorganisms are essential for evolution and a successful life strategy of terrestrial plants [1, 2]. Benefits acquired by plants as a result of symbiosis are a facilitated uptake of minerals from soil and the enhanced tolerance to infections and abiotic stressors [3, 4]. Arbuscular mycorrhiza (AM), an interaction between plant roots and *Glomeromycota* fungi, is the most common endosymbiosis typical of 80-90 % land plants. AM provides

nutrient assimilation (mainly phosphorus and nitrogen-containing substances) from soil [4]. The symbiosis between legume *Fabaceae* plants and rhizobia (*Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*) is best known among the nitrogen-fixing plant-microbe symbioses. In legume-rhizobium symbiosis (LRS) the specialized structures (nodules) colonized by bacteria that fix atmospheric nitrogen are developed on plant roots [3]. AM and LRS have a lot of similarities, pointing to their evolutionary relationship [5, 6]. High genetic and metabolic integration of the partners is characteristic of both symbioses. AM and LRS development involves certain molecular and cellular events which are clearly interrelated among partners and accompanied by a constant exchange of signals and metabolites [6, 7].

Experimental mutagenesis is widely used to elucidate the mechanisms underlying formation and the functioning of mutualistic plant-microbe symbioses. In particular, extensive collections of bean mutants allow to identify several dozen regulatory symbiotic genes [8-11], also called *Sym* (symbiosis) genes [12]. Many of them have been cloned and sequenced, and various legume species show the similarities in structure and activity of orthologous *Sym* genes. The functions of these genes vary and comprise the control of reception of the signaling molecules produced by microsymbionts, activation of common signaling cascade for AM and LRS (CSC) and the subsequent changes in transcriptome of the root cortex cells [6, 7, 13].

Garden pea (*Pisum sativum* L.) belongs to the most important legume crop in Russia and in the world [14]. There are over 100 independent pea mutants defective in LRS (rarely AM) development with 44 *Sym* genes affected [1, 8, 9, 15-21]. A promising approach to identification of the sequence of mutated pea genes is based on a comparative genetic mapping and search for candidate gene in the genome of closely related legume plant barrel medic *Medicago truncatula* Gaertn. and *Lotus japonicus* (Regel.) K. Larsen [8, 22-24].

The purpose of this paper is to overview current data on the genetic mapping of pea *Sym* genes, including the results obtained in ARRIAM.

Genetic mapping technique. Genetic mapping is based on the classical genetics including determination of linkage groups and estimation of recombination frequency. The distances between genes are measured in map units (m.u.)/Centimorgan (cM) equal to one percent recombinant phenotypes [25]. Genetic mapping involves statistical analysis of massive data on marker allele segregation in a certain generation after the crossing. To this end, at least a dozen of software has been developed, for example, MAPMAKER 3.0, MapL98, JoinMap 4.1, etc. [26-30].

Both morphological and molecular markers (protein- and DNA-based) allow pea gene mapping, however, only the involvement of DNA markers in genetic analysis made it possible to develop large-scale genetic maps with high resolution [31]. Over the past 25 years, various versions of the pea genome genetic maps have been constructed with the use of molecular markers [22, 32-43].

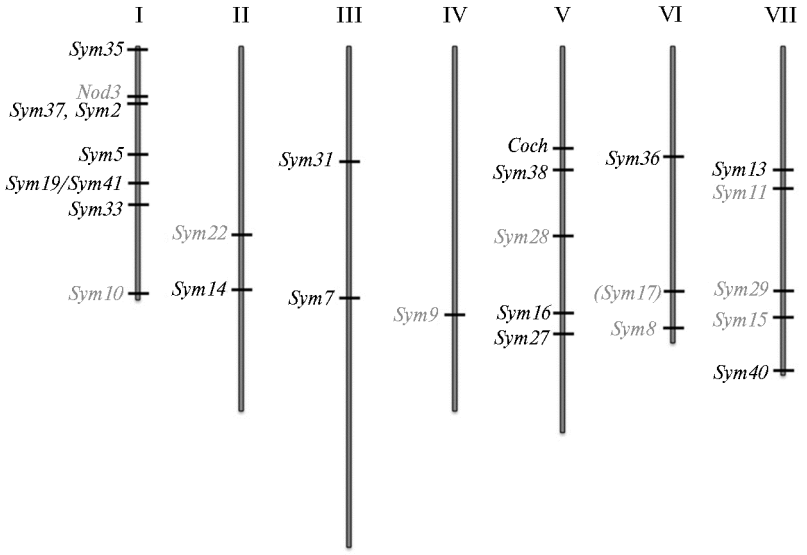
Gene-specific EST (expressed sequence tag) markers identified by amplification of the expressed sequence fragments are considered the most appropriate for genetic mapping pea genes after their detection by experimental mutagenesis. To design primers, the known EST sequence is commonly used. If the restriction site of the endonuclease applied to detect an allelic state of the amplification product coincides with the single nucleotide polymorphism site, the markers are referred to as CAPS (cleaved amplified polymorphic sequences) type markers. Allelic polymorphism of such markers is manifested as different numbers and sizes of the resulting restriction fragments separated by agarose gel electrophoresis [44].

Gene-specific markers in legumes allow to exploit the phenomenon of genomic synteny to transfer knowledge between model (*Lotus japonicus*, alfalfa) and crop legumes including pea [24, 45]. Synteny is referred to as the same order of homologous genes on the chromosomes of different species. This is micro-synteny in case of synteny within a cluster of several closely located genes, and this is macro-synteny if such a pattern is typical of the whole chromosomes or their extended sections.

Research aimed at developing new gene-specific markers and construction of genetic maps for legumes disclosed both macro- and micro-synteny in their genomes [22, 40]. Detection of high synteny among pea and alfalfa *Medicago truncatula* genomes generated a new impetus to the pea genetics development, especially after the successful sequencing of the alfalfa genome. Due to high genome similarity, the knowledge about the alfalfa genes can be used in studying pea genes, developing their gene-specific markers and gene cloning, once homologous mutations (i.e., mutations with similar phenotypic manifestation) found [24]. The Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute (USA, http://www.phytozome.net) [46] represents current information on alfalfa genome sequencing using interface of a genome browser that facilitates the search for homologous genes and candidate gene analysis.

Genetic mapping as a tool for identification of pea symbiotic gene sequences. Over the past 10 years, the set of more than 100 gene-specific molecular markers has been designed in ARRIAM for mapping pea *Sym*-genes of all linkage groups. In this, we used known ESTs which positions in the genome are described in literature (so-called anchor markers).

Oligonucleotide primers for PCR and amplification of target DNA fragments in different pea lines have been designed using nucleotide sequences of selected ESTs from GenBank (NCBI, http://www.ncbi.nlm.nih.gov). The resulting set has been allowed us to define first or clarify the localization of ten symbiotic genes [20, 47, 48]. The figure shows a generalized diagram summarizing all the data, including those obtained in ARRIAM, on the genetic mapping of pea symbiotic genes [9, 20, 47-49].




**Pea (*Pisum sativum* L.) symbiotic (*Sym*) gene mapping:** I-VII — the linkage groups**.** For *Sym17* (in parentheses) the position should be additionally confirmed [50]. The genes which position has been determined or confirmed in ARRIAM (St. Petersburg, Russia) are marked black and those reported

by other researchers are marked gray. The relative position of these genes is not defined: *Coch—Sym38*, *Sym16—Sym27*, *Sym14—Sym22*, *Sym11—Sym13*, *Sym15—Sym29*.

Genetic mapping of mutants using gene-specific markers allowed us to identify positions of 13 *Sym*-genes in pea. Many of them were determined simultaneously with that of orthologous genes in other legumes, including model species [20, 48, 51-57]. Generalized scheme to determine order of the pea genes included four steps. These are the gene localization on pea genetic map; search for known genes in the syntenic region of the model legumes (*Medicago truncatula* or *Lotus japonicus*); selection of candidate homologous genes for the target pea gene by phenotypic comparison between the mutants in pea and the model legume species; amplification and sequencing the pea gene with the primers designed based on the sequence of a model legume candidate gene [8].

The seven pea genes have been sequenced in ARRIAM independently or in collaboration with world's leading laboratories. Of them, the pea *Sym33*, *Sym35* and *Sym37* genes have been sequenced based on the homology to alfalfa gene *IPD3* [56], *Lotus japanese* gene *NIN* [52] and *NFR1* gene [54], respectively. The *Sym33* gene encodes the protein that interacts with $Ca^{2+}$-calmodulin-dependent protein kinase (CaM kinase), the central component of common signaling cascade (CSC), *Sym35* gene encodes the LRS-specific CSC-dependent transcription factor, and *Sym37* genes encodes the Nod factor (bacterial signal) receptor [6]. The pea *Sym40* gene was identified as a homologue of previously known gene *EFD*, encoding a transcription factor of the ethylene response factor (ERF) group in alfalfa [48], and the mutation corresponding gene *Sym41* is a variant (weak allele) of another symbiotic pea gene *Sym19*, encoding the CSC-involved receptor kinase [20]. The orthologous genes *Cochleata* and *NOOT* controlling nodule meristem have been recently identified as homologs of *Arabidopsis thaliana* (L.) Heyhn. *BOP1* and *BOP2* genes involved in leaf and floral morphogenesis [57]. Six of these pea genes have been sequenced based on the gene maps except *Sym34* with unknown localization. For *Sym34* sequencing the homology to the alfalfa *NSP1* gene which encodes CSC-dependent transcription factor common to LRS and AM was assumed solely on the basis of the phenotype of the pea mutant [21].

Thus, in pea the nucleotide sequences of 14 *Sym*-genes are presently identified which is only a third of that identified by mutational analysis, i.e., many *Sym*-genes have yet to be mapped and sequenced.

Next generation technologies — an outlook. In recent years, with the development of next-generation sequencing and massive genotyping, the information on gene-specific makers in pea is increasingly accumulated. Next generation sequencing (NGS) allows a high speed to read a great number of fragments, which makes it possible to analyze polymorphism of thousands sequences considered as potential markers. Massive genotyping using GoldenGat microchips (Illumina, USA) allows a one-time analysis of thousands designed markers. Due to these approaches, several thousand gen-specific markers have been mapped in pea in the past two years [43].

The whole genome sequencing in *P. sativum* undoubtedly will facilitate genetic mapping of the symbiotic genes. However, pea, like many other legumes, is still waiting for a chance to be among the species with sequenced genomes [58], while its studies are mostly limited to transcriptome sequencing [59-62].

Thus, the use of gene-specific markers for genetic mapping allows to exploit genome synteny to transfer data obtained for model legumes (*Lotus japanese*, alfalfa) to economically significant crops, including pea. Design of new-type "interspecies" markers derived from pairs of orthologous genes from different leguminous plants and saturation of pea genetic map obviously can greatly

assist in the implementation of a complete pea genome assembly based on the next generation sequencing (NGS). In turn, the mapping of symbiotic genes and identification of their sequences will help in better understanding of how the pea genes can control plant interaction with beneficial soil microorganisms.

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# PARTICULARITY OF BREEDING AND PERSPECTIVES ON THE USE OF MOLECULAR GENETIC METHODS IN FLAX (*Linum usitatissimum* L.) GENETICS AND BREEDING RESEARCH
## (review)

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Abstract

Breeding and genetic researches of flax (*Linum usitatissimum* L.) started more than 100 years ago, but their relevance does not decrease. More than 200 cultivars of this culture are offered in the international seed market for oil and fiber production with yearly amount of the harvested area about one million hectares and 0.3 million hectares, respectively. Different agro-climatic conditions in the countries, in which flax is being cultivated, and the progress in technologies of processing and new ways of application of products made of the fibers and other parts of the flax plant determine the necessity to accelerate breeding process. Now duration of breeding work for creating a new variety is up to 10-15 years. The dominating method for flax breeding is intraspecific hybridization with the subsequent selections. Basic part of breeding work is ecological trials and intensive using in the crossing the best new cultivars, old popular varieties («kriazhee»), local samples and breeding forms (S.N. Kutuzova et al., 2010; A. Diedrechsen et al., 2013). Prebreeding work focuses on the overcoming of limits of traditional hybridization methods by using some peculiarities of combinative variability (L.N. Pavlova, 2010). Recombination induction can be achieved by using the stress conditions for flax plant (hybrid) growing (A.A. Zhuchenko Jr. et al., 2009). Physical (γ-radiation) and chemical (N-nitrosomethylurea, ethyleneimine, dimethyl sulfate) mutagens considerably raise an output of mutant forms which possess economically valuable traits (M.I. Loginov et al., 2005; I.V. Uschapovsky, 2013). The article examines the examples of the use of cell and tissue culture techniques that extend somaclonal variation and allow to produce flax lines with desirable properties, including disease resistance (fusariosis, anthracnose) (V.A. Lyakh et al., 2008; N.V. Proletova et al., 2010). The review describes DNA marking technology which allows us to group genetic material of flax according to its genetic proximity and to optimize matching the genotypes to crossbreeding in view of saving the maximum genetic diversity (Y.B. Fu et al., 2003; V.A. Lemesh et al., 2006). SSR analysis is examined as the perspective direction for genetic identification of flax lines and cultivars (V.A. Lemesh et al., 2013), identifications of inter- and intraspecific genetic linkages (J. Vromans, 2006), possible linkages between molecular markers and economically important traits (V.A. Lemesh et al., 2012) and establishments of linkages groups between marker pairs (S. Cloutier, 2012). The directions of possible integration of traditional breeding methods and methods of molecular biology for creation of new flax cultivars with the set of the parameters of economically important traits are considered.

Keywords: fiber flax, linseed, breeding, hybridization, selection, mutagenesis, tissue culture, genetic variability, primers, markers, SSR, PCR

Common flax (*Linum usitatissimum* L.), because of the unique properties of its fiber and oil, is being used since the dawn of human civilization and still is of interest. Flax is an annual plant with a short growing period (80-120 days), a small number of agronomic and morphological traits, low rate of reproduction and small flower in which pollination, as a rule, occurs before the opening. Despite the fact that the crop is studied deeply enough, its phylogenesis, ontogenesis, genetic control and the inheritance of the traits remain topical for researchers [1-4].

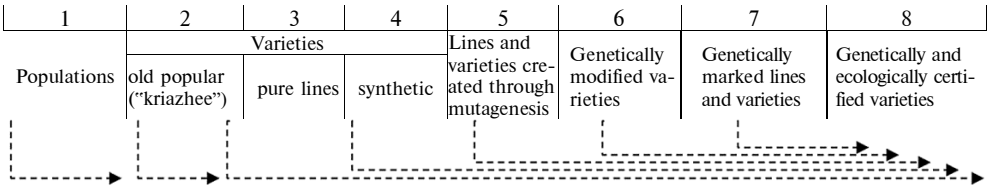
Flax domestication and breeding resulted in the two types of pure-line varieties grown for fibers taken from the stems (fiber flax varieties) and for lin-

seed oil and other components of the seeds (oilseed varieties). To date, more than 250 flax varieties are recorded in the world [5]. FAO UN reports the flax crop area remains relatively constant in recent years accounting for over 2.3 million hectares for oilseed flax and over 0.3 million hectares for fiber flax [6]. Since interest in the flax production is growing due to its use in various industries and the development of the doctrine of environmentally friendly economy, the area of flax crops will expand and the flax breeding will be increasingly important. Note, two flax morphotypes, due to intraspecific similarity, should be considered as a whole in the genetic studies and breeding.

The purpose of our review is to generalize the results and give an outlook on the prospects of molecular genetic techniques for the flax study and breeding.

Historical overview of flax breeding. As a common practice, flax breeding started in Holland and in Russia since the beginning of the XX century. First it was an experimental individual selection within local flax populations [7-9]. Since then, the flax breeding was largely based on a combination of simple and complex crosses followed by pure line selection [10, 11]. Practical experience of the breeder and a large number of parental forms checked under various conditions were essential to create the crop varieties [12]. In flax, the difficulties are mainly related to the fact that the genetics of this crop is poorly understood, a set of Mendelian genes used is small, the major valuable traits are controlled by QTLs, and different national research programs are not well coordinated.

Retrospectively, a scale of flax breeding methods is as follows (Fig.).




**Evolution in flax (*Linum usitatissimum* L.) breeding techniques and the types of varieties created:** 1 — unconscious selection (from the introduction in the VII-V centuries BC); 2 — massive selection (XIII-XIX centuries); 3 — individual selection (since the beginning of the XX century); 4 — hybridization based on meiotic recombination (since 1920); 5 — induced mutagenesis (since 1960); 6 — cell culture technique and genetic transformation (since 1980); 7 — molecular markers (since 1990); 8 — an integrated approach (forecasted from the 2020s).

The flax cultivation started with unconscious selection by man of plants for fiber and food use. It is believed to occur in the heyday of the Ancient Egypt civilization (7-5 thousand years ago), but earlier dates (10-30 thousand years ago) are supposed for flax introduction in the Colchis territory [13, 14]. Resulting flax populations were little influenced by selective factors during thousands of years of evolution and still can be found in the territories of cultivated flax origin, presenting a valuable material for its study. The world's flax collections preserve specimens of most these populations [4, 15-18]. Russian old landraces of flax (so-called kriazhee"), the polymorphic local populations known in the major regions of flax cultivation in Russia since XII-XIII centuries, are a classic example of mass selection from natural flax populations. These landraces produced more fiber of better quality in the place of origin, but did not possess the genetic stability and uniformity [1]. The first research schools engaged in flax breeding appeared at the end of the XIX century in Western Europe and Russia. For the first time, pure line flax varieties were obtained based on individual selection and crosses [12]. Flax breeding is mainly focused on productivity (straw output, fiber, seed and oil yields, fiber content in the stem), quality (fineness and breaking load of fiber, fatty acid composition of the oil, the content of bioactive substances in the seeds), resistance to fungal diseases, edaphic and climatic factors, ontogeny peculiarities (the length of vegetation period and the transi-

tion periods between ontogenetic phases, a synchronization of generative and vegetative growth). A high yield of fiber or oil, disease resistance and lodging resistance remain the priorities for nearly a century.

Currently, from 300 thousand to 1 million USD and more than 10 years are required to create a flax variety [19]. The most successful breeding groups manage to offer up to three varieties annually, but practical introduction of the novelties faces with many economic and organizational difficulties [20]. Stable varieties with acceptable economic value and agronomic characters (L-1120, Orshanskii 2, Belinka, Mogilevskii, etc.) can be cultivated for decades. However, intensive agriculture necessitates varieties which perform stable yielding, high quality [21, 22] and possess new traits or their combinations (e.g., dual purpose or winter flax).

Combination selection and sources of genetic diversity. Genetic (meiotic) recombination remains the most effective in breeding oilseed and fiber flax and involves simple hybridization, backcrossing, pollination of three or more varieties, sequential crossing. The vast majority of modern flax varieties have been created due to genetic (meiotic) recombination [21-24]. The identification of transgressive makes it possible to form a selection fund on the parameters of efficiency and quality of flax fiber [25]. However, the in-depth study of the flax recombination system is neglected [26].

Important sources of agronomic traits are working, national and international (under the auspices of FAO) flax collections [15, 17, 27-29], numbering more than 25 thousand items in total. From 1930s to 2000s, the flax breeding resulted in a narrowed genetic diversity, leading to increased undesirable correlations between productivity, earliness and quality [30]. It should be recognized that the genetic study of the flax collections is not deep enough, and scientific cooperation of the major collection holders is rare, while current characterization of the gene pool, prevention of sample duplication, reliable preservation and effective use of the resources require a correct assessment of genetic diversity [16, 31, 32]. Local landraces and forms resulted from a long natural and artificial selection are considered the "Golden Fund", as they are better suited to locale conditions, including the length of growing season [27]. Knowledge of the basic features of local varieties allows establishing their phylogenetic relationships and geographic distribution of alleles (gene geography).

Among the molecular genetic methods [33], we note the molecular marking. That is particularly effective for species which, like flax, are characterized by weak intravarietal differences. Molecular techniques revealed that in modern flax varieties the frequency of rare and unique alleles of microsatellite loci decreased while that of the fixed recessive RAPD (random amplification of polymorphic DNA) loci increased, indicating a narrowing of genetic variation [34]. SSR (simple sequence repeats) analysis is effective to detect unique loci that increase the genetic diversity. Based on the evaluation of microsatellite loci polymorphism, the valuable source populations with rare and unique alleles have been isolated among fiber and oilseed flax varieties and Belarusian landraces [35].

Induced mutagenesis. γ-Irradiation (200-900 Gy) of flax seed allows to get the progeny with positively altered valuable traits [36], and chemical mutagens (N-nitrosomethylurea, ethyleneimine, dimethyl sulfate), stimulating genetic variability, lead to an increased (over 30 %) output of mutant families in the progeny of treated plants [37]. Stressor (herbicide) enhances the effect of ethyl methane sulfonate (EMS) in flax [38]. However, mutational variability is not used wide enough. For more than half a century only 3 % of the flax varieties were developed using radiation and chemical mutagenesis [39]. An example of successful experimental mutagenesis in oilseed flax breeding is a commercial variety Solin with modified fatty acid composition. It is shown that the low α-

linolenic acid in flax is control by two independent recessive gene loci. EMS-induced mutants altered in two these genes were found in $M_2$ [40]. By self-pollination of $M_2$ plants a low-linolenic-acid line was developed with less than 2 % α-linolenic acid compared to 49 % level in the wild-type parent variety McGregor. EMS was also used to isolate two mutants [41] characterized by 30 % linolenic acid content. Their recombination generated plants with less than 2 % α-linolenic acid, and a sharp reduction in the linolenic acid content in seeds was not accompanied by its change in leaves [42]. There is a large collection of oilseed flax γ-ray- and EMS-induced mutations in the genes affecting growth, flowering, etc. [43].

A codominant DNA marker MutFad3, able to detect a mutant allele of gene *fad3B* encoding ω3/Δ15-desaturase in flax has been designed the Institute of Genetics and Cytology (Belarus) to reveal genotypes with a decreased α-linolenic acid content in the seed oil [44].

Cell and tissue culture. Doubled haploids, transformants and somatic mutations can be involved in breeding via in vitro culture.

Interestingly, flax embryos were among the first embryoids cultured in vitro [45, 46], and initiation of growing point in the flax hypocotyl explants was first reported in 1946 [47]. In 1970s there were publications on the effect of various components of culture media on flax morphogenesis in in vitro culture [48, 49]. Ability of flax hypocotyl pieces to form shoots, developing into full plants, and rhizogenesis in calluses derived from protoplasts were discovered in 1976 [50]. The same researchers found the dependence of in vitro regeneration on the genotype of explants [50].

First explanation of differences between in situ and in vitro processes was also experimentally obtained on flax. In early studies, flax immature and mature zygotic embryos were used to induce somatic embryogenesis [51]. Then somatic embryos were derived from hypocotyl segments of seed flax, germinated in vitro [52], and form protoplasts of *L. alpinum* L. [53]. A number of factors (source of carbon, total nitrogen content, free sterol, the ratio of calcium to zeatin) has been reported to influence somatic in vitro embryogenesis in flax hypocotil explants [54]. Various agents (6-benzylaminopurine, etc.) are tried to stimulate callus growth and organogenesis in vitro from hypocotil tissue [55]. Plants regenerated from hypocotyl had changed valuable traits, including improved resistance to fusarium [56]. Study of callus formation and regeneration showed different genetic mechanisms underlying these processes [57].

Haploid and dihaploid lines were regenerated from anther and microspore cultures in fiber flax [58, 59] and crown flax [60]. Currently, there are a significant number of domestic lines resistant to fusarium and anthracnose produced due to in vitro selection on the growth media containing toxins [61, 62). In vitro selection for resistance to Al and temperature is quite effective in flax [63, 64]. Obviously, somaclonal variation extends the opportunities of practical breeding. Additionally, in vitro experiments give deeper understanding of the fundamentals of cell metabolism.

Transgenesis in flax. The first successful *Agrobacterium tumefaciens*-mediated genetic transformation of flax with the use of strain carrying a non-oncogenic Ti plasmid-derived vector containing a chimaeric *npt-II* gene and a wild type nopaline synthase gene occurred in 1987 [65]. In 1988, the transformation of flax via other pathogenic bacteria, *A. rhizogenes*, was first reported [66]. In the same year, data on genetically modified linseed flax resistant to glyphosate were published [67, 68]. These plants, after the hypocotyl explant transformation, acquired an insertion of gene encoding 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. The same authors also developed the transgenic

flax plants resistant to herbicide chlorsulfuron [68]. Laboratory tests and field trials at elevated herbicidal load showed the ability of the plant progeny to produce the forms that combined high yield and herbicide resistance [69, 70]. There was an attempt to transfer the *pat* gene encoding phosphinotricine-N-acetyltransferase which confers resistance to the nonselective herbicide glufosinate, however, field trials were not successful [71]. The β-1,3-glucanase gene from potato was introduced in flax to improve tolerance to *Fusarium oxysporum* and *F. culmorum*. The resistance to *Fusarium* in the resulting transgenic plants was 3-times higher as compared to unmodified forms [72].

Appearance of escapes, the "false transformants" which have adapted to antibiotic- or herbicide-containing selective medium in the absence of inserted foreign DNA in their genome, is a side effects at transgenesis. In vitro conditions could be considered as stress, resulting in enhanced rate and range of genetic variability (poly- and aneuploidy, point mutations, transposition of mobile elements, somatic crossing-over, etc.). Due to somaclonal variations in flax in vitro culture, it was possible to derive forms resistant to extreme environmental factors [73], salinity [74], stress [75], the herbicide chlorsulfuron [76], and fungal diseases [59

False transformants, surviving despite the stress factors, apparently possess higher physiological adaptiveness, can extend the spectrum of genetic variation and, therefore, be useful to create forms with a valuable combination of traits. Based on false transformants, four fiber flax populations were first created in the Institute of Genetics and Cytology (Belarus). Certain plant groups significantly exceeded the initial variety in the number of bundles on a cut and the number of elementary fibers per slice (the latter by 12.2 %), indicating a well-developed fibrous tissue and, consequently, higher quantitative and qualitative characteristics of the fiber. Two-year field trials of false transformant-based populations of fiber flax showed a significant excess in total plant height, a technical length, and the number of seed capsules and seeds compared to those of the initial varieties [77].

Protein markers. Hampering flax breeding, despite a success of hybridization and selection, is due to the fact that the biological limits for major improved valuable traits have been almost reached. Therefore, the assessment of genetically determined characteristics, their comparison and phased monitoring of breeding process necessitate a transition to molecular level. For this end, different marker system can be used, including DNA and protein markers [78-81].

Identification and certification of flax varieties and biotypes based on the storage protein polymorphism are not widely used. There are two types of flax seed proteins, the 2S albumins (conlinins) encoded by two independent loci *Cnll* and *Cnl2* [82], and 11S globulins [83]. The patterns of storage proteins (and total seed proteins) in Russian [84, 85] and foreign [86] flax varieties differed slightly with different methods of extraction and electrophoresis, so their use in genetics, phylogenetics, taxonomy, breeding and seed production remains questionable.

Isozymes may also be the biochemical markers. It is shown that in fiber flax cultivars there are two isoforms of 6-phosphogluconate dehydrogenase, two isoforms of acid phosphatase, and four patterns of aspartate amine transferase isozymes due to the lack of one or more isoforms [79]. However, the homogeneity found in majority of the samples does not allow to use these isoenzymes for intraspecific and varietal differentiation. Nevertheless, the horizontal electrophoresis showed high genetic diversity in three enzyme systems (PGD — 6-phosphogluconate dehydrogenase, GPI — glucose phosphate isomerase and MDH — malate dehydrogenase) both between the varieties of different types (fiber flaxes, oilseed flaxes and landraces), and within each sample [87].

DNA markers. DNA markers facilitate differentiation of both individuals and taxons. Marker assisted breeding (or marker assisted selection), when the DNA markers tightly-linked to target loci are used, greatly improves the breeding programs.

It should be noted that the analysis of structure and function of flax genome is limited by insufficient knowledge of its karyotype. The difficulties in kariological studies started more than half a century ago arise from a small size of the chromosomes, complicated chromosome identification and cytological study of meiosis. In early publications the chromosome counts, reported for *L. usitatissimum*, differed ($2n$ = 30 and $2n$ = 32) [88-91], and only recent investigations confirmed that the diploid number of chromosomes in *L. usitatissimum* is 30 [92]. Differential staining pattern of flax chromosomes visualized by fluorescence in situ hybridization with DAPI (4′,6-diamidino-2-phenylindole) was similar to C-staining. The largest C/DAPI bands are located mainly in the precentromeric regions, while medium and small C/DAPI bands are located in the telomeric and intercalary regions. In the flax genome there are one or two basic 26S rDNA loci, co-localized with 5S rDNA loci, and one to three individual 5S rDNA loci [92].

Flax genome consists of ~ 35 % highly repetitive nucleotide sequences, ~ 15 % moderately repetitive nucleotide sequences and ~ 50 % low-copy sequences [93]. Early cytogenetic study revealed two nucleolar organizer regions with rRNA genes organized in tandem repeats 8.6 kb in length, encoding 45S rRNA and spacer DNA. 45S rRNA is a precursor of 25-, 5,8S and 18S rRNAs [94]. The 5S rRNA loci are evenly distributed along the flax chromosomes as a tandem repeats 350-370 bp in length, consisting of transcription units (120 bp) and spacers (230 bp) [90]. The multigene 5S rRNA family (~ 117,000 copies per diploid genome) accounts for about 3 % of the genome [95], whereas in *Arabidopsis* the 5S rRNA genes comprise only 0.7 % of the genome [96].

According to the earlier data, flax genome ranged from C = 538 million bp to C = 685 million bp in size, although the reassociation kinetics indicated C = 350 million bp [93, 97]. As to recent estimates, the genome of oilseed flax variety CDC Bethune is ~ 373 million bp in size [98]. This genome was sequenced with the use of WGS (whole genome shotgun) method [98]. The sequencing resulted in 116,602 contigs (a total length of ~ 302 million bp) assembled in 88,384 scaffolds (~ 318 million bp in size) that made ~ 81 % of the flax genome. The remaining 70 million bp were the gaps within and between scaffolds. To date, after correction the total length of assembled scaffolds makes ~ 271 million bp. The estimated size of flax chromosomes ranges from 15.1 to 32.8 million bp [99].

In 2011 [100], the first flax genetic map was constructed which included 24 linkage groups with 113 markers, covering ~ 833.8 cM. QTL analysis revealed two main QTL loci for linoleic acid (LIO, *QLio.crc-LG7*, *QLio.crc-LG16*), two main QTL loci for linolenic acid (LIN, *QLin.crc-LG7*, *QLin.crc-LG16*), two main QTL loci for iodine value (IOD, *QIod.crc-LG7*, *QIod.crc-LG16*) and one main QTL locus for palmitic acid (PAL, *QPal.crc-LG9*). Mutant allele *fad3A* mapped to the chromosomal segment inherited from the parent SP2047 form, underlies the QTL on linkage group 7 and was positively associated with high LIO level but negatively associated with LIN and IOD values. Physical map of the flax genome [101] contains 416 contigs averaged ~ 368 million bp in length. Of known fractions of the genome (54.9 %), 20.7 % are dispersed repeats, including LTR-Copia elements (3.4 %), LTR-Gypsy elements (1.8 %), LINEs and SINEs (0.4 %), unclassified LTR elements, DNA transposons (0.4 %), ribosomal DNA (13.8 %) and microsatellites (0.2 %). The coding regions occupy 26.8 % of

the flax genome, and functions of 45.1 % are unknown.

Integration of genetic and physical maps [102] showed that the haploid chromosome number in *L. usitatissimum* corresponds to 15 linkage groups. In sum, the consensus genetic map length is 1551 cM. A total of 670 markers are located on physical map within 204 of 416 contigs, covering ~ 274 million bp, or 74 % of the flax genome (370 million bp).

RAPD-analysis revealed the genetic diversity within and between varieties of flax and landraces [80], and the genetic diversity and geographical distribution of Canadian flax that facilitated the understanding of flax domestication [103]. It is shown that the flax varieties are very similar in genetic markers and constitute a homogeneous group, whereas oilseed flax forms several groups with nine landraces. It is also noted that in Canada, flax breeding has led to greater loss of genetic diversity than in the US. These findings concern most loci involved in the Canadian breeding programs. Geographically distant samples showed 84.2 % variability between the countries with 15.8 % variability within one country. The samples from East Asia and Europe had the highest genetic diversity, and those from Africa and India were more genetically homogeneous [103, 104].

Experts, when testing flax varieties according to the DUS (distinctness, uniformity, stability) criteria, noted that the morphological variability of new varieties decreased significantly [104], indicating their narrow genetic basis. AFLP (amplified length fragment polymorphism) analysis of a large number of samples revealed inter- and intragroup genetic diversity of fiber flax and oilseed flax under the growth conditions. Although the analysis failed to distribute the flax varieties in clusters, fiber flax population was found to be a subgroup of the oilseed flax population. This confirms separation of fiber flax from oilseed flax due to recent breeding for fiber quality, and, in turn, is consistent with the hypothesis of the origin of fiber flax from oilseed flax [78, 105]. Based on the submitted hypothesis, older flax varieties should have more similarities with the varieties of oilseed flax than modern varieties.

Due to 1306 SSR markers described to date, flax plants occupy a prominent position among other major crops [106]. According to various publications, trinucleotide repeats predominate among flax microsatellites [107-111]. However, these data may be misleading, since ESTs (expressed sequence tags) characterized by trinucleotide repeats were used in most studies to design corresponding primers. In flax, trinucleotide SSRs are the most common, but dinucleotide SSRs are more polymorphic [102] that is in line with the data for some other crops. It is shown [112], that a trinucleotide repeat AGC-GCA-CAG is most abundant in taro, ginseng, tangerine, millet, ginger, persimmon, lawn grass and foxtail Italian, AGG-GGA-GAG prevails in Chinese cabbage, Eleusine coracana, radish and sesame, a dinucleotide repeat AG-GA is the most frequent in Chinese cabbage, Eleusine coracana, cuckoo tears, railings, amaranth, sesame, radish, persimmon, apricot, and AC-CA is characteristic of sesame, mandarin, ginger, taro. In flax, trinucleotide GAA-AAG-AGA and dinucleotide AT-TA motifs were the most common, indicating the unique composition of its genome [102].

Trinucleotides are common for EST-derived SSR markers (68.7 %) rather than for those BES (BAC-end sequences)-derived (54.6 %) [102], probably due to suppression of non-trimeric SSRs in the coding regions which can change reading frames. Polymorphism positively correlates with the number of repeats per locus and the total length of the locus. In this, BES SSR markers can reveal significantly higher polymorphism (58 %, PIC = 0.39) compared to EST SSR markers (42 %, PIC = 0.34). Interestingly, polymorphism in flax varieties, even at 42 % effectiveness of EST SSR markers, is higher than that of wheat, barley,

soybean and cotton [106, 108, 113-115]. However, the polymorphism level may vary substantially depending on the number of investigated genotypes and their genetic diversity (Table).

**SSR markers described in flax (*Linum usitatissimum* L.)**

| Source of SSR[1] | Number of | | | | alleles per locus | PIC[2] (limits) | Authors |
|---|---|---|---|---|---|---|---|
| | geno-types | polymorphic primer pairs | loci | | | | |
| | | | in total | per primer pair | | | |
| Genome | 8 | 10 | – | – | 3.7 (2-8) | 0.60 (0.25-1.0) | I. Wiesner et al. (2001) |
| Genome | 93 | 23 | 28 | 1,22 | 3.3 (2-10) | 0.33 (0.02-0.73) | C. Roose-Amsaleg et al. (2006) |
| ESTs | 23 | 248 | 275 | 1,11 | 2.3 (2-7) | 0.35 (0.08-0.82) | S. Cloutier et al. (2009) |
| Genome | 60 | 60 | 66 | 1,10 | 3.0 (2-8) | 0.39 (0.06-0.87) | B.J. Soto-Cerda et al. (2011) |
| Genome | 8 | 35 | 37 | 1,06 | 3.5 (2-6) | 0.60 (0.23-0.84) | X. Deng et al. (2010) |
| ESTs | 61 | 23 | 23 | 1,00 | 2.3 (2-4) | 0.38 (0.08-0.55) | B.J. Soto-Cerda et al. (2011) |
| Genome | 8 | 38 | 38 | 1,00 | 3.4 (2-12) | 0.43 (0.20-0.88) | X. Deng et al. (2011) |
| Genome | 27 | 9 | – | – | – | – | S.M. Kale et al. (2011) |
| Genome | 19 | 42 | 42 | 1,00 | 3.3 (2-8) | 0.47 (0.10-0.86) | C.L. Bickel et al. (2011) |
| ESTs | 16 | 145 | 149 | 1,03 | 2.4 (2-6) | 0.34 (0.12-0.70) | S. Cloutier et al. (2012) |
| BESs | 16 | 673 | 720 | 1,07 | 2.8 (2-9) | 0.39 (0.12-0.85) | S. Cloutier et al. (2012) |
| In total | | 1306 | 1400 | 1,07 | | | |

N o t e. 1 — gene library or published sequences, expressed sequence tags (ESTs), BAC-end sequencing (BESs) ; 2 — polymorphism information content (индекс информативности). The dashes indicate no data reported.

Trinucleotides are common for EST-derived SSR markers (68.7 %) rather than for those BES (BAC-end sequences)-derived (54.6 %) [102], probably due to suppression of non-trimeric SSRs in the coding regions which can change reading frames. Polymorphism positively correlates with the number of repeats per locus and the total length of the locus. In this, BES SSR markers can reveal significantly higher polymorphism (58 %, PIC = 0.39) compared to EST SSR markers (42 %, PIC = 0.34). Interestingly, polymorphism in flax varieties, even at 42 % effectiveness of EST SSR markers, is higher than that of wheat, barley, soybean and cotton [106, 108, 113-115]. However, the polymorphism level may vary substantially depending on the number of investigated genotypes and their genetic diversity (Table).

Integration of molecular and traditional approaches. Breeding programs necessitate estimation of genetic variability occurred in present and primitive old cultivars of flax naturally or due to recombination and mutagenesis. Molecular genetic methods are effective to identify lines and varieties, inter- and intraspecific relationships, to find markers associated to quantitative traits, and to assign markers to linkage groups. Cluster analysis, based on grouping forms according to their genetic proximity, makes it possible to optimize hybridization in order to maintain the maximum genetic diversity of flax breeding material.

Molecular and traditional breeding methods, when integrated, are also important in the certification of varieties (for copyright, control of purity and matching to reference standards). Presently, use of molecular markers in DUS testing in the countries of UPOV (International Union for the Protection of New Varieties of Plants) remains debatable for technical (choice of methods of molecular marking) and legal aspects. UPOV's official view is summarized in a number of documents relating to the development of appropriate methodological approaches. Recommended molecular markers should be associated to traditional traits of the variety and convenient to determine disease resistance, genetic modifications (e.g., tolerance to herbicides), etc. Molecular markers are the most helpful n species with weak intravarietal differences, such as flax and rape.

DNA-markers have several advantages. They are high-resolution and, therefore, enable more precise revealing differences between the specimens. Molecular methods are applicable at all stages of plant development, being especially indispensable, when traditional approaches do not reliably allow distinguishing the samples. DNA analysis, along with pedigree method and information about

the key valuable polygenic traits of a variety give the most characteristic features for its description and certification. It is also important that modern technologies allow development of the DNA markers to assess the conformity of new varieties to standard criteria under DUS testing. Based on polymorphism, a panel of 11 pairs of SSR primers for genotyping flaxes poorly distinguishable or indistinguishable morphologically and a system for reliable molecular genetic certification have been developed in the Institute of Genetics and Cytology (Belarus) [116, 117]. The allele *Lu8207* which can serve as SSR marker of low linolenic oilseed flax was revealed by microsatellite analysis of low and high linolenic varieties carried out in the Institute of Genetics and Cytology (Belarus) [43].

Thus, step-by-step algorithm for flax breeding and genetic improvement by combination of traditional and molecular techniques involves 1) screening potential parental forms for commercially valuable traits under different environmental conditions; 2) molecular genetic analysis of the sources of valuable traits found; 3) hybridization and selection based on bioinformatic models (QTL analysis, cluster analysis, etc.); 4) DUS UPOV testing of the derived lines; 5) national or international registration of a new variety based on its genetically fixed characteristics with a Plant Variety Protection Certificate

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# BREEDING GRAIN CROPS TO INCREASE ADAPTABILITY
(review)

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Abstract

The most essential feature of promising varieties is their adaptability, i.e., an ability to withstand environmental effects, which reduce productivity. Adaptation under plant–environment interaction, and the use of self-regulation mechanisms of yield formation and habitat-forming environmental processes influenced by plants take a central place in the evolution theory and plant breeding (Z.V. Andreeva et al, 2014). The review considers the adaptive potential of grain crops and the main concepts, characteristics and tasks of plant breeding for adaptability. The purpose of such breeding is to obtain the varieties with high resistance to unfavorable conditions. The concepts of stability, plasticity, homeostasis and resistance to stressors are discussed. In a broad sense, the genotype–environment interaction reflects plant response to any changes of the environment. In a narrower sense, the concept is used to describe the productivity change of genotypes in different environmental conditions. This interaction is crucial to increase crop production. The interactions and interrelations of genotype and environment are various and complicated. They greatly depend on genotype and a factor chosen as environmental agent or conditions (V.A. Zykin et al, 2005). A slight difference between genotypic potential and its phenotypic manifestation indicates a less response of a particular genotype to the environmental factors. Various methods are helpful to identify potential productivity and adaptability of the varieties. The estimates of adaptability and plasticity allow to determine reliably the differences among breeding material and gives an additional information to select valuable parental forms possessing adaptive traits. Special breeding methods which depend on environmental conditions and a phase of plant ontogenesis are necessary to create crop varieties and hybrids that have a good adaptability (A.P. Golovchenko, 2001).

Keywords: plant breeding, adaptability, stability, plasticity, homeostasis, tolerance (resistance) to stressors

One-size-fits-all approach to environmental management systems and violation of requirements to planting of cultivated species and varieties in strict compliance with their adaptability, i.e. in edaphoclimatic macro-, mezo- and microzones optimal for their cultivation, contribute to decline in agriculture. Progress in this area is attributed to extensive use of methods of plant breeding for adaptability and introduction of modern seed breeding techniques [1]. Plant breeding plays a crucial role in crop production with a focus on steady increase in yield, sustainability, efficient use of resources and energy, and nature conservancy [2]. This generally makes sense, as only 10 % of more than 14 billion ha of agricultural lands worldwide are characterized by favorable conditions for cultivated crops [3]. With regard to other lands, high yield is constrained by various limiting factors.

Domestic and foreign literature offers various definitions of a norm of response to a range of changes of environmental conditions with regard to a variety, but all of them come down to adaptability. According to N.A. Lykova (2008), adaptiveness means preservation of viability and seed formation ability under non-optimal (up to extreme) conditions [4]. According to A.A. Zhuchenko (1988), increase in yield is inextricably connected with resistance of crops to effect of factors, causing decrease in productivity. It has been argued that the contribution of plant breeding to increase in yield of major agricultural crops has reached 40-80 %

over the last 30 years. Biological component, most significantly the selective improvement of varieties and hybrids, will play an increasingly important part in yield gain and promotion of its quality [5]. Plant breeding for adaptability formed the basis for local inhabitant plant breeding, when resistance of plants to adverse climatic conditions and diseases was considered more valuable than record-breaking harvest. Modern plant breeding is primarily focused on varieties with high adaptive potential. Such potential represents an inherently determined ability of adaptation to changing environmental conditions [6]. It can be also defined as a limit of resistance of cultivated plants to adverse factors, such as insect pests, weed infestation of crops, diseases, drought, soil salinization, or low temperatures [1].

Violation of adaptability requirements leads to dramatic rise in prices of agricultural products or prevents survival of introduced plants, like attempts of corn cultivation further north than its area, or tea plant cultivation in Transcarpathia [7].

Monitoring of ecological plasticity, stability and adaptability of varieties and hybrids is the key feature of breeding adaptive plants. The average value of a trait and environmental sensitivity of a trait are under independent genetic control [8-10]. Adaptability of a variety is assessed through decrease in average productivity trait. Resistance of varieties and lines to stressors, determined by difference between the minimum and the maximum trait ($Y_{min}$-$Y_{max}$), is an important indicator of adaptability and ecological plasticity. This is a negative indicator, and decrease in its absolute value leads to increase in stress resistance, i.e. broadens the range of adaptive abilities of a variety [11-13].

According to V.O. Ostroverkhov [14], ecological plasticity of plants means the ability to adapt to changing growing conditionsand, with regard to varieties, the ability to produce a good and high-quality yield under various edaphoclimatic and agrotechnical conditions. V.N. Mamontova [15] and others [16-18] define plasticity as a persistently high yield under various environmental conditions. When considering ecological plasticity as a relation between productivity and resistance, a somewhat different interpretation is offered: in this case genotype plasticity shall mean the degree of its response to improvement of growing conditions [19]. V.A. Zykin et al. [20] define this concept as an ability to consistently produce high yield (as compared with other varieties or hybrids) with genetically determined quality within a broad area, under sufficiently diverse weather and agrotechnical conditions. Other authors use this definition as well [21, 22].

Homeostasis theory as an ability of plants to maintain internal equilibrium and to fulfill the genetically determined potential of a variety at the phenotypic level under abnormal conditions is an integral part of breeding ecologically plastic varieties [23]. A state of homeostasis can be used as the main genotype evaluation criterion. Homeostasis of a variety is measured through its ability to less significant decrease in yield in case of impairment of cultivation conditions. The above mentioned is critical to obtaining both maximum and steady yield within a wide range of growing conditions [24-26]. Steady grain yield indicates high homeostaticity, while increased variability indicates low homeostaticity of a genotype with regard to the same limiting factors of the environment [24].

Genotypes with high homeostaticity provide less volatile productivity of plants within an agrocenosis and insignificant decrease in yield in case of close planting or adverse factors combined with close planting [27]. Homeostasis is associated with physiological «buffer capacity» (tolerance to adverse environmental factors) [28]. A variety shall be considered as buffer (stable) in case of stability of its yield with regard to a wide range of acting factors [29]. In a narrow sense, stability is inherent in varieties, where changes of environmental conditions do not affect the development of traits (stability regardless of yield or another trait).

In a broad sense, it is the degree of deviation from mean with regard to response to change of conditions within a system (group) of genotypes in question (stability of high productivity). Stability is also considered as steady manifestation of a trait under various conditions [30], ability of genotypes to maintain a certain phenotype under various conditions, using specific regulatory mechanisms [31], adaptive response of a genotype, leading to correlation between changes of status of traits and properties of an organism, and changes of environmental conditions [32]. A.A. Zhuchenko [5] highlights a close connection between stability and adaptive potential of plants, which is manifested under various environmental conditions with various physiological, morphological and other adaptive responses.

During ontogenesis and phylogenesis plants are affected by various abiotic and biotic factors, complicated in terms of their combination, intensity and time of manifestation. At that, genotype–environment interaction of a certain nature is established [33]. It was demonstrated that, when comparing genotypes grown under different conditions, their degrees of productivity are not constant, but vary depending on conditions, i.e. in a narrow sense, genotype—environment interaction effect is observed [34]. A slight difference between genotypic potential and its phenotypic manifestation in contrast environments indicates a less response of a particular genotype to their change [35).

Various aspects of genotype—environment interaction and methods of its evaluation in plant breeding are the subject of wide speculation in professional literature [36-39], with regard to ecological breeding issues [40] and epistasis [41], resistance to diseases [42], in terms of various cultures [43-45] and depending on physiology of a variety [46].

Genotype—environment interaction is common biological phenomenon, statistically represented by nonadditivity of genotype and environmental effects, observed in determining various genetic parameters. Statistical approaches for evaluation of this interaction have been suggested, when testing varieties in several environments (variety × year, variety × area, variety × year × area), and when assessing general and specific combining ability of genotypes and a degree of dominance. Interaction and relations between a genotype and environment are significantly diversified and complicated by nature and the character of manifestation, and depend on a genotype and the factor considered as an environment or conditions [35, 47]. It is believed that evaluation of genotype—environment interaction gives an insight into stability and plasticity of genotypes in question. At that, plasticity and stability characterize modification abilities with regard to certain traits of plants [48]. It is established that a) adaptability is mostly related to plasticity or lack of stability; b) plasticity of various traits differs, but is related to the time of manifestation of a trait during ontogenesis; c) traits formed during long-term meristematic activity periods (habitus, the number of leaves, etc.) are more prone to environmental impacts and more plastic than quickly formed ones; d) plant breeding can be performed with regard to both high and low plasticity of a certain trait; e) special attention should be given to examination of traits responsible for adaptability of a variety to the conditions of cultivation area and thus affecting sustainable yield [17].

It has been argued that stability and response of a trait are opposite features of a genotype (i.e., a genotype can not be stable and responsive with regard to the trait in question at the same time) [31], and stability of yields limits the possibility of increasing yield by improvement of conditions, including agrotechniques, which is not universally true. Thus, strong drought-resistance genetic systems will support productivity under drought conditions, and a variety will provide a bigger yield gain in case of irrigation. At that, a variety which is not drought-resistant will provide no yield, but will show the same yield gain in case of irriga-

tion, as compared with a drought-resistant variety. These abilities play an important role in adaptability and maintenance of homeostasis in plants. Maximum adaptability of genotype is ensured by plasticity of certain traits which, in their turn, determine stability of other traits [35]. Plasticity is related to adaptation mechanisms: ability to respond to environmental signals is genetically determines, while the degree of response may evolve due to effects of natural and artificial selection [49].

An ecological test shall be performed in order to determine the response of genotypes to environmental changes. An extended ecological test is particularly useful with regard to search of adaptability gene sources [50]. However, such test is expensive. The use of various preceding crops, planting dates, forms and doses of fertilizers, as provocative conditions, can be helpful. An ecological test of breeding material provides a large amount of data which can hardly be analyzed without the respective methodological approach and processing. Some approaches may only complement the data on biological properties of a genotype, which shall form the basis for decision on future use of samples in question [22, 51].

The works of K.W. Finlay and G.N. Wilkinson [52] is among those first focused on phenotypic stability analysis of genotypes during testing under various agroecological conditions. These authors have developed the statistical method to compare yield for a set of corn varieties, grown in several areas during several years. An improved mathematical model and a calculation procedure for interaction between genotypes and environmental factors were offered upon its adjustment [19]. This approach was rather commonly used in plant breeding to evaluate ecological plasticity and remains relevant both in Russia [35, 53-55] and worldwide [53-59]. Stability evaluation allows us to describe the ability of a genotype to combine high potential yield with minimum decrease in yield under adverse conditions. Later this method was partly updated 60, 61]. The approach offered by G.C.C. Tai [62] comes down to evaluation of genetic stability based on structural relations analysis. Genotype—environment interaction effect is considered as two components: linear response to environmental effects (statistical $\alpha$) and deviation from linear response (statistical $\lambda$). Ecological plasticity parameters $\alpha$ и $\lambda$ correspond to bi and $\sigma d2$ parameters according to S.A. Eberhart and W.A. Russell [19, 30, 62]. Both methods provide for graphic representation of results which makes the data easier for understanding. A method of behavioral evaluation for a set of genotypes in various environments has been developed as well. This method allows us to identify general and specific adaptability, to perform target breeding, to specify environments as breeding conditions and to divide a phenotypic population variance into general and specific adaptability variances to choose breeding methods [31, 63].

A so-called ecovalence [64] was offered for each variety representing to characterize the variability. The more this indicator differs from 0, the stronger response of a variety to environment change it provides [64, 65].

A coefficient of response to improvement of growing conditions is used in evaluation of varieties. It represents a fraction, where the value of a studied trait of a genotype grown in improved conditions is a numerator, and the value of a studied trait of a genotype grown in less favorable conditions is a denominator [20]. V.A. Dragavtsev et al. [66] used a multiplicative coefficient to determine a homeostaticity, which is calculated using a ratio between increment in the average trait of a variety for several geographic points and the average trait under ecological gradient. Homeostaticity of varieties can also be calculated using a ratio between the average trait and the difference between its values under optimal and limited (severe) conditions [67].

Suggested identification of potential productivity and adaptability of vari-

eties [68] is based on a ratio between an implemented general species adaptive response to certain conditions and the average yield for the compared varieties. This value is a response indicator for a certain set of varieties with regard to environmental factors in every certain year. Response of each tested variety to these factors can be determined by comparing its yield with the average yield for the variety using a percentage ratio, where the average genotype value is taken as 100 % [68, 69].

Some researchers assume that evaluation of genotypes using one or two methods is not sufficient for representation of their stability and plasticity. Application of several methods provides the most detailed information, but ranking with regard to all parameters shall be used in this case, and evaluation shall be performed using the rank sum obtained by each method [70, 71].

When using various methods to evaluate a variety ecological plasticity, awareness of their compatibility is crucial [72]. The character of correlations between parameters and relation between the same parameters and yield are reported to vary significantly. Strong positive relations allow us to choose one of these indicators without detriment to outcomes [20]. Lack of significant relations between yield and the indicators of response and stability suggests a possible combination of these parameters within one genotype [71]. When comparing three known methods [60, 67, 19], it was suggested to use the last one to evaluated ecological plasticity by the trait with mandatory consideration of its average value and the limits of its variation [21].

For the foreseeable future, agricultural progress will determine improvement in adaptation of agricultural systems and cultivated lands to time- and spatial-varying environmental factors. These breeding trends are characterized by agroecological targeting, related to better adaptation of new varieties and hybrids to local conditions, as well as knowledge-intensive cultivation techniques. Their main distinctive feature is a combination of high potential productivity (yield and its quality) and resistance to the most common local abiotic and biotic stressors, as well as dominance of a genotype over uncontrolled environmental factors [73). Effectiveness of such breeding is ensured by use of various methods for evaluation of ecological plasticity which differ by complexity of calculations and result interpretation. As an approach, creation of environments for identification of adaptive traits and features has been proposed. A number of problems should be solved in order to obtain highly adaptive genotypes for certain area, including a) determination of mechanisms of response to environment changes; b) choose of desired traits and properties; c) identification of effective methods for breeding material evaluation; d) specification of environments to identify such genotypes, etc. In developed countries, yield increase depends on genetics and breeding by 90-95 %, and on improvement of agricultural technologies only by 5-10 % [74].

Adaptability of a variety (hybrid) is a balanced combination of numerous traits, where the most valuable are preferred. Adaptability degree depends on both adaptation of a variety and specific conditions within an agrocenosis. The following requirements are applied to an adaptive variety: a) ecological plasticity (ability to provide at least average yield within a wide range of climate variations); b) heterogeneity of agro-populations (presence of plants differing in height, rooting depth, drought resistance, blooming period, etc.); c) early maturity (ability to grow and develop quickly); d) intensity (ability to respond quickly to improved growing conditions, for example, to precipitation); e) resistance to fungal and other diseases; f) resistance to insect pests and high regrowth in case of infestation [8].

Spring wheat variety Lutescens 62, released as early as in 1929 and cultivated on large areas in eastern, southern and central regions of the country, as well as winter wheat varieties Bezostaya 1 and Mironovskaya 808, grown on sev-

eral million hectares in the USSR and cultivated in a number of countries, may serve as an example of ecologically plastic varieties. Genotype plays an increasingly important role in yield gain and steadying, and the contribution of a variety during regionalization amounts to 30-50 %, according to some sources [75].

Globally, continuous increase in yield is based on improvement of growing technologies and the achievements in plant breeding. Breeding of complementary varieties is considered rather challenging [1]. Modern varieties shall be adapted to mechanized sowing, planting, handling, and harvesting. Non-shattering grain crops varieties resistant to lodging are highly valuable. Mexico, India and other countries have made meaningful progress in wheat grain production over 15-20 years using short-stalked varieties, and have refused from its import completely. The highest yield achieved in field trials in India with improved technology) amounted to 10.2 t/ha at 4.5 t/ha on average. Early-ripening, drought resistant, and winter-hardy varieties, or varieties for irrigated or reclaimed lands may be required, depending on conditions in the area of cultivation. Reclamation of marginal lands (acid, salinized, or bogged soils, sands) also depends on results in plant breeding. This aspect is a matter of particular concern in some countries. In Brazil, the varieties which are not resistant to acid soils are not allowed to production [1]. Adaptations under moisture deficiency [76-78], drought [79, 80], thermal stress [81], and salinity [82] have been studied at various levels of plant organization in recent years.

A concept of habitat-forming and resource reclaiming (nature reclaiming) role of varieties, formulated by A.A. Zhuchenko [83], is especially noteworthy. In fact, this resource of plant breeding, despite its value, is undeesstimated. Decrease in reclaiming properties upon achievement of high potential yield is observed. Adaptation of modern varieties and hybrids is often insufficient to create high-yielding and environmentally sustainable agricultural systems and cultivated lands. As a result, only 25-40 % potential yield is obtained because of insufficient (often decreasing) plants resistance to abiotic and biotic stresses. Effectiveness increases when several varieties are cultivated, differing by maturation timing, intensity of growth processes, and response to environmental challenges, soil fertility, and the preceding crops [1].

Thus, adaptability, as the most essential feature of promising varieties, shall be considered in breeding programs. The environment for variety cultivation should also be specified, considering the role of genotype—environment interaction. Estimation of potential productivity and adaptability, as well as evaluation of stability and plasticity of crop varieties allow us to determine the significance of observed differences and to reveal the sources of valuable traits for breeding programs.

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# THE THEORETICAL JUSTIFICATION OF INTENSIVE GENETIC POTENTIAL OF THE VARIETIES OF SOFT WHEAT

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Abstract

Wheat, a hexaploid originated in Anterior (N.I. Vavilov, 1926) and the Central Asia (P.M. Zhukovsky, 1971), was widely spread and grown beyond the Fertile Crescent northerly and southerly. In this, cold resistant wheat and drought resistant spring forms have been originated in the course of unconscious selection (N.I. Vavilov, 1926; Qing-Ming Sun et al., 2009), and nowadays bread wheat is a dominant cereal crop cultivated worldwide (N.P. Goncharov, 2013). Analysis of origin of the soft wheat intensive breeds shows that more than 150 years, they were formed on the basis of the genetic material the secondary (P.A. Gepts, 2002; G.M. Paulsen, J.P. Shroyer, 2008), induced the peripheral centers with huge potential (R. Vencovsky, J. Crossa, 2003; S. Cox, 2009). Akagomughi, a short-strawed Japanese variety became the basis of intensive selection (N.I. Vavilov, 1987). A successful combination of genetic associations in derivatives of Hungarian (Banatka), Russian (Krymka), local Galician, English squarehead wheat and Chinese dwarfish wheat made it possible to create a high-yielding, adaptive intensive winter wheat variety Bezostaya 1. It was widely involved in the breeding for intensive yield production resulting in the best intensive high-yielding varieties of winter soft wheat. In the 1970s, these varieties were used to produce new spring wheat intensive varieties, such as winter-and-spring wheat Kazakhstanskaya 10 and spring variety Ikar. Note that the use of vernalized seeds of winter wheat for hybridization is not desirable because of temperature-induced mutations reducing the genetic value of the original forms. In crossing winter crops with spring crops it is necessary to allow them to pass flowering phase simultaneously (V.V. Novohatin et al., 2014). Discrete inheritance in each variety leads to certain changes in its biological, morphological, physiological and bio-climatic properties reflecting evolutionary direction in plant breeding. For example, Kazakhstanskaya 10, created by hybridization of 39 varieties of which 23 one were winter wheat varieties possesses a well-developed, deep penetrating root system (243 cm), is tolerant to salinization, pre-harvest sprouting and fusariosis. Its potential yield under irrigation is 8.02 t/ha. The variety is common in Central Asia and the south-east Kazakhstan, Bashkortostan, Kurgan and Tyumen regions. The variety Kazakhstanskaya 10 is involved in many breeding programs. As a result, a middle-ripening, medium-height, resistant to lodging and pre-harvest sprouting, intensive Ikar variety (winter wheat Bogarnaya 56 × Kazakhstanskaya 10) (Pyrotrix) been created which genealogy includes 59 varieties of different ecological origin. Its distinctive features (the pubescence and dark-colored ears) contribute to the accelerated maturation of the grain, which is very important for Siberia and Trans-Ural Region. Data on full genealogy of created varieties allows us to control the hybridization of parental pairs when creating the desired genotypes and ecotypes.

Keywords: wheat, environment, genome, population, breeding, variety, area, primary and secondary centers, mutations, hybridization, transgressions, evolution

Spring bread wheat combines the genetic potential of the *Triticum* L. and *Aegilops* L. genera thanks to natural hybridization followed by amphidiploidization of genomes of monoploid *Triticum urartu* (einkorn) and two diploid species, the *Aegilops speltoides* and *Ae. squarrosa*. Einkorn *T. urartu* became the source of genome A, and *Ae. speltoides* of genome B. Their amphidiploid *T. dicoccoides* participated in the formation of a tetraploid *T. dicoccum* (Emmer, or hulled wheat), carrying the BBAA genome, and further attachment of the genome D from *Ae. squarrosa* allowed to produce a wheat hexaploid with the AABBDD genome [1-3].

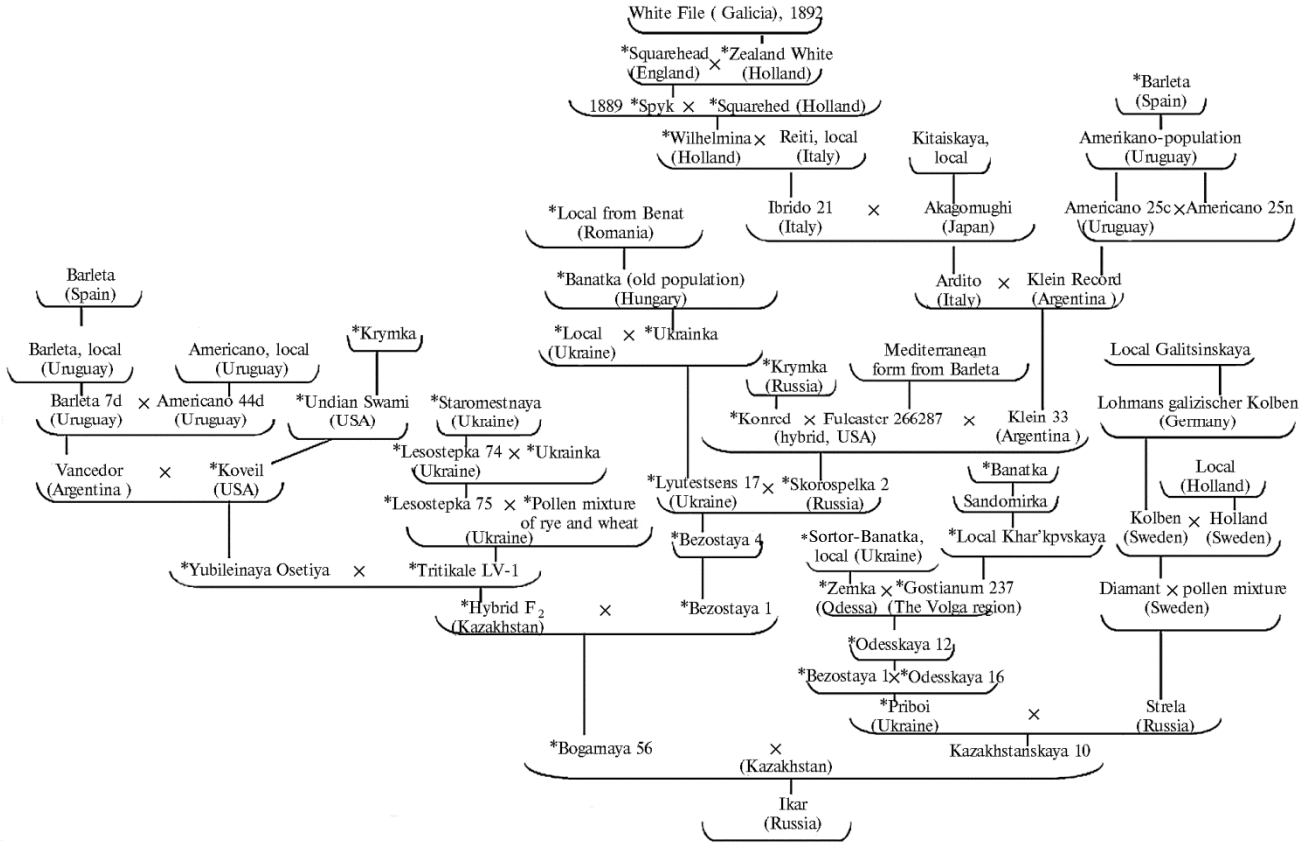
According to N.I. Vavilov [4], the origin of bread wheat is associated

with the Persian center of origin, while according to P.M. Zhukovsky [5] its birthplace was the Central Asian Center. This hexaploid became widespread in the territory of the Fertile Crescent, i.e. from Asia Minor to the Iran-Iraq frontier and from Palestine to the Turkish Caucasus, where its domestication completed up to the II millennium BC. Here started the original evolution of the species [3], which became the basis for its further changes [5, 6] that are ongoing till present [7, 8], which is confirmed by modern molecular methods [6].

The area of bread wheat expanded from the primary center of origin to the suburbs, where the recessive genes accumulated. To the north cold resistance was formed, while to the south — drought hardness [4] and springiness [9]. The long-term (hundreds of thousands of years) diffuse spread had led to the ecocentric principle and development of secondary [10, 11], and, later, of induced and peripheral [12, 13] centers of origin of species. Indeed, on the outskirts of the area, in the numerous valleys, mountain labyrinths of the Carpathians and the Pyrenees, with contrasting climate variations, increased ultraviolet insolation, extended days, and favorable water and mineral regime, there developed the secondary morphogenetic centers of origin. Thermal phytomutations and spontaneous hybrids developed, which accumulated biotypes with modified systems of *Vrn* and *Ppd* genes, contributing to their winter character. This expanded the possibilities of using agro-climatic factors and producing high yields [14]. The latter is largely driven by the genotype—environment interaction [15-17] which unequally manifest itself under different conditions [18]. Moreover, it is much more pronounced in new varieties [19, 20], and in the future it will be controlled and adjusted [21]. Wintering forms of bread wheat of the Carpathian origin (local Galician form, Banatka- and Taika-based varieties) emerged in the III-II millennium BC [22], and, due to the evolutionary variability, the crops accumulated extensive genotypes which were closely linked to environmental factors and ensured crop dissemination [23).

It is very important to engage valuable genetic sources from secondary and induced centers of origin [24-26]. In the secondary centers of origin, wheat for centuries was subjected to natural and artificial (unconscious and conscious, or systematic) selection. Species from other foci of morphogenesis penetrated here. The resulting populations were characterized by a high heterozygosity and a large number of subvarieties. Natural mutations and hybridization in the secondary foci contributed to the emergence of new transforms. These populations spread throughout Europe, and later penetrated into Russia. The proximity of Russia to the major centers of origin of the wheat had a beneficial effect on the formation of complex populations, consisting of various biological forms. For example, the Hungarian wintering variety Banatka became widespread in the southern regions of Russia and was the basis for drought-resistant Krymka varieties, tracing their origin from the X-VIII centuries BC [5, 22]. Through the Black Sea they penetrated into Turkey, and from there, acquiring the name Turkeus, into the Apennines, in Australia and North America, and also became the basis of all varieties in Central Europe.

The Banatka population gave rise to cultivated in the south-west of Ukraine (XVIII-XIX centuries) local winter populations. It had a high variety-forming ability, thereby becoming the basis of winter varieties produced by means of analytical selection, such as Ukrainka, Lesostepka 74, Lesostepka 75, Lutescens 17 (the female parent form of the Bezostaya 1 variety), as well as Zemka and Gostianum 237 (the parental forms of the drought-resistant high-quality variety Odesskaya 16) (see Fig.). Local varieties from Western Ukraine replenished the genetic resources of the crop in Germany, Italy, and the Czech Republic [24-26].




**Pedigree and origin of intensive bread wheat (*Triticum aestivum* L.) varieties** (winter forms marked with asterisks).

At the beginning of the XVIII century, the Spanish population Barletta spread along with the settlers to South America. Under new conditions, during the transition from winter to spring forms, the impact of local factors on the populations led to the elimination of poorly-adapted biotypes. New phytomutations and spontaneous hybrids with an altered genetic structure appeared which greatly expanded the range of variability. Because of transgressions, in a setting of high pathogenic load, populations tolerant to rust and Fusarium fungi stood out against the remaining ones. When a directed formative process was applied, new associations between genetic systems were created within them, which led to the emergence of high-yielding forms, adapted to local conditions. The artificial selection of the best of them (the end of XIX—beginning of the XX century) gave rise to genetically diverse varieties which became the basis of the South American selection. The intensive Vancedor and Klein Record varieties, resistant to abiotic factors, spread through the region.

From the Krymka form, in the United States [11] the Koveil and the Konred varieties were selected, as well as a hybrid produced with the participation of the latter and the Mediterranean variety Fulcaster 266287 (see Fig.). Crossing the Argentinian variety Vancedor and the American variety Koveil allowed to produce the winter variety Yuzhnaya Ossetia (the Caucasian southern forest ecotype), which became the parent form of a strong, winter, drought-resistant cultivar Bogarnaya 56 of the dry steppe ecotype.

A local high-yielding plastic Galician population was widely used in the analytical selection and creation of breeding material in the UK, the Netherlands, Germany and Scandinavia. The participation of this population and the derivatives of high-yielding, resistant to lodging, low-quality English Squareheads allowed to release a well-known Dutch form Wilhelmina which was used as a female parent for producing the Italian variety Ibrido 21. In the latter, a local form Rieti was the initial one (♂). Crossing Rieti with the Japanese cultivar Akagomughi [27], having the recessive genes of the short stature [28], gave a high-yielding form Ardito common in Italy which became the basis for intensive breeding [29]. In Argentina, with the participation of the Ardito variety (♀) and a local variety Klein Record (a direct descendant of the Barleta), a short, high-yielding, resistant to lodging variety Klein 33 was developed [27], bringing together a pool of genes from seven varieties of different geographical origin, such as the Netherlands, Argentina, Italy, Japan (their basis included Barleta, a local Galician wheat, English Squareheads and Chinese dwarfish wheat).

The American winter hybrid (♀) Konred × Fulcaster 266287 and the Argentinian intensive variety Klein 33 (♂) participated in the creation of the Kuban' winter variety Skorospelka 2, which lacked winter hardiness and plasticity. These properties are characteristic of Lutescens 17, the variety of the same ecotype and a direct descendant of the variety Banatka in which the intensity was limited due to lodging. A combination of Lutescens 17 × Skorospelka 2 was initial in creating the Bezostaya 4 variety, from which, by means of recurrent selection, the Bezostaya 1 of the southern forest-steppe ecotype was produced [30].

A successful combination of genetic associations in derivatives of Hungarian forms (Banatka), Russian forms (Krymka), local Galician, English Squareheads and Chinese short wheat made it possible to create a famous winter variety Bezostaya 1. This is mid-season, medium-grown, high-yielding, plastic, intensive variety, resistant to lodging, with high grain quality, having a high variety-forming ability. Bezostaya 1 is a male parent form of the heat and drought-resistant, winter-hardy variety Bogarnaya 56 (Pyrotrix subvariety) with high grain quality. Its female parent form was an interspecies hybrid produced by crossing the winter variety Yuzhnaya Ossetia (♀) (a direct descendant of the Barleta and

Krymka forms through Argentinian and American breeding) with a triticale LV-1 (created with the participation of the Ukrainian variety Lesostepka 75, a derivative of the Banatka variety and a mixture of wheat and rye pollen) [31]. The selection of intensive cultivars involves as a parent form the winter drought-resistant and tolerant to leaf rust Priboy variety, of the steppe ecotype, which was produced with the participation of the Bezostaya 1 (♀) and Odesskaya 16 (a drought-resistant Banatka derivative) varieties. Its distinctive features include a well-developed, deep penetrating root system, resistance to Fusarium root rot [32] and a high grain quality [33].

The Priboy variety is involved in creating intensive varieties of spring bread wheat [34]. Success was a combination, in which the male parent was a spring, mid-season, high-yielding form Strela (Milturum subvariety), a spontaneous hybrid of Swedish variety Diamant, derived from the Swedish variety Kolben, local Galician wheat and the Swedish variety Holland (selected from a local Dutch form, produced with the participation of English Squareheads and a Galician population). In cool environment, the Strela variety is capable of forming a grain resistant to pre-harvest sprouting, and may actively ripen. The variety intensity is limited due to its tall stature and a tendency to lodging with its yield exceeding 3.0 t/ha.

In a combination Priboy × Strela (the $F_2$ generation), the best plants were selected (Milturum subvariety) and reproduced for 3 years. From a segregating family, by means of recurrent selection, the 15612-13-77 line (Lutescens subvariety) was isolated which became the female parent of the spring mid-season, medium stature, resistant to lodging, intensive variety Kazakhstanskaya 10 (winter-and-spring wheat). The variety possesses a well-developed, deeply penetrating root system (up to 243 cm), is tolerant to fusariosis and pre-harvest grain sprouting, is common in Central Asia and the south-east Kazakhstan, where it is cultivated in saline rice rotations. Its maximum yield (8.02 t/ha) was obtained by the autumn sowing in the irrigated field of the Przhevalsky seed-trial ground in Kirghizia (now Kyrgyzstan), in 1987. As a spring variety, it became widespread in Bashkortostan, Kurgan and Tyumen regions. It is included in the list of valuable varieties. The genealogy of Kazakhstanskaya 10 involves 39 intensive varieties, including 23 winter ones, and covers all the secondary and most of the induced centers of origin of the culture. The variety boasts a well pronounced plasticity and combining ability, it is involved in many breeding programs. As a result, from a hybrid combination Bogarnaya 56 (winter) × Kazakhstanskaya 10 in the $F_7$ generation, the Pyrotrix 365 line was selected which became the Icarus variety (Pyrotrix subvariety). The obtained variety is mid-season, intensive, medium stature, resistant to lodging, hardy to pre-harvest sprouting, with a potential yield of 6.0-7.0 t/ha [10, 20]. The presence of pubescence and dark-colored ear contribute to the accelerated passage of a milky ripeness—full ripeness phase, which is very important for the environmental conditions of Siberia and Transurals.

Hybridization with winter forms and varieties is considered of great importance for creating the intensive and highly productive breeding material of spring wheat. Meanwhile, the winter forms should be taken as the female parent ones, as they are able to transmit with a cytoplasm the genetic material that allows to form a well-developed secondary root system in the hybrids being created. It is undesirable to use vernalized seeds of winter varieties for the hybridization, as this gives rise to thermal phytomutations, which reduce the genetic value of the initial forms. When crossing winter and spring plants, it is required to combine such climatic conditions and technologies that allow both of the plants to simultaneously pass the flowering phase. This is feasible in southern Kazakh-

stan (in the foothills of the Ala-Tau and in the Adler Microdistrict), as well as in the phytotron.

Intensive varieties of bread wheat have been formed for more than 150 years by selection based on the material from the secondary, induced and peripheral centers of origin, possessing a huge genetic potential for selection for adaptability. Such a selection is possible because of a discrete nature of inheritance manifestation, leading to changes in species diversity in terms of biomorphological and physiological and biochemical characteristics, and also has an evolutionary direction [6, 23]. The complete parentage of the varieties being produced allows to theoretically justify the selection of parental pairs for crossing [28, 35], and to conduct a directed formative process and selection of genotypes of a desired ecotype. In addition, according to the law of homologous series by N.I. Vavilov (1987) and data on the crop genesis, one can plan the engagement of certain samples for the creation of the initial material to produce transgressions with a manifestation of characters, exceeding the parental forms. Cold and drought tolerance as integrated physiological parameters are controlled in spring wheat by many initial genetic systems. For example, in the setting of fierce dry-farming in the southeast Kazakhstan, cold-resistant intensive Scandinavian varieties have the same productivity as a drought-resistant variety Saratovskaya 29. This should be considered when creating new varieties with engagement of the genetic resources of drought resistance [36-38].

A large number of forms of different ecological and geographical origin are involved in the creation of modern varieties. Crossbreedings are effective, when varieties and forms, similar in biology and morphology, but genetically different, are used as parents. Regrouping the genes of the original forms that control quantitative and qualitative traits can result in the selection of genotypes with a discretely improved manifestation in the first case, and with a systematically accumulated manifestation in the second case. Intensive modern breeding is accompanied with microevolutionary processes [8], caused by recombinogenesis [39], the impact from environmental factors [40, 41], infectious load [42, 43] and the artificial selection [44, 45], which results in the formation within the populations a limited number of high-yielding, locally adapted biotypes with a pronounced synergism. Some of them become the parents of intensive varieties. This directed process leads to the impoverishment of species due to absence of demand and loss of many individuals and varieties. Therefore, it is required to develop a program for preserving the produced hybrid material (the $F_2$ generation), derived in systematic breeding and genetic studies of varieties and forms with established genetic parameters, combining and variety-forming ability. In the future, this will allow for a more targeted and rapid production in the phytotron of the initial material and varieties with predetermined properties for any ecological niche. Creating the industrial-based varieties and their time-limited use will significantly reduce financial and human costs spent for the original seed growing.

Therefore, the parentage of the intensive varieties of spring bread wheat allows to justify the manifestation of biological and economically valuable traits and characteristics, and to choose parental forms for crossing, taking into account also the genetic potential of winter varieties. Crossbreedings are effective, when varieties and forms, similar in biology and morphology, but genetically different, are used as parents. Selection of genotypes with a discretely improved manifestation and a systematically accumulated manifestation of a trait are possible when regrouping the genes of the original forms that control quantitative and qualitative traits, respectively.

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## Plant breeding: tissue cultures, molecular markers

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# SUGAR BEET (*Beta vulgaris* L.) HAPLOID PARTHENOGENESIS in vitro: FACTORS AND DIAGNOSTIC CHARACTERS

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Abstract

Traditional obtaining of inbred lines and hybrids in sugar beet breeding requires a long time and is labour-consuming because of 2-year cycle of plant development, self- and cross-incompatibility, and inbreeding depression. To induce genotypic diversity in initial population, biotechnology methods including haploid parthenogenesis are promising. We have shown that, when inducing sugar beet (*Beta vulgaris* L.) haploids in vitro, express-diagnostics using phenotypic and embryological characters that are representative of periods of flowering shoots, organs and buds development, and stages of embryo sac, ovule and pollen grains formation is effective. The regenerative activity is observed in ovules of bud 1 to bud 25 located on ear part of pleochasium upward from the open flower. The nuclei and cells of female gametophyte of isolated ovules under in vitro conditions are capable of neoplasm at all stages of development, but the 8-nuclear or 7-celled embryo sacs are the most appropriate to morphogenesis and switching of development program from gametophyte to sporophyte type. Critical period of embryo sac development has been beforehand determined from the accompanying embryological characters — the presence of single-nuclear and two-three-celled pollen grains of anthers located in the same bud as ovules. The results we obtained indicate that hormonal composition of the Gamborg's B-5 (B5) medium is an important factor that effectively regulates direction of morphogenetic development in isolated ovules through direct regeneration (embryoidogenesis) or via callus (hemorhizogenesis) that is the evidence of totipotency of both sexual and somatic cells in the explant. The obtained data on in vitro reproduction of haploid regenerants add to available scientific notion of morphogenetic potential specificity in sugar beet plants. Stabilizing selection used to produce double haploids promotes detection of valuable morphological features of the regenerants. Determination of chromosome and chloroplast numbers in stomata guard cells as well as isozyme electrophoretic mobility (for *Adh-1*, *Mdh-1*, *Mdh-2*, *Me-1*, *Idh-1*, Idh-2, *Gdh-1* loci) can serve as markers when inducing haploidy and producing homozygous restitution lines of sugar beet. Efficiency of RFLP-analysis method using Hind III restrictase that has allowed for the first time to identify haploid microclones according cytoplasm type is shown. Molecular markers have indicated that regenerants with normal cytoplasm (N) have one PCR-product of 800 bp in length not digested by Hind III. Two fragments (320 bp and 480 bp) of 800 bp product digestion are found in cytoplasmic male sterile (CMS) forms (S) that reflects combination of recessive and dominant genes. Obtaining haploid regenerants with sterile cytoplasm from initial population is of great theoretical and practical importance for sugar beet breeding thus facilitating the problem of producing homozygous lines with CMS and high-productive hybrids on the sterile basis.

Keywords: sugar beet (*Beta vulgaris* L.) haploid parthenogenesis, female gametophyte, doubled haploid, embryoids, organogenesis, isozymes, RFLP-analysis

The modern domestic gene pool of sugar beet is sufficient enough to create varieties with the desired properties, however, traditional obtaining of inbred lines and hybrids requires a long time, and is labor-consuming, due to a 2-year cycle of plant development, self- and cross-incompatibility, and inbreeding depression [1]. A biotechnology method of haploidy, involving the in vitro cultivation of plants [2], may be effectively used to induce genotypic diversity in the

initial population, although its use is limited due to the narrow specificity of these conditions not only for different genotypes of the same species, but also depending on the cultivation stage. The variation observed when cultivating reproductive organs of plants may significantly extend the limits of variability and facilitate the derivation of forms for breeding purposes (including those obtained based on the doubled haploids) [3].

To improve the method of haploid parthenogenesis in sugar beet, the conditions for inducing unfertilized ovules and cultivating haploid regenerants with valuable genetic recombinations are optimized, and techniques of including restitution lines in the selection process are practiced [4-8). The most challenging problem is the hormonal composition of the growth media for the in vitro cultivation (of note, the principle of empirical choosing conditions for the induction of haploidy remains dominant) [9-10]. Several methods appear to be of interest, such as the use of liquid media, which significantly increases the output of haploid regenerants [11), as well as spontaneous haploid diploidization in long-term (over 1 year) cultivation [12], pretreatment of ovules with different agents (e.g. colchicine, trifluralin, etc.) that promote the antimitotic activity of the female gametophyte cells and increase the frequency of haploid regeneration [13]. The comparison between morphology of haploids and doubled haploid regenerants revealed the advantages of the first ones in the rate of shoot development and propagation in vitro. Based on this, it is proposed to use the sugar beet haploids both to produce doubled haploids and in specialized projects on genomic analysis, or in genetic transformation, when haploid tissues serve as the starting material [14].

In sugar beet, in vitro cultivation of unfertilized ovules and obtaining haploids from them allowed to directionally produce homozygous breeding lines [9, 15]. However, the commercial application of these technologies is limited by a low output of haploid regenerants and the lack of methodical research in regard to a) the critical periods in the development of the embryo sac and the establishment of the morphogenetic competence of its elements in order to switch from a gametophyte to sporophyte development program, and b) biochemical and molecular genetic evaluation of the produced homozygous lines.

For the first time, we analyzed the traits associated with the morphogenesis and in vitro reproduction of regenerated plants of sugar beet in case of haploid parthenogenesis; also, we demonstrated the possibility of their use as a diagnostic tool to assess the critical periods in the development of the female gametophyte during the transition to a sporophytic morphogenesis. Furthermore, the application of the RFLP analysis using the Hind III restriction enzyme allowed to identify for the first time haploid microclones from sugar beet variety populations based on the cytoplasm type (N or S), thus facilitating the problem of producing lines with the cytoplasmic male sterility (CMS).

Our objective was to identify traits that improve the efficiency of in vitro haploid parthenogenesis in sugar beet, involving biochemical and molecular markers.

*Technique*. The organ and tissue explants of *Beta vulgaris* L. hybrids (the collection of A.L. Mazlumov All-Russian Research Institute of Sugar Beet and Sugar) were used for plant tissue cultures. A conventional technique for sterilization and preparation of growth media was as described by R.G. Butenko [2].

The activity of the NADP isocitrate dehydrogenase enzyme was determined in plant tissue homogenates [16]. Electrophoresis of isoenzymes in the starch gel and PAGE was performed as described [17].

A Hind III RFLP (restriction fragment length polymorphism) [18] was used to identify sterile and fertile haploid regenerants. The products were separated by 1 % agarose gel electrophoresis with ethidium bromide.

Ploidy levels were determined by flow cytometry on a Ploidy Analyser

PA-2 (Partec GmbH, Germany) [11].

The cytoembryological data was processed using the coefficient of variation *Cv* [19].

*Results.* The in vitro cultivation of sugar beet unfertilized ovules revealed a unique ability in embryogenic cells to implement the developmental potential influenced by exogenous factors [20], which enabled to develop a technology for producing homozygous lines. This technique allows to obtain genetically and morphologically diverse material from donor plants without repeated self-pollination. The proposed technique provides an output of up to 38 haploid regenerants from a single donor plant, with overall production of homozygous sugar beet plants over 2-3 years (instead of 8-10 years with traditional methods). Currently, more than 100 of such homozygous lines are used in breeding programs [20]. Different experiments revealed a number of morphological, cytoembryological, biochemical and molecular genetic features that enhanced the efficiency of haploid detection when inducing in vitro haploid parthenogenesis, thus accelerating the creation of homozygous lines.

When introducing ovules into the culture, a period of flowering shoot development in donor plants is among critical factors affecting the efficiency of haploid regeneration. In sugar beet, budding at the end of an early generative stage of inflorescences (VII and VIII steps of the organogenesis) is an optimal phase [21]. Buds with ovules, more capable of inducing haploidy and most suitable for tissue culture, are located in the central stem of the pleiochasium raceme. Regeneration activity is observed in ovules from buds 1 to 25 (located on the spicate raceme upward from the opened flower).

For a successful ovule culture, it is also required to select the optimum stage of the embryo sac development. Cytoembryological observations indicate that nuclei and cells of the female gametophyte in sugar beet are capable of in vitro neoplastic processes at all stages of development. This appears to be related to the peculiarities of the female gametophyte, primarily to its well-defined polarity, differentiating divisions and totipotency of its elements, that together promotes, under in vitro conditions, the initiation of the program of haploid regenerant formation. Our findings demonstrated that the polarization started in the mononuclear embryo sac which, with its development, increased in size and stretched out toward the micropyle. As a result of the next three rounds of mitotic divisions, an eight-nucleus embryo sac is formed. After the last round of mitosis, one polar nucleus leaves each pole and migrates toward the center. Around the nuclei, cytoplasm is localized and cytokinesis occurs, which ends with the formation of the seven cells of the embryo sac [22].

Culture of isolated ovules, containing eight-nucleus (or 7-cell, but eight-nucleus) embryo sacs, is considered the most favorable for the induction of haploidy and regeneration [8]. One can assume that the stringent polarity of the embryo sac in this period, as well as totipotency of its nuclei and non-specialized cells of female gametophyte, ensure the greatest capacity for morphogenesis. This is a crucial factor for switching to sporophytic type of development.

Determining stages of the embryo sac development is complicated and long. In this case, embryological traits, such as the presence of mononuclear microspores and 2- or 3-cell pollen grains of anthers located in one bud with ovules are used to indicate a critical period. Depending on the weather conditions at flowering, these traits can be observed 1-5 days before the opening of buds.

Anomalies of the male and female gametophytes, which were apparent in some genotypes, resulted from self-pollination and polyploidy, and in the CMS hybrids with the apomixis frequency up to 40 %, may also serve as the diagnostic signs. Recessive genes, involved in the apomixis control [23], including in the

haploid parthenogenesis, may possibly accumulate in different ways during the selection. Genotypes with the greatest anomalies are more likely to induce haploidy in vitro.

A significant disadvantage of the in vitro haploidy technology is a low rate of regeneration (1.7-10.5 %). It may be increased by stress factors, e.g. pretreatment of ovules with cold (4-6 °C, 2-4 days) or X-rays (1000-5000 P, 50 min) [24]. In sugar beet ovules (similar to wheat microspores) [25], under in vitro conditions, low positive temperatures, altering metabolism, may delay the development of the embryo sac and induce sporophytic morphogenesis.

Hormonal composition of the growth media when culturing ovules was also a key factor in determining the type of morphogenesis, i.e. through regeneration (embryoidogenesis) or via a callus (gemmorhizogenesis) [26].

In our experiments, induction and morphogenesis of the haploid regenerants toward sporophyte were limited by Gamborg (B5) medium supplemented with vitamins by White and various hormones [26]. For example, the addition of gibberellin (2 mg/l) induced the embryoidogenesis of the haploid regenerants. The cell division in the egg apparatus was observed in the ovule embryo sac from the first days, with subsequent development of a multicellular (5-8 tiers of cells) proembryo on day 3. By day 5, the embryo took the form of a ball, and increased in size due to divisions transversely and longitudinally, filling all the space of the embryo sac by days 8-12 (while the haustorial outgrowth in the nucellus did not increase). The central cell which did not started dividing, remained until the days 5-8, and then degenerated. Sometimes several endosperm nuclei were formed, which also degenerated. The starch in the cells of the nucellus was not accumulated, perisperm and seed coat were not formed. On day 28, the embryo ruptured the integuments, and a seedling appeared on the surface of the ovule, with subsequent formation of small cotyledonary leaves, hypocotyl and the primordial root. This indicates the similarity between haploid embryoidogenesis and embryogenesis of the zygotic embryo, although an embryoid is formed using only mineral salts from growth medium (without nutrients from the endosperm which does not form under in vitro conditions).

Auxins stimulated the growth of callus along with gynogenetic embryos. Gibberellin (2 mg/l), 6-benzylaminopurine (0.1 mg/l) and indolyl-3-butyric acid (0.1 mg/l) induced haploid embryoids and then secondary regenerates from hypocotyl callus tissues that increased 6- to 10-fold the output of haploids [20]. As totipotency of the heterogeneous callus cells varies, two major types of callus may probably form, i.e. morphogenic and non-morphogenic. A non-morphogenic callus consists of parenchymal cells and can not regenerate. Root formation (rhizogenesis) or gemmogenesis (the appearance of the buds), or gemmorhizogenesis occurred in morphogenic callus which had more dense fine grain structure. Gemmorhizogenesis began in the subepidermal layer. In vitro, initial cells appeared having a thickened cell membrane and a larger nucleus, from which meristem zones developed de novo as pimples. Following the periclinal divisions on the outside of the pimples, an apex (sickle-shaped fold) and the first leaf primordium (the growth bud) were formed. Root apexes were set in the lateral or basal part of the callus. A conducting bundle was formed between the root and the bud. Plant reproduction via callus occurred under gemmorhizogenesis or gemmogenesis, followed by root induction in 3-4 weeks.

Culture of unfertilized ovules in liquid medium resulted in the increased proliferation of the female gametophyte cells and maintained their viability for 4-6 months. The transfer of these explants onto the agar medium of the same composition stimulated regeneration (13.7 and 18.9 % for calluso- and embryoidogenesis, respectively). Note that the callus is a system of indirect organogen-

esis which extends the time of regenerant formation, therefore, the direct in vitro regeneration is a more promising for breeding.

The stage of stabilization involves the selection of normally developed haploid regenerated plants [27] with a high ability to form adventitious shoots and micropropagation. To improve the efficiency, we used morphological and cytological characteristics at this stage. Accordingly, the height and size of organs in haploid regenerants are usually lower than in diploids. Depending on the genotype, normally developed haploid forms have more narrow leaf blades with long petioles or, on the contrary, broad leaves with wavy edge and short petioles. A typical cytological trait of haploids is 9 chromosomes, which are revealed through microscopic examination or cytophotometry based on the content of nuclear DNA [28]. The latter method is faster, more reliable and can be widely used for identifying plants with altered ploidy. The presence of 8-10 chloroplasts in the stomata guard cells, among which there are abnormal or non-forming chloroplasts, or those having one bean-like cell, should be considered cytoembryological features. As cytoembryological signs in haploids varied only slightly, they can be used as morphological markers for identification and selection of haploid regenerants. This allows to make an assessment at the earliest stages of the regenerated plant development, when the analysis of the chromosome set is an extremely difficult and leads to the death of the explants.

Colchicination of haploids [27] resulted in polyploidization with the formation of diploids, triploids and tetraploids. With increasing ploidy level, the number of chloroplasts in a pair of the stomata guard cells also increased, e.g. from 9-11 in haploids to 12-14, 15-17 and 18-21, respectively, in di-, tri- and tetraploids. The ratio of the area of guard cells in haploids vs. di-, tri- and tetraploid forms was also modified (1:1.56; 1:1.87; 1:2.68, respectively) (Table 1).

**1. Cytomorphological characteristics of pairs of stomata guard cells in sugar beet (*Beta vulgaris* L.) plants at different ploidy levels**

| Ploidy | Pairs of cells, examined | Number of chloroplasts, pcs. | | *Cv*, % | Area, μm$^2$ | *Cv*, % |
|---|---|---|---|---|---|---|
| | | mean | min-max | | | |
| Haploid (×) | 250 | 10.2 | 9.4-10.9 | 9.3 | 0.95 | 0.9 |
| Dihaploid (2×) | 250 | 13.2 | 11.8-14.5 | 9.7 | 1.48 | 1.6 |
| Triploid (3×) | 250 | 15.5 | 14.4-16.6 | 5.8 | 1.78 | 1.8 |
| Tetraploid (4×) | 250 | 19.7 | 18.5-20.9 | 6.4 | 2.55 | 1.3 |

It should be noted that some doubled haploids during the reproduction (both in vitro and in soil) recovered their haploid state, which may probably arise from a shorter mitotic cycle in haploid regenerants. Therefore, colchicination and intracellular selection may contribute to an increased proportion of haploid cells in the mixoploid meristem of the regenerants which adversely affects the diploidization. To increase the effect of diploidization in haploids in vitro, we modified the hormonal composition of the growth medium by adding cytokinins and gibberellin, which inhibit the growth of haploid tissues. Kinetin, when added at 0.25 mg/l, demonstrated its high ability to stimulate cell division in diploids or to slow it down in haploids, and that allowed to produce up to 90 % of regenerated plants with a constant diploid set of chromosomes.

A comparison of isozyme spectra is a convenient tool for identifying haploid regenerates of sugar beet. When studying the electrophoretic mobility of the isoforms of NADP-isocitrate dehydrogenase (NADP-IDH, Rf 1.1.1.42), we revealed differences between haploid regenerants and the original diploid plant. Previously, we demonstrated the presence of two isoforms of NADP-IDH, such as cytoplasmic (~ 95 % activity) and mitochondrial (~ 5 % activity), in leaves of control plants, which was consistent with the results of electrophoresis: the control plants had two isoforms of NADP-IDH ($R_f$ 0.31 and 0.39), with bands of equal

intensity, while in haploids the enzyme was represented by two areas of activity, i.e. monomorphic ($R_f$ 0.37) and polymorphic ($R_f$ 0.26 and 0.31).

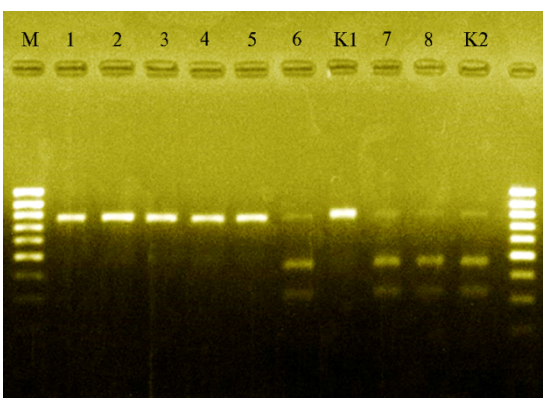
The isozyme analysis can also be used to determine the degree of homozygosity of the restitution lines obtained as a result of the stabilizing selection. Theoretically, all the lines of doubled haploids must be homozygous. However, on average 4 % heterozygous loci are revealed in the restitution lines produced via treatment with colchicine [29]. It cannot be ruled out that such polymorphism can be explained by epigenetic variability in the doubled haploids of sugar beet [30, 31]. The index of isozyme homozygosity (Iiz) in the tested lines, defined as the average percentage of homozygosity by seven isozyme loci, ranged from 0.81 to 1 [29] and averaged to 0.96 (Table 2). Homozygosity of these restitution lines is probably quite high, despite the possible epigenetic changes.

**2. Indicators of isozyme homozygosity in restitution lines of sugar beet (*Beta vulgaris* L.)**

| Line | The proportion of homozygotes based on isozyme loci, % | | | | | | | Index of isozyme homozygosity (Iiz) |
|---|---|---|---|---|---|---|---|---|
| | *Adh-1* | *Mdh-1* | *Mdh-2* | *Me-1* | *Idh-1* | *Idh-2* | *Gdh-1* | |
| 1 | 100 | 87 | 93 | 88 | 100 | 100 | 100 | 0.95 |
| 2 | 100 | 97 | 100 | 68 | 100 | 100 | 96 | 0.94 |
| 3 | 100 | 40 | 93 | 42 | 100 | 100 | 93 | 0.81 |
| 4 | 100 | 87 | 93 | 100 | 100 | 100 | 100 | 0.97 |
| 5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 1.00 |
| 6 | 100 | 100 | 100 | 96 | 100 | 96 | 100 | 0.99 |
| 7 | 100 | 100 | 100 | 96 | 93 | 96 | 100 | 0.98 |
| 8 | 97 | 79 | 84 | 96 | 100 | 100 | 100 | 0.94 |
| Mean | 99 | 86 | 95 | 98 | 99 | 99 | 99 | 0.96 |

Molecular markers are neutral with respect to the phenotype, they are not tissue-specific, and can be detected at any stage of plant development. They allow to control the genetic transfer from the donor plants and screen for the desired trait, such as CMS [32, 33]. It is known that there are plants with normal (N) and sterile (S) cytoplasm in the sugar beet populations. Pollen is viable and fertile in N-plants, while in S-plants it can be either fertile or sterile, depending on the interaction between sterile (S) cytoplasm and recessive nuclear genes $rf_1$ and $rf_2$. The sterility of cytoplasm in sugar beet is caused by a change in the nucleotide sequence in the mitochondrial and chloroplast genomes [34, 35].

Our studies showed that the PCR and Hind III RFLP analysis enabled to identify the type of cytoplasm in the produced haploids by the restriction fragment pattern. In haploid microclones with normal and sterile cytoplasm, a single fragment (800 bps) was amplified, which was digested only in sterile (S) forms (see Fig.; two restriction fragments and the residues of the 800 bps fragment can be observed).




**An electrophoregram of restriction digests of the amplified DNA fragments** (Hind III RFLP analysis**) in haploid regenerated plants of sugar beet (*Beta* vulgaris L.):** K1 — control fertile plants; K2 — control sterile plants; 1-5 — forms with normal (N) cytoplasm, 6-8 — forms with sterile (S) cytoplasm; M — molecular weight markers (MassRuler™ DNA ladder, 80-1031 bps, SM0383, Thermo Scientific, USA).

Haploids, in which this fragment was not digested with Hind III, were represented by completely fertile forms with normal cytoplasm (N) and nuclear genes in the recessive state (*rf*). In the remaining samples, polymorphism of

fragments was observed which appeared to imply that corresponding haploid forms had the sterile cytoplasm (S) and various combinations of recessive and dominant alleles of the $Rf_1/rf_1$ and $Rf_2/rf_2$ nuclear genes. Note that the PCR profiles of all sterile regenerants (both haploids and doubled haploids) are identical. Therefore, the identification of regenerated plants with sterile cytoplasm at the different stages of cultivation is of great interest for the sugar beet breeding, thus facilitating the production of lines with the CMS and highly productive sterile-based hybrids.

Thus, we demonstrated the efficiency of a combination of diagnostic features for improving the technique of in vitro haploid parthenogenesis in sugar beet. The identified phenotypic, morphological, cytoembryological markers reliably characterize the critical periods for the development of generative organs in donor plants and the induction of haploidy, as well as the stage of in vitro morphogenesis, which are considered the most favorable during the stabilizing selections of haploids and doubled haploids. Determination of the isozyme electrophoretic mobility and molecular marking make it possible to evaluate the degree of homozygosity in the produced lines. RFLP analysis provides an opportunity to select the haploid regenerants possessing a desired trait, such as the cytoplasmic male sterility (CMS), and produce lines homozygous for the CMS.

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# IDENTIFICATION OF CHARACTERISTIC ORGANIC MOLECULES IN KERNELS OF MAIZE (*Zea mays* L.) HYBRID GRAIN USING INFRARED SPECTROSCOPY

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Abstract

Modern biophysical methods have provided a breakthrough in investigations of the status and functions of the intact plants at the molecular level. The infrared (IR) spectroscopy allows us to analyze the molecular composition and structure by recording the absorption of infrared radiation as a function of frequency of valent and deformation vibrations (wavenumber, $cm^{-1}$) for chemical bonds. We used the IR Fourier spectroscopy method (IR spectra with Fourier Transformation,) to investigated the grain composition in maize hybrids — ZP 341, ZP 434 and ZP 505 created at Maize Research Institute (Zemun Polje, Belgrade, Serbia). The resulted spectra differed in peak number and intensity, and in oscillation frequency. Particularly, there are 20 to 23 peaks and characteristic spectral bands within the wavenumber range of 400 to 4000 $cm^{-1}$. Characteristic spectral bands were analyzed for each hybrid with regard to absorption intensity in %, experimentally determined wavenumber in $cm^{-1}$, and published wavenumber range. A comparison of these peaks to reference IR spectra from databases revealed biogenic organic molecules: alcohols, amines, esters, alkanes, carboxylic acids, alkenes, aldehydes, ketones and esters in the studied grain hybrids. In a typical IR spectrum of maize hybrid ZP 341 there were three most distinct bands with wavenumbers of 3400, 2900 and 1000 $cm^{-1}$. Four peaks (3400, 2950, 1700 and 1000 $cm^{-1}$) were characteristic of the ZP 434 hybrid, and eight major peaks of 3400, 2900, 2850, 1750, 1700, 1450, 1150 and 1000 $cm^{-1}$ were observed in ZP 505. That is, the grain charterisics in ZP 505 slightly differ from those in ZP 341 and ZP 434, whereas in ZP 341 and ZP 434 the grain structure is more similar. In general, Serbian hybrids are characterized by high quality, productivity and technological suitability. The developed methodology for IR spectra recording and analysis in grain allows to reveal the composition and structure of biogenic compounds. It is important not only for diagnosis and breeding, but also for the development of biotechnological screening methods, or the estimation of grain storage time.

Keywords: maize hybrids, grain, molecular structural characteristics, infrared spectra, spectral bands

Modern biophysical study methods have provided a significant breakthrough in the diagnostics of physical and functional condition of intact plants on the molecular level [1-9]. IR and Raman spectroscopy is a practical and effective tool in studying organic compounds, which allows to identify vibrational characteristics of molecules. Analysis of IR spectra allows to obtain important data on structural features of systems undergoing study, and the properties conditioned by said features [2-5, 10].

Earlier [9, 11-16] we have compared the structure of carotenoides in the grain of hybrid and inbred lines of corn (*Zea mays* L.) with high photosynthesis efficiency, enriched pigment composition and increased nutritious value. We have shown that the structure may be used as a molecular marker for agronomic efficiency assessment in studied forms. The IR spectroscopy method is known to allow to analyze composition and structure of molecules by register-

ing infrared absorption depending on frequency (Wavenumber, $cm^{-1}$) of stretching and bending vibrations of chemical bonds [3, 4].

The current study is the first to present the results of using the IR spectroscopy method for the molecular diagnostics of quality and functional characteristics in Serbian selection corn hybrids.

The goal of the study is to develop a methodology of recording and analyzing of the IR spectra of grain, which would allow to reveal the structure of biogenic compounds.

*Techniques.* Grain from three high yield corn hybrids ZP 341, ZP 434 and ZP 505 (originator and owner is Maize Research Institute, Zemun Polje, Belgrad, Serbia) was used for biological samples.

An IR Prestige-21 (Shimadzu Corp, Japan) spectrometer was used to record IR spectra in the 40-4000 $cm^{-1}$ range with a Fourier transform (Fourier Transform Spectrometry). The samples were prepared by homogenizing the grain and embedded into tablets containing potassium bromide (KBr). All IR spectra reflecting the dependency of infrared absorbance (abs. units) on the wavenumber were obtained in no less than 3 consequent tests with 3-5 repetitions each.

Standard methods described in more detail in classical works and by ourselves [10, 17] were employed to determine grain chemical composition.

The yielding, quality and technological suitability of hybrids for growing for grain and ensilage (selection and seed growing features, characteristics and parameters) were assessed in ecogeographic studies during 2008-2011 in the majority of areas of Serbia — Loznica (western Serbia), Sakule (South Barat), Smederevo (Podunavye), Zmaevo (South Bachka), Jarkovac (East Srem), Batosh (Middle Banat), Divosh (North Srem), Bechei (East Bachka) etc. using the standard agrotechnologies [11-13, 18].

Statistical analysis was performed using software.

*Results.* ZP 341, ZP 434 and ZP 505 hybrids, cultivated in Serbia, are used both in Serbia and in Russia. More than 1 mln ha is sown in Serbia annually. The studied ZP corn hybrids are also successfully cultivated in Romania, Bosnia and Herzegovina, Croatia, Montenegro, Macedonia, Bulgaria and parts of Hungary.

The results for ZP 341, ZP 434 and ZP 505 in this study have been obtained using both common standard and modern instrumental methods.

Infrared spectra of the grain of studied hybrids. The Fourier Transform Spectroscopy that we used (Fig. 1) [4-16, 19] is known as a method of identification and feature analysis of chemical substances by their molecular IR absorbance spectra. Such a spectrum is unique for every individual compound and serves as its «molecular fingerprint», by which it can be found in respective databases. These spectra can also be employed as means of calculating the content of an element in the sample, due to absorption intensity being directly proportional to the quantity of a substance.

The spectrophotometers used for the IR spectrum bear no principal differences from such for visible and UV spectra. They do, however, have certain minor differences. These are based on IR specifics, particularly in the medium- and long-wave parts of the spectrum [1, 3, 20] and related to the nature of IR, its energy, the features of IR emitters, the usage of temperature sensors etc.

Currently, a specific type of spectrophotometers is used, based on the interferometer principle. These devices record not the spectrum itself, but an interferogram, which is later converted into a standard spectrum via computer processing. The process is called the Fourier transform, and the whole method is the Fourier Transform Spectroscopy — FTS. These instruments are the most suitable for analyzing the far ranges of the IR spectrum and possess high resolution.

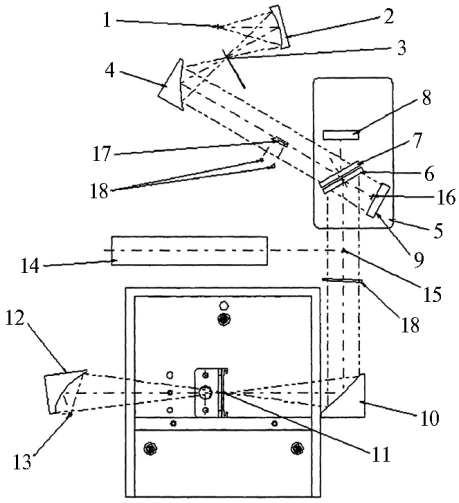


**Fig. 1. Schematic diagram of the IR Prestige-21 IR-Fourier spectrometer optical system** (Shimadzu Corp, Japan): 1-13 — elements of the diagram. The beam from the emitter (Position 1) is reflected by the spherical mirror (2) focused on the diaphragm (3). Going through the diaphragm, is reflected by the collimator (4), transformed into parallel, and gets into the Michelson interferometer with 30° angle of incidence (5). The IR beam reflected to the interferometer is sent by the beam splitter to the rotating mirror (8) and static mirror (9). Each beam obtained that way becomes interfered by the splitter and sent to the collimator mirror (10). The parallel interfered beam projects the image of the emitter in the center of the sample compartment. The beam passes through the sample, is reflected by the collimator mirror (12) to the sensor (13), where it's recorded in the form of an interferogram.

The analysis of the obtained IR spectra (Fig. 2) has revealed approximately 20-23 spectral bands in the wavenumber range of 400 $cm^{-1}$ to 4000 $cm^{-1}$. The spectral bands differed in peak height, kinetics and base width. There were three to five most pronounced bands, two to three moderately pronounced and four to five bleaker bands. Several merged bands were impossible to separate, which shows an unstable condition. This condition may manifest if the vibration ranges of chemical bonds are so little for some reason, that they are practically indistinguishable. There are other explanations for this phenomenon and the system's unstable condition [15, 21].

The IR spectra represent the dependence of infrared absorbance on the frequency of stretching and bending vibrations of chemical bonds for multiple functional groups within biogenic organic molecules of vitamins, pigments and dietary fibers. Analysis and comparison of revealed spectral profiles first with the samples in the standard library (obtained in reference IR studies of especially clear organic substances) and then with the results of studies of organic, natural and polyatomic substances, described in literature [3-6] allowed to characterize the chemical composition and molecular structure of the grain of the three study corn hybrids with a certain level of probability. It is of note, that besides the mentioned references, the IR-spectra (about 150 thousands) are described in The American Society for Testing and Materials (ASTM) library (http://www.hellers.com/steve/resume/p101.html).

The typical ZP 341 corn hybrid IR spectrum (see Fig. 2, A) was characterized by three pronounced spectral bands with wavenumbers of 3400, 2900 and 1000 $cm^{-1}$. The bands with wavenumbers of 2850, 1650, 1175, and 1145 $cm^{-1}$ may also be considered pronounced here. At a closer look bleak bands with wavenumbers of 3780, 2300, 1550, 1145, 1100, 925, 825, 775, 700 and 600 $cm^{-1}$ could also be seen. In the whole 4000-400 $cm^{-1}$ wavenumber range an unstable condition was recorded for wavenumbers 3000, 1900 and 650 $cm^{-1}$.

For the ZP 434 hybrid four spectral bands stood out in the IR spectrum of the grain with wavenumbers of 3400, 2950, 1700 and 1000 $cm^{-1}$ (see Fig. 2 B). Besides that, the bands with wavenumbers of 2825, 1775 and 1185 $cm^{-1}$ were clearly pronounced. The bands with wavenumbers of 3750, 1500, 1225, 1100, 975, 900, 800, 700 and 600 $cm^{-1}$ appeared to be bleak. Wavenumbers of 3900, 2300, 1900, 1400 и 450 $cm^{-1}$ [2-4] conformed to the system's unstable condition for this hybrid.

The ZP 505 hybrid, unlike the others, has eight most pronounced spec-

tral bands with wavenumbers of 3400, 2900, 2850, 1750, 1700, 1450, 1150 and 1000 $cm^{-1}$ (see Fig. 2, B). And 12 more peaks (wavenumbers of 3750, 3025, 2350, 1550, 1300, 1100, 1000, 900, 775, 700, 575 and 500 $cm^{-1}$) were bleak. The system's unstable condition was recorded for the wavenumbers of 3850, 1900, 1800, 1460 and 1430 $cm^{-1}$.

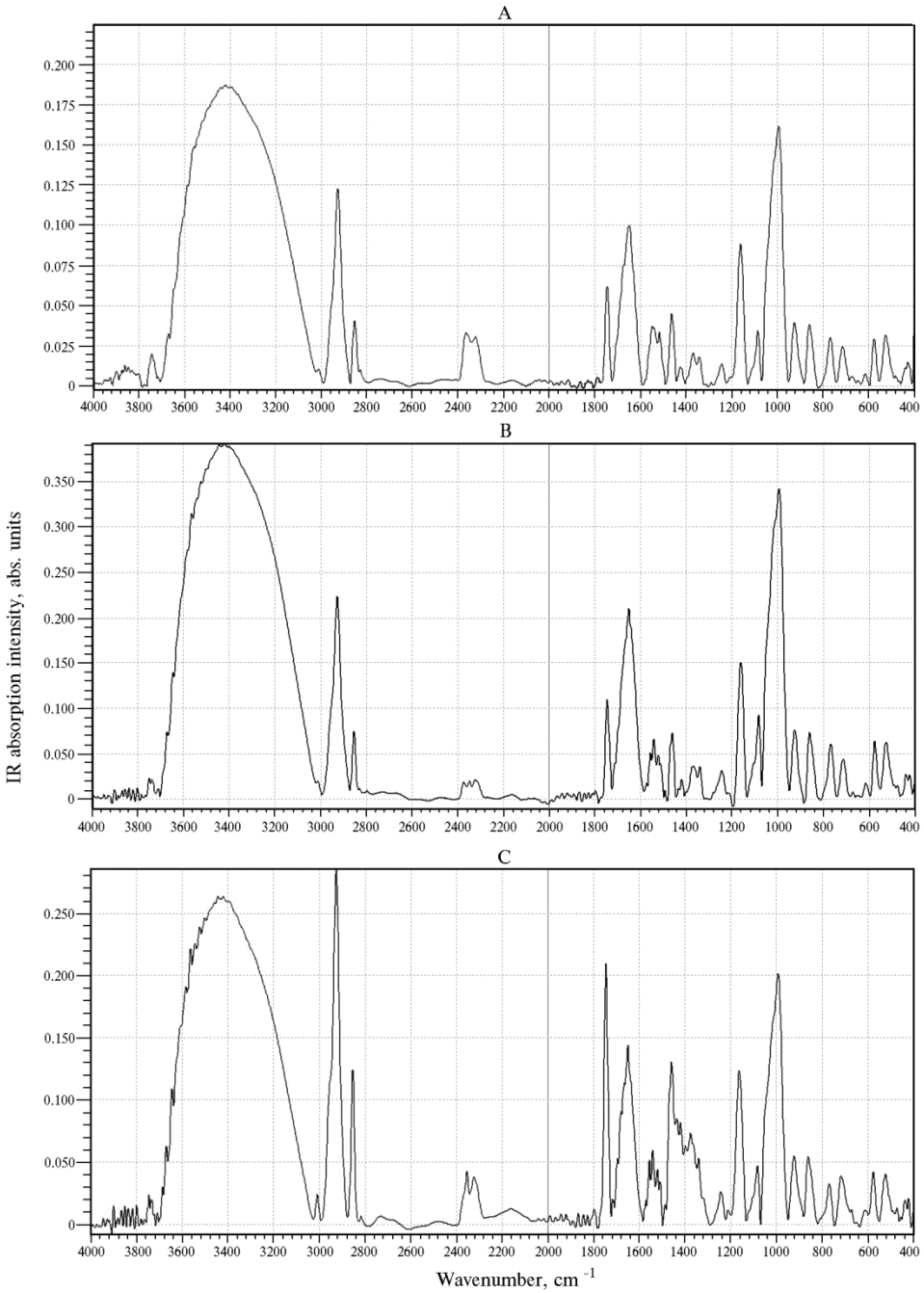


**Fig. 2. The typical** IR **spectrum of Serbian selection corn hybrids grain (*Zea mays* L.) ZP 34** (A)**, ZP 434** (B) **and ZP 505** (C) (IR Prestige-21IR-Fourier spectroscope Shimadzu Corp, Japan)**.**

It should be noted that along with the most typical and pronounced spectral bands, obtained for corn hybrids grain, minor peaks, representing excited states in organic molecules, may also be of interest [6, 19].

We tried to find the answer to the pending question, if the individual class of biogenic organic molecules can be revealed by knowing the characteristics of spectral bands of respective functional groups, and whether or not there is a difference between the IR spectra of grain of ZP 341, ZP 434 and ZP 505 corn hybrids. Apparently, the data obtained in our study allow to reveal the changes in the molecular structure of the components of the grain of the hybrids.

The analysis of each IR spectrum (see Fig. 2) by five most pronounced bands allowed us to test for the presence of stretching symmetrical and asymmetrical vibrations of a C−H group, and bending vibrations of C−H groups. Beside that, an attempt was made to detect bending (N−H, C−N) and stretching (C=O) vibrations for amide bands (amide I and amide II) in molecules of proteins, peptides and α-amino acids, stretching vibrations of C−H bond of alkenes, stretching vibrations of S−O, C−P, C−S bonds, bending vibrations of the C−H bond in a molecule of terminal carbohydrates, stretching vibrations of C=O bonds in aldehydes and ketones, O−H bonds of secondary and tertiary alcohols (Table 1).

**1. Characteristics of the main peaks of IR absorbance spectrum of the components of the grain of Serbian selection corn hybrids (*Zea mays* L.)**

| Band peak relative to the maximum of the hybrid, % | Wavenumber of the band peak, $cm^{-1}$ | Substance class |
|---|---|---|
| | ZP 341 hybrid | |
| 87.5 | 3400 (3200-3650, 3250-3500) | Alchohols, amines |
| 70.5 | 1000 (1000-1260) | Alcohols, ethers |
| 56.0 | 2950 (2840-3000, 2500-3300) | Alkanes, carboxylic acids |
| 44.0 | 1650 (1620-1680, 1690-1750) | Alkenes, aldehydes, ketones |
| 38.0 | 1150 (1000-1260) | Alcohols, ethers |
| | ZP 434 hybrid | |
| 100 | 3410 (3200-3650, 3250-3500) | Alchohols, amines |
| 88.5 | 1000 (1000-1260) | Alcohols, ethers |
| 60.5 | 2925 (2840-3000, 2500-3300) | Alkanes, carboxylic acids |
| 50.0 | 1625 (1620-1680, 1690-1750) | Alkenes, aldehydes, ketones |
| 39.0 | 1175 (1000-1260) | Alcohols, ethers |
| | ZP 505 hybrid | |
| 93.0 | 3410 (3200-3650 3250-3500) | Alchohols, amines |
| 100 | 2975 (2840-3000, 2500-3300) | Alkanes, carboxylic acids |
| 73.0 | 1775 (1735-1750, 1690-1750, 1710-1760) | Ethers, aldehydes, ketones, carboxylic acids |
| 67.0 | 1000 (1000-1260) | Alcohols, ethers |
| 49.5 | 1650 (1690-1750, 1620-1680) | Aldehydes, ketones, alkenes |

Note. Substance classes and their respective reference spectral characteristics (in brackets) are presented according to the description (6).

During the analysis of the IR spectra (see Fig.2, Table 1) the absorption intensity for the most pronounced spectral bands, wavenumbers, at which such peaks manifest, the data on wavenumber ranges in literature [6], absorption intensity and frequencies of stretching and bending vibrations of functional groups of biogenic molecules were taken into account. This allowed us to characterize the composition of biogenic molecules and their structural features in the grain of corn hybrids ZP 341, ZP 434 and ZP 505 [21, 23]. We identified alkanes, alkenes, amines, alchohols, ethers, carboxylic acids, aldehydes and ketones [21, 23] in the grain of ZP 341, ZP 434 and ZP 505 by comparing our data to the data on various substance classes in the literature [6, 20, 24]. According to the results obtained (see Table 1) we can suggest that the charcteristics of ZP 341 and ZP 434 grain are consimilar, while such of ZP 505 are different from the other two hybrids.

The quality, productivity and technological suitability of the hybrids. Table 2 represents the chemical composition of the grain of ZP 341, ZP 434 и ZP 505 by the primary characteristics. The data presented corresponds to the results of studies of vitamin and dietary properties of the grain,

obtained by foreign authors [6, 8, 9, 16] and was discussed by us in detail earlier [21].

**2. The results of the analysis of the chemical composition of the grain of study Serbian selection corn hybrids (*Zea mays* L.)** (the mean of 3 years, experimental corn field of Zemun Polje Corn Institute, Belgrad, Serbia)

| Indicator | The data in literature (16) | | The mean of the experiment | | |
|---|---|---|---|---|---|
| | limits | mean | ZP 341 | ZP 434 | ZP 505 |
| Humidity, % | 7-23 | 16.0 | 11.96 | 11.56 | 11.14 |
| Starch, % | 61-78 | 71.7 | 70.40 | 72.04 | 73.38 |
| Proteins, % | 6-12 | 9.5 | 9.75 | 10.15 | 9.88 |
| Lipids (oil), % | 1.0-5.7 | 4.3 | 6.28 | 6.02 | 6.38 |
| Ash, % | 1.1-3.9 | 1.4 | 1.34 | 1.40 | 1.31 |

ZP 341, ZP 434 and ZP 505 hybrids are characterized by significantly higher quality, and are generally meant to be grown in the European corn zone, where they have shown high productivity [15, 21] (Table 3). The genetic productivity potential of ZP 341, ZP 434 and ZP 505 was studied in Serbia at 38 geographical points in 2008, at 35 points in 2009m, at 41 in 2010 and at 37 point in 2011. A standard growing technology with no irrigation was used.

**3. The productivity of study Serbian selection corn hybrids (*Zea mays*L.) at a standard growing technology with no irrigation in ecogeographic studies** (Serbia, 2008-2011)

| Hybrid | 2008 | 2009 | 2010 | 2011 | Mean productivity of the hybrid | |
|---|---|---|---|---|---|---|
| | | | | | ton/ha | % |
| ZP 341 | 7.299 | 9.318 | 8.389 | 7.626 | 8.158 | 100.0 |
| ZP 434 | 7.432 | 9.522 | 8.393 | 7.788 | 8.284 | 101.6 |
| ZP 505 | 7.580 | 9.706 | 8.752 | 7.918 | 8.489 | 104.1 |
| Mean by year: | | | | | | |
| ton/ha | 7.437 | 9.515 | 8.511 | 7.777 | 8.310 | |
| % | 100.0 | 127.9 | 114.4 | 104.6 | 111.7 | |

N o t e. The study fields were situated in Loznica (western Serbia), Sakule (South Banat), Smederevo (Podunavye), Zmaevo (South Bachka), Jarkovce (East Srem), Batoshe (Middle Banat), Divoshe (North Srem), Bechee (East Bachka) etc.

It has been established that the hybrids differed only slightly in productivity. That being said, if ZP 341 is 100 %, ZP 434 has 1.6 % higher productivity (0.126 ton/ha) and ZP 505 is 4.1 % higher (0.331 ton/ha), which suggests a sufficiently close selective productivity potentials of study forms. The higher selective potential of ZP 505 can probably be explained by its longer vegetation period. The dynamics of changing of study parameters, however, were significantly different. Taking the 2008 productivity minimum as 100 %, we have a 4.6 % (0.340 ton/ha) excess in 2011, 14.4 % (1.074 ton/ha) in 2010, and 27.9 % (2.078 ton/ha) in 2009. That data demonstrates the degree of influence of climate conditions on corn productivity in different years.

The main selection, seeding and technology characteristics of the hybrids are generalized in Table 4. Preliminary results of studies (see Tables 2-4) clearly prove high quality, productivity and effectiveness of biotechnological application of Serbian selection hybrids.

Therefore, that was the first recording of IR spectra of ZP 341, ZP 434 and ZP 505 hybrids grain. The spectra obtained bear characteristic bands and peaks (20 to 23) in the wavenumber range of 400 to 4000 $cm^{-1}$. It is established, that the recorded spectral bands may be pronounced at different degrees (strong, moderate, bleak) and have different absorption intensity (%), different kinetics and base width. Five spectral bands were tested for each of the study hybrids, which have specific characteristics (absorption intensity, %, experimentally established wavenumber, $cm^{-1}$, wavenumber range by the data in literature). Analysis of the peaks and comparing them to the known databases

(reference IR spectra) allowed to reveal biogenic organic molecules in study hybrides grain: alcohold, amines, ethers, alkanes, carboxylic acids, alkenes, aldehydes, ketones and athers. The structural characteristics of ZP 505 grain differ slightly of such for ZP341 and ZP 434, being consimilar for the two latter. In general, the study samples of Serbian selection hybrids are characterized by high quality, productivity and technological suitability for cultivation.

**4. Agronomy and morphology characteristics of Serbian selection corn hybrids (*Zea mays*L.) during field study**

| Parameter, growing region | ZP 341 | ZP 434 | ZP 505 |
|---|---|---|---|
| Agronomy characteristics | | | |
| Hybrid type | TC | SC | SC |
| Ripeness group (by FAO) | 300 | 400 | 500 |
| Stem height, cm | 210 | 220 | 230 |
| Stem height up to the cob, cm | 100 | 105 | 110 |
| Weight of 1000 seeds, g | 350 | 350 | 400 |
| Seed type | Odontoid | Odontoid | Odontoid |
| Planting density, $\times 10^3$ plants/ha | 70 | 70 | 60-65 |
| Leaf position | Vertical | Vertical | Vertical |
| Drought resistance | Good | Good | Good |
| Disease resistance | Good | Good | Good |
| Leaf color during harvest | Stay-green | Stay-green | Stay-green |
| Height above sea level in the growing areas, m | Up to 600 | Up to 600 | Up to 500 |
| Silage productivity, ton/ha | 50 | 50 | 60 |
| Cob morphology characteristics | | | |
| Grain humidity, % | 11.96 | 11.56 | 11.14 |
| Cob length, cm | 21.53 | 21.53 | 23.05 |
| Cob weight, g | 281.43 | 296.62 | 309.13 |
| Cob rows number, pcs | 14.60 | 14.70 | 15.70 |
| Cob seeds number, pcs | 604.10 | 599.50 | 706.20 |
| Germ weight, g | 41.31 | 43.07 | 45.18 |
| Cob seeds weight, g | 240.33 | 253.56 | 263.95 |

Note. SC — Single Cross, TC — Triple Cross (hybrid obtained from three parent plants). The studies were performed at experimental fields of the Zemun Polje Corn Institute for 4-8 years (depending on the hybrid) and at agricultural farm in Voyevodino and in central Serbia (in river valleys). After the trials were finished, some combines dedicated more than 100 fields up to 10 ha in square for commercial planting of these hybrids.

Thus, IR spectroscopy was used to study the molecular composition of grain of different hybrids for the first time, which is important not only for diagnostics and selection, but also to develop a methodology of biotechnology screening or determining grain storage time limits. The approach developed, making revealing of biogenic structures possible, allows to control changes of content or synthesis of different components of the grain and its composition at the genetic level.

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# METAGENOMIC CHARACTERISTIC OF RHIZOSPHERE EFFECT ON CEREALS IN BLACK AND SOD-PODZOLIC SOILS

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Abstract

Changes in the composition of microbial communities under the influence of root exudation of plants (rhizosphere effect) is widely reported in the scientific literature. A number of studies clearly show the rhizosphere effect of external factors such as soil type, species and plant variety, etc. The aim of this work is to study the effect of soil type and plant species using modern high-throughput sequencing techniques. This effect has been studied well by foreign counterparts, but such work on Russian soils and crops used in the domestic agro-industry, is carried out for the first time. We used two soils contrasting by their agrochemical parameters, black earth (Voronezh region), and sod-podzolic soil (Pskov region). Rye (*Secale cereale* L., k-6469) and wheat (*Triticum aestivum* L., k-54609**)** seeds obtained from VIR collection (St. Petersburg) were grown in a greenhouse on both soils for 42 days. Using NGS-V4 variable region sequenced 16S rDNA gene, microbial community composition in bulk soils and the rhizospheres formed on them was analyzed. Despite the short period of the experiment, clear rhizosphere effect was revealed in both soils. The strongest factor was the type of soil. Communities of bulk soil as well as rhizosphere communities on these soils, were significantly different from each other. Both soils show the same effect in the formation of rhizosphere communities of rye and wheat. Type of plant is the second largest (after the type of soil) factor in determining taxonomic composition of the rhizosphere microbiome. Communities of rye rhizosphere in general are closer to the communities of bulk soils than wheat rhizosphere communities. Also, the rhizosphere communities of rye on sod-podzolic soil according to the cluster analysis are closer in structure to the original communities of the soil. The taxonomic analysis of the communities at the level of phyla revealed several groups. They are most responsible for the rhizosphere effect. Formation of rhizosphere communities was accompanied by an increase in the number of *Betaproteobacteria* class sequences, while reducing the part of the bacteria of *Verrucomicrobia* phylum. Significant changes in the community occurred in wheat-cultivated sod-podzolic soil. According to the results of all analyzes, these communities differ significantly from the original communities of soil and rhizosphere communities of rye on sod-podzolic soil. Perhaps this can be attributed to an increased proportion of the genus *Flavobacterium* (phylum *Bacteroidetes*) bacteria in these communities. Using the method of high-throughput sequencing it has been clearly demonstrated the presence of rhizosphere effect on rye- and wheat-cultivated soils, as well as the features of the interaction of individual factors responsible for rhizosphere effect. However, to confirm rhizosphere effect, as well as for more detailed studies of the mechanisms underlying it, it is necessary, in addition to the taxonomic analysis carried out, to elucidate how the rhizosphere microbiome is influenced by the plant exudate composition. To do this a series of model experiments with introduction into the soil of certain root exudate substances of rye and wheat are already scheduled.

Keywords: rhizosphere effect, rhizosphere microbiom, metagenomic analysis, rye rhizosphere, wheat rhizosphere

The rhizosphere of plants represents a special niche, where microbial community specific for each species of plant is formed [1-4]. The structure of this community is largely determined by the composition of plant exudates, performing both the role of the substrate and regulatory functions [3-6]. Owing to the exudation process, a plant actively cooperates with the soil microbiota, forming the microbial environment which provides the plant with a number of adap-

tive advantages, such as protection from pathogens, mineral nutrition, adaptation to abiotic stresses, and regulation of the development [3-6]. The development of a plant, the growth of its roots and root exudation is a powerful biotic factor contributing to the formation of rhizosphere microbiome [6-9]. Qualitative and quantitative modification in the composition of microbial community under its influence became known as the rhizosphere effect [6, 8]. It was shown that it manifested differently in different soils [10, 11], at different stages of plant development [12], and in different plant species and even varieties [10-14]. Significant differences in the rhizosphere effect were also identified when cultivated plants were compared with initial wild relatives [14, 15].

For a long time, the rhizosphere effect was studied using conventional microbiological methods, which thereby provided a wealth of scientific experience with respect to both physiological and genetic properties of the main representatives of the rhizosphere microbiome [2, 6, 8]. However, as it is known, only a small part of the diversity was covered in these studies [16, 17]. Modern molecular techniques, such as high-throughput sequencing, made possible a more detailed examination of the rhizosphere microbiome, including not only cultivated, but also its uncultivated representatives. This method is widely used by foreign scientists [18-22], but in the domestic soil investigations there is obviously a lack of such research. The use of high-throughput sequencing when investigating the rhizosphere effect allowed to show conclusively the role of the soil type, the duration of growth and plant variety [10, 12-14] in determining the taxonomic composition of the rhizosphere microbiomes. All the studies indicated that it was the type of soil, which had the greatest influence on the rhizosphere effect [10, 13, 22]. The rhizosphere effect was examined in a number of plants, i.e. from model objects, such as *Arabidopsis* sp. [17, 18, 21], to the species of major agricultural importance, e.g. rice (*Oryza sativa*) [23], and lettuce (*Lactuca sativa*) (22). In these cases, the rhizosphere effect manifested differently for different plants. It should also be noted that in most reports the rhizosphere effect was studied for soil types which were similar in structure and genesis [11, 17, 21].

Considering that the rhizosphere effect manifests itself depending on the characteristics of plants and soil types, our primary objective was to expand the circle of diversity of the tested subjects. This is the first paper to report a study of the rhizosphere effect for the agricultural crops common in Russia (rye and wheat varieties), and in commonly occurring contrastive soils (chernozem and sod-podzolic).

The objectives of the study included the evaluation of the rhizosphere effect in model experiments on the cultivation of rye and wheat plants in soils of different types, with concurrent identification of specific taxonomic groups of bacteria.

*Technique.* The soil samples for the experiments were taken in the agriculturally used areas (division edges on the fields free of crops over the last 50 years) at a depth of 3-15 cm. Sod-podzolic soil samples were provided by Pskov Agricultural Research Institute and the state owned farm «Rodina» (Pskov Province, the coordinates of the sampling point: 57°50'44,2"N, 28°12'03,7"E).) Chernozem samples were obtained from Voronezh Province (the nature preserve Kamennaya Steppe: 510°01'41,6"N, 400°43' 39,3"E). The soil samples were sieved on a 5 mm soil screen, dried and filled into plastic containers (by 5.0 kg for chernozem and 5.5 kg for sod-podzolic soil) and then humidified at the rate of 75 % of total moisture capacity.

One day after, seeds were introduced in each pot to a depth of 3-5 cm in regular rows, 25 pcs per pot. We used rye seeds of a local variety seeded only in Pskov Province (k-6469 in the VIR catalog — N.I. Vavilov All-Russian Institute

of Plant Genetic Resources, St. Petersburg), and wheat seeds (the Volshebnitsa variety, k-54609 in the VIR catalog). Two pots with each type of soil were used per each variety. The experiment was carried out for 42 days (from September 23 to November 4, 2014) in the greenhouse covered with a plastic wrap (the end wall was covered with a mesh to provide gas exchange), while maintaining a constant soil moisture (75 % of the total moisture capacity). The average daily temperature during the experiment was 13 °C and nighttime temperature 4 °C. At the end of the experiment, two samples of roots were taken from each pot. The roots, as soon as separated from the soil, were divided into two roughly equal portions, then placed in vials with water (50 ml) and shaken vigorously for 1 minute to obtain a homogeneous suspension of soil. A 2-ml aliquot of the suspension was collected into a microtube, centrifuged, and the pellet was used to isolate the rhizosphere DNA.

DNA was isolated using the method developed in the All-Russian Research Institute for Agricultural Microbiology [25]. The resulting DNA concentration was on average 18 ng/ml. The purified DNA was used as a template for the PCR with universal primers targeting variable region 4 of the 16S rRNA gene, the F515 GTGCCAGCMGCCGCGGTAA and R806 GGACTACVSGGGTATCTAAT [26], with the addition of the oligonucleotide identifiers for each sample and supporting sequences required for pyrosequencing technology. NGS-sequencing (next-generation sequencing) was carried out using a GS Junior system (Roche, USA) according to the manufacturer's recommendations.

The data were processed in QIIME, v.1.8.0 (http://qiime.org/) [27]. The sequences of the 16S rRNA gene were analyzed in several stages. The first stage involved quality control of the sequences to exclude from the analysis those with length less than 200 nucleotides, with a quality score of less than 25, with misread sequences of primers and multiplex identifiers, extensive homopolymer repeats (more than 8 nucleotides) and unidentified nucleotides. After excluding all non-bacterial and chimeric sequences, the resulting libraries were normalized according to the number of sequences in the smallest library. As a result of all the procedures performed, 19,440 sequences were selected (810 in each library). The sequences with a $> 97$ % similarity were combined into operational taxonomic units (OTUs), using the de novo algorithm (based on the «uclust» method). One sequence was selected from each OTU to produce a set of representative sequences. The next stage was the classification of representative sequences using the RDP naïve Bayesianr RNA Classifier, and the alignment using the PyNast algorithm [27], where a specially designed Greengenes coreset of sequences served as a matrix for alignment [28]. After aligning, the sequences were used to construct gene distance matrix and the phylogenetic tree.

To characterize biodiversity and carry out a comparative analysis of the communities, the parameters of α- and β-diversity were calculated. The α-diversity was assessed using species richness indices (the OTU value in the sample) and the Shannon index (Shannon, H). The significance of differences in the α-diversity indices between the microbiomes was determined using *t*-test. To assess β-diversity the Weighted unifrac method was used, allowing to identify the percentage of similarities between all pairs of the microbiomes being compared [29]. The results were presented using methods of the PCoA multivariate statistics (principal component analysis) and data were visualized in the Emperor program (is a part of QIIME) (http://emperor.colorado.edu). For calculating the indices of diversity and performing the cluster analysis, the Bray-Curtiss criterion was used and calculations were carried out in the PAST software (http://folk.uio.no/ohammer/past/) [30]. Statistical support for clusters was calculated via the bootstrap method (1,000 replacements).

The differences between the samples in terms of the taxa frequency were determined using the Fisher's exact test adjusted for multiple comparisons by the Benjamini-Hocberg FDR procedure at the 5 % significance level.

*Results.* We used primers which were designed based on the analysis of nucleotide sequences of both bacteria and archaea, and allow to amplify the 16S rRNA gene fragment of approximately 400 bps. The paper analyzed the microbiome communities in six variants, such as the sod-podzolic soil (SP), chernozem (ChZ), the rye rhizosphere in the sod-podzolic soil (rSP), the wheat rhizosphere in the sod-podzolic soil (wSP), the rye rhizosphere in the chernozem (rChZ) and the wheat rhizosphere in the chernozem (wChZ).

Indices of diversity. Indices of diversity calculated for soil communities and rhizosphere communities were not significantly different. Significant differences in the values of the Chao-1 and Shannon indices were demonstrated for the community of wheat rhizosphere in the sod-podzolic soil (see Table). As can be seen, the values of both indices were significantly lower than in communities of the sod-podzolic soil or the rye rhizosphere on the same soil.

**Indices of α-diversity for soil and rhizosphere microbiome communities, depending on the soil type and plant species** ($X \pm x$, wheat *Triticum aestivum* L. and rye *Secale cereale* L.)

| Microbiome | Indices of diversity | | |
|---|---|---|---|
| | Species richness | Chao-1 | Shannon (H) |
| Chernozem (Voronezh Province) | | | |
| No plants | 277±35 | 360±41 | 4.94±0.17 |
| Rhizosphere: | | | |
| of wheat | 219±21 | 310±31 | 4.78±0.13 |
| of rye | 297±23 | 426±18 | 4.96±0.04 |
| Sod-podzolic soil (Pskov Province) | | | |
| No plants | 287±33 | 401±28 | 5.20±0.11 |
| Rhizosphere: | | | |
| of wheat | 248±26 | 335±31 | 4.78±0.07 |
| of rye | 290±22 | 393±10 | 5.09±0.05 |

Note. Rye (k-6469 in the VIR catalog, N.I. Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg), a local variety from Pskov oblast) and wheat varieties (the Volshebnitsa variety, k-54609 in the VIR catalog) were used.

The observed effect is of particular interest in relation with the available literature data, i.e. previous reports indicated that indices of diversity of rhizosphere communities were not significantly different from those of the initial soil communities both in case of different soils and plant varieties [9, 10] and when analysing the rhizosphere of plants of different age [9].

Cluster analysis and principal component analysis (PCoA). In the dendrogram (Fig. 1), «chernozem cluster» (including the initial chernozem and the rhizosphere of both plants formed on this soil) and "sod-podzolic cluster" clearly stand out. The first one demonstrated a pronounced division of communities into two separate groups which corresponded to the initial soil and rhizosphere. In this case, communities of rye and wheat rhizospheres in chernozem in terms of their taxonomic structure were more similar to each other than each of them to the initial soil community. In the «sod-podzolic cluster» another trend is observed, i.e. the initial soil and rhizosphere of rye on sod-podzolic soil appeared to be in a separate clade. These data correlate well with diversity indices, further suggesting an expressed rhizosphere effect in the cultivation of wheat. However, the observed effect, associated with a reduced diversity in the rhizosphere of wheat on the sod-podzolic soil, can be considered only as a trend, since the corresponding cluster on the dendrogram had a relatively low statistical support (no more than 64 %). However, it was reproduced in the principal component analysis.

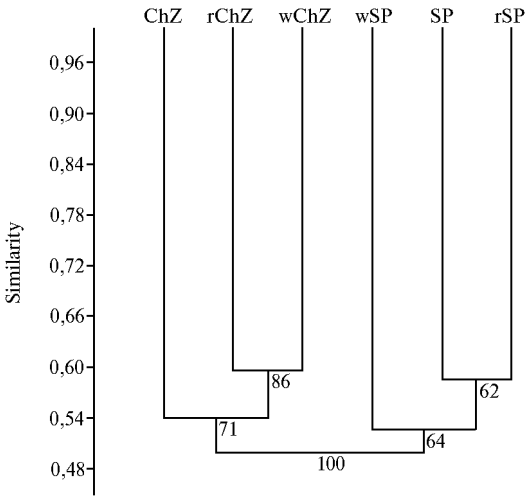


**Fig. 1. Cluster analysis of microbiomes of soils and rhizosphere communities formed in these soils:** ChZ and SP — chernozem and sod-podzolic soil; rChZ and rSP — rhizosphere of rye (*Secale cereale* L., a local variety from Pskov Province, k-6469 in the VIR catalog, N.I. Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg;), wChZ and wSP — rhizosphere of wheat (*Triticum aesivum* L., the Volshebnitsa variety). Greenhouse pot experiments. The similarity dendrogram was constructed using the Bray-Curtiss similarity measure, bootstrap = 1,000.

The graph, representing the results of the PCoA, clearly shows marked differences in the structure of clusters corresponding to the chernozem and sod-podzolic soils, i.e.

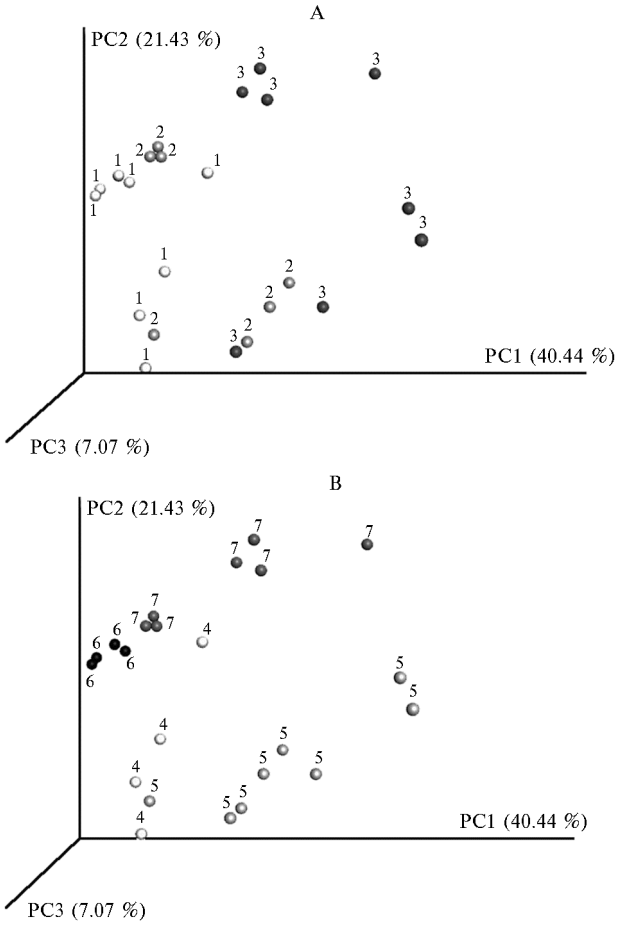


**Fig. 2. Principal component analysis** (PCoA) **of soil communities and based on them rhizosphere microbial communities:** A — compared to the initial soil, B — in initial soils and rhizospheres based on them; 1 — initial soil, 2 — rhizosphere of rye (*Secale cereale* L., a local variety from Pskov Province, k-6469 in the VIR catalog,) 3 — rhizosphere of wheat (*Triticum aesivum* L., the Volshebnitsa variety); 4 — chernozem, 5 — rhizospheres on chernozem, 6 — sod-podzolic soil, 7 — rhizospheres on sod-podzolic soil. Greenhouse pot experiments.

in the first case, we observed a significant variation in experiment replications,

while in the second one the variation was practically absent (Fig. 2). The reason may lie in the high heterogeneity of the soil. Meanwhile, it is possible that the sequencing depth was inadequate for the community. Also, the graph shows that communities of the rye rhizospheres on the sod-podzolic soil were similar in structure to the communities of the initial soil, and the differences were seen only in communities of the wheat rhizospheres which constituted a separate group (see Fig. 2, A). There were no clearly separate groups determined in the chernozem communities. However, as we can see, the communities of rye rhizospheres have been generally closer to the communities of the initial soils than the communities of wheat rhizospheres.

The taxonomic composition of communities. An analysis, carried out at the phyla-level (classes for the phylum *Proteobacteria*), showed significant differences ($p < 0.05$) in the number of sequences between the experiment variants (Fig. 3).

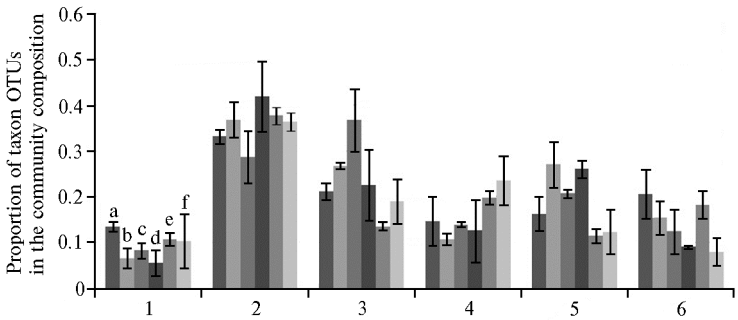


**Fig. 3. Taxonomic composition** (presented in part) **of soil communities and based on them rhizosphere microbial communities:** OTU — operational taxonomic unit; 1 — *Acidobacteria*, 2 — *Actinobacteria*, 3 — *Bacteroidetes*, 4 — *Verrucomicrobia*, 5 — *Betaproteobacteria*, 6 — *Gammaproteobacteria*; a — rye (*Secale cereale* L., a local variety from Pskov Province, k-6469 in the VIR catalog,), sod-podzolic soil; b — rye, chernozem; c — wheat (*Triticum aesivum* L., the Volshebnitsa variety), sod-podzolic soil; d — wheat, chernozem; e — control, sod-podzolic soil; f — control, chernozem. Greenhouse pot experiments, $p < 0.05$.

All rhizosphere communities showed an increase in the number of sequences belonging to a class of *Betaproteobacteria* (non-significant for SP and rSP communities), as well as a reduced number of representatives of the *Verrucomicrobia* phylum (non-significant for rSP—SP and wChZ—ChZ communities). A significantly increased number of sequences belonging to a class *Gammaproteobacteria* was evident for the sod-podzolic soil community as compared to the chernozem community, although this trend was lost in the communities of the rhizospheres of these soil types. In the communities of the wheat rhizosphere on the sod-podzolic soil, it was observed an increase in the proportion of representatives of the *Bacteroidetes* phylum. The proportion turned out to be significantly lower in the community of the rye rhizosphere in the sod-podzolic soil, and even lesser in the very sod-podzolic soil. It has also been shown a decrease in the number of representatives of the *Actinobacteria* phylum in the wheat rhizosphere on the sod-podzolic soil compared to the initial soil. The communities of the rye rhizosphere on the sod-podzolic soil differed from wheat rhizosphere communities on the same soil with a significantly higher number of representatives of the *Actinobacteria* phylum.

As it is known from the literature data, at different developmental stages, plants possess mostly different groups of microorganisms. Based on the study of root exudation in *Arabidopsis*, the major differences were found in four phyla, such as *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Cyanobacteria*, moreover, a positive correlation was revealed between the number of the *Bacteroidetes* phy-

lum representatives with the number of amino acids released by the roots of this plant, and an inverse correlation with the phenolic compounds [11].

Most likely, it is an increase in the number of the *Bacteroidetes* phylum representatives in the wheat rhizosphere community on the sod-podzolic soil, which led to a marked difference in terms of α-diversity in comparison to the initial soil. The statistical analysis revealed that differences in the composition of communities in this case were associated with an increase (more than 12.6-fold, $p < 0.05$) in the number of microorganisms from the *Flavobacterium* genus.

A more detailed statistical analysis of the taxonomic structure of microbial communities also allowed to identify the group of microorganisms which caused differences in the formation of rhizosphere microbiomes in other variants of the experiment. In particular, compared to the initial soil (ChZ), in the rChZ rhizosphere, there was a significant increase in the proportion of the *Pedobacter* (by 47.6 times) and *Chitinophaga* (by 76.9 times) genera, and in the wChZ rhizosphere — of the *Pedobacter* (10.0-fold) and *Kaistobacter* (14.2-fold) genera. In the rSP rhizosphere (as compared to SP), there was an increase in the proportion of the *Pseudomonas* (by 48.6 times) and *Achromobacter* (by 25.0 times) genera, and in the wSP rhizosphere — of the *Mesorhizobium* (40.0 times) and *Chitinophaga* (53.7 times) genera. Therefore, the composition of the rhizosphere community varied in different soils. These results are consistent with the literature data indicating that the rhizospheres of plants cultivated on soils of different types vary significantly in composition at the genera-level. For example, growing lettuce on three different soils showed an increase in the number of members of the *Sphingomonas* and *Rhizobium* (α-proteobacteria), *Pseudomonas* (γ-proteobacteria), *Variovorax* (β-proteobacteria) and *Flavobacterium* (*Bacteroidetes*) families, however, unique families are typical for each of the soil type which alter the number of their representatives only in rhizospheres on this type of soil [11].

It should be emphasized that currently there is no single internationally recognized method both for separating communities of the rhizosphere and rhizoplane, and for the isolation of DNA from the soil and its further analysis. Therefore, our findings are difficult to be compared with those described in the literature. Note, for example, that a more thorough analysis of the communities of the root zone, separating the rhizosphere and the rhizoplane, indicates that the rhizosphere community has a similar taxonomic composition with the community of the initial soil, while the major differences are observed in the rhizoplane, which are limited to a decrease in the proportions of the *Acidobacteria*, *Planctomycetes*, and *Gemmatimonadetes* phyla [24]. It is now clear that, for a more comprehensive assessment of the rhizosphere effect, a universal sampling technique should be developed which would include additional methods enabling a more precise localization of some representatives of the rhizosphere community (e.g., FISH techniques — fluorescence in situ hybridization, etc.).

Therefore, despite the relatively short duration of the experiment (42 days), the communities of the initial soils and rhizospheres varied greatly, i.e. a pronounced rhizosphere effect was revealed in both types of soil. The formation of the rhizosphere community is greatly influenced by both a soil type and the plant species. The strongest factor appeared to be the type of soil, since rhizosphere communities, generated in various types of soil, and the initial soil communities significantly differ from each other. As it was demonstrated, these differences were preserved for both soils during the formation of different rhizosphere communities. The plant species was the second most important (after the type of soil) factor in determining the taxonomic composition of the rhizosphere microbiome. In general, the communities of the rye rhizosphere are somewhat closer to the communities of the initial soils than the wheat rhizosphere communities are.

The taxonomic analysis of the communities at the phyla-level allowed to reveal groups, most responsible for the rhizosphere effect, i.e. the formation of rhizosphere communities was accompanied by an increase in the number of sequences from the *Betaproteobacteria* class along with reductions in the number of the *Verrucomicrobia* phylum representatives.

The combination of features of the sod-podzolic soil and characteristics of the wheat, cultivated on it, resulted in significant changes in the community. According to the results of all analyzes, these communities differ significantly from the initial soil communities and the communities of the rye rhizosphere on the sod-podzolic soil. This may be due to an increase in the proportion of bacteria from the *Flavobacterium* genus (the *Bacteroidetes* phylum) in these communities.

Thus, using high-throughput sequencing method with its high resolution and the ability to examine even non-culturable microorganisms, the rhizosphere effect was shown to exist in the soil when growing cultivars. However, to confirm the presence of this effect, as well as for more detailed studies of mechanisms underlying it, our taxonomic analysis should be supported by further studies that would characterize the association between the structure of the rhizosphere microbiome and the composition of plant exudates. To do this, model experiments are projected on introducing into the soil the components of plant exudates of the tested rye and wheat varieties.

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# THE ALIGNMENT OF SOIL'S CONDITIONS FOR PLANT'S DEVELOPMENT DURING MICROBIAL DESTRUCTION OF PLANT'S RESIDUES BY MICROBIAL PREPARATIONS

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## Abstract

Modern agriculture is developing in the direction of producing consistently high yields and high quality seed production. In this regard, the precision agricultural technologies are develop for the leveling a soil conditions. Individual phenotypic characteristics of plant are determined by the local soil conditions near their root's systems. As a result, the variance of plant's height is dependent on the spatial distribution of energy resources and nutrients in the soil. The variance of plant's height restricted to the genetic norm for this characteristic and it can been reduced when a soil conditions are leveled. In experiments with planting alfalfa was been shown, that the variance of plant's mass may decrease with an increase in the efficiency of plant-microbial symbiosis. Perhaps the plant-microbial symbiosis is able to level a soil conditions and selectively stimulate the plants by using of the microbial metabolites. We assume that the effect of microbiological leveling soil conditions (MLSC) may be observed during destruction of plant's residues using microbial preparation. Previously, MLSC effect has not studied. Therefore, these theoretical and experimental researches are new. In addition, we have obtained new practically important results thanks to the use of the original fractal analysis of molecular-genetic data of the soil microbial community. The goal of this work was experimental and theoretical study of MPSC effect arising after destruction of plant's residues with the using of the microbial preparations, which was been developed in the All-Russian Research Institute of Agricultural Microbiology. To achieve this goal were used the data of two experiments. In the first experiment, the variances of the barley plant's height were been investigated after the destruction of plant residues using a microbial preparation Barkon. Out the second experiment used data of the molecular-genetic analysis of soil microbial communities after the destruction of plant's residues using three microbial preparations: Barkon, Bags and Omug. The preparation Barkon contains the consortium of bacteria and fungi; Bugs is a consortium of cellulolytic organisms, derived from biologically active soil; Omug is the microbial fertilizer obtained after biotechnological processing of poultry manure. The functional activity of microbial networks arising during the destruction of plant's residues with using of microbial preparations was been studied using fractal analysis of molecular genetic data of microbial communities in soil. With using of the fractal analysis was been obtained the fractal taxonomic portrait of microbial communities and the index of the functional efficiency of microbial network formations, which were formed during destruction of plant's residues. The first experiment showed that the using of preparations for the destruction of plant's residues leads to a gradual leveling of the soil's conditions and to reducing of the variance of plant's heights, which were been grown on these soils. Without these preparations, the dispersion of plant's heights increases with each successive year. From this, it follows that these preparations may initiate the effective microbial networks that are able to save the energy resources and the nutrients distributing them evenly in the soil. The molecular-genetic data from second experiment confirmed that the functional efficiency of the microbial networks after using preparations significantly increases due to better organization of destructive processes. The results of this study suggest that the destruction of plant's residues by using of the special preparations is a necessary and effective complement of the modern precision agro technologies. Thus, the microbial

preparations for destruction plant's residues start processes which lead to the restoration of the required level of energy resources and nutrients in the soil, to the leveling of resources in soil's space, to the increasing the stability of yields and to the improving the quality of plant's products.

Keywords: the destruction of plant's residues with using of the microbial preparations; the microbial destructive communities in the soil; the dispersion of the individual heights of plants, the fractal-taxonomic portrait of the microbial community; the index of the functional efficiency of microbial networks

Modern agriculture is developing toward sustainable high yields and high quality seed production. In this regard, the precision agricultural technologies for leveling soil conditions are being developed [1-5].

The growing season of plants, starting from seed germination, depends on the local soil conditions in a small area surrounding the seeds and roots. Each plant is characterized by individual parameters of stem height, the number of vegetative organs, the weight of the above-ground and root parts, etc. As a result, there is dispersion (variability) of quantitative phenotypic traits in the populations. On the top it is limited to the allowable normal plant response to changes in the environmental factors [6], and may decrease in the setting of the soil conditions leveling [7-10]. A decrease in the variability of phenotypic traits of plants was found in the 3-year experiments in alfalfa (*Medicago sativa*) [11]. Each year, the ratio of genotypic and phenotypic components of the plant biomass dispersion changed. The observed increase in the variety-strain (genotypic) component and a decrease in a random (phenotypic) one may have been caused by the leveling of the soil conditions as a result of increased efficiency of microbial-plant symbiosis, although it could also be the result of targeted stimulation with microbial metabolites [12, 13] in those plants in the population, which were in a less favorable environment compared to the remaining plants.

We assume that the effect of microbiological leveling of soil conditions (MLSC) is associated with the destruction of plant residues by microbial-based products. In this case, there is a selective accumulation of nutrient resources by microorganisms in soil microniches which initially lack them. MLSC is possibly a consequence of the diffusion of low molecular weight nutrients, resulted from the plant residue degradation, to areas with a low content of nutrient resources. It is obvious that in such leveled soils plants must demonstrate a lower variability of quantitative phenotypic traits and the high yield quality [14].

To date, the question whether the MLSC processes are affected by microbial products that cause the destruction of plant residues in the soil in the pre-growing period of the year (without plants), has not been investigated. We are the first to describe these effects, having received significant for the practical application results by using a genuine fractal analysis of molecular genetic frequency data for soil microbial communities, and the coefficient of variation of the individual heights of plants grown on the respective soils.

The aim of this work was an experimental and theoretical study of the effect of microbiological leveling of the soil conditions using microbial-based products which initiate the functioning of microbial networks in soil.

*Techniques.* In the experiment 1 (carried out in 2011-2014), we used an experimental sample of a microbial-based product Barkon for humification of plant residues (developed by the All-Russian Research Institute for Agricultural Microbiology, ARRIAM), which consisted of a consortium of bacteria and fungi [15]. In pots containing the sod-podzolic soil (3.5 kg, $C_{hum}$ 2.0 %, $N_{tot}$ 0.19 %, $pH_{sol}$ 5.6), barley straw (BS) was laid at a depth of 0-3 cm. This experiment included the following options: 1 — control (without addition of BS), 2 — addition of BS without inoculation of Barkon, 3 — addition of BS, inoculated with Barkon (1 ml/10 g of straw). The crushed BS (air-dry weight 10 g per pot) in

the options 2 and 3 was introduced into pot 3 times, i.e. in autumn of 2011, 2012 and 2013. There were 4 pots used in each option. Fertilizers were not added in the pot during the experiment. Barley plants (*Hordeum* L.) were planted into the pots in the spring 2012, 2013 and 2014 (30 pcs per pot), and at the end of the growing season (autumn 2012, 2013 and 2014) the height of the aboveground parts of plants (100-120 pcs per experiment option) was measured. The data were processed using the analysis of variance [16], and the coefficient of variation of this phenotypic trait per each experiment option was calculated.

In experiment 2, molecular genetic data on the microbial communities were used to study the network organization of microorganisms, which constituted the basis of the biologicals for the destruction of cereal straw [17]. Per 1 kg of cultivated sod-podzolic soil ($C_{hum}$ 4.02 %; $N_{tot}$ 0.316 %; $pH_{sol}$ 5.63) in each pot, 3 g of crushed rye straw (RS) was added in three ways, i.e. either superficially or laying down the surface at a depth of 0-3 cm and 9-12 cm. To accelerate the decomposition, the straw was treated with Bags, Barkon and Omug, according to the the developers' recommendations (ARRIAM). Bags is a consortium of cellulosolytic microorganisms based on the biologically active substrate [18], and Omug is a microbial fertilizer obtained after biotechnological processing of poultry manure [19].

On day 60, the molecular genetic analysis of 12 soil samples was performed using a standard technique [19] and a CEQ 8000 genetic analysis system (Beckman Coulter International S.A., Switzerland). Data on the frequency of occurrence of operational taxonomic units (OTE) in the soil samples were subjected to a modified fractal analysis [21-26] in order to obtain information about the characteristics of the resulting microbial network formations. Taxonomic data were not used in the study. Data on the taxonomic analysis of the same data were presented earlier [17].

*Results.* In the experiment 1 (options 1 and 2), the coefficient of variation of plant heights (VPH) in barley plants increased from 1st to 3rd year of observation (Table 1). The total amount of nutrient resources in soil was likely to decrease, thereby increasing the variability of the soil conditions and individual plant heights.

**1. The coefficient of variation of plant heights** (VPH) **in barley plants when grown on soil with barley straw** (BS) **added as plant residues, and depending on the inoculation of the microbial-based product Barkon**

| Option | Year | Coefficient VPH, % |
|---|---|---|
| Control (without addition of BS) | 2012 | 19.8 |
| | 2013 | 25.5 |
| | 2014 | 27.0 |
| Adding BS without inoculation of Barkcon | 2012 | 13.2 |
| | 2013 | 14.9 |
| | 2014 | 16.3 |
| Adding BS with inoculation of Barkon | 2012 | 17.1 |
| | 2013 | 14.5 |
| | 2014 | 16.0 |
| Confidence interval | | ±0.2 |

In the option 3, the coefficient of VPH over the years, on the contrary, decreased (see Table 1). This suggests that using a microbial-based product led to a gradual increase in the efficiency of soil destructive microbial community. The accumulation of nutrient resources for plants and leveling of soil conditions can take several years [27].

It is probable that low molecular weight nutrients are formed during this period, diffusing into microniches with their low concentration, which might also lead to fading initial differences between the microniches.

Microbiological degradation of plant residues can be represented as a gradual division of organic molecules into a growing number of fragments of decreasing size. As a mathematical object displaying the destruction of the molecules, it is proposed to use the power series of non-integral numbers, belonging to

the fractal numerical series [28, 29]. For example, a sequence (1, $^1/_2$, $^1/_4$, $^1/_8$, ...) is a fractal set as it is formed obeying the rule fixed for all numbers in the set: each following number in a series is twice as little as the previous one.

Numbers from fractal series can be roughly compared with the size of fragments of molecules. Then the position in the fractal series will mean a number of the destruction stage, and the current number of a series will indicate the size of fragments of molecules, which are subjected to destruction at an appropriate stage. Actual degradation processes take place with the change of molecular fragmentation rules. At some stages the number of fragments can be doubled, in other — tripled, i.e. we assume that the microbiological degradation processes are multifractal [30-34].

Our studies have shown that for the fractal series-based mathematical modeling of complex destructive processes of organic molecules it is sufficient to use only the first three members of the fractal series. This restriction considerably simplifies the identification of complex destructive processes with unknown beforehand rules of fragmentation and number of stages of destruction.

We believe that the destruction of organic molecules of plant residues by microorganisms is carried out along with the formation of multifractal network microorganism structures [35, 36]. Moreover, the process takes place with the involvement of the required number of microorganisms which is proportional to the number of fragments undergoing degradation. Such an assumption allows for fractal analysis of microbiological destruction processes based on the OTU frequencies of occurrence using the following mathematical rule that binds the frequencies of three OTUs:

$$\frac{ln(p_k)}{ln(p_{\max})} = \frac{ln(p_0)}{ln(p_{\max})} + a \cdot k\,, \tag{1}$$

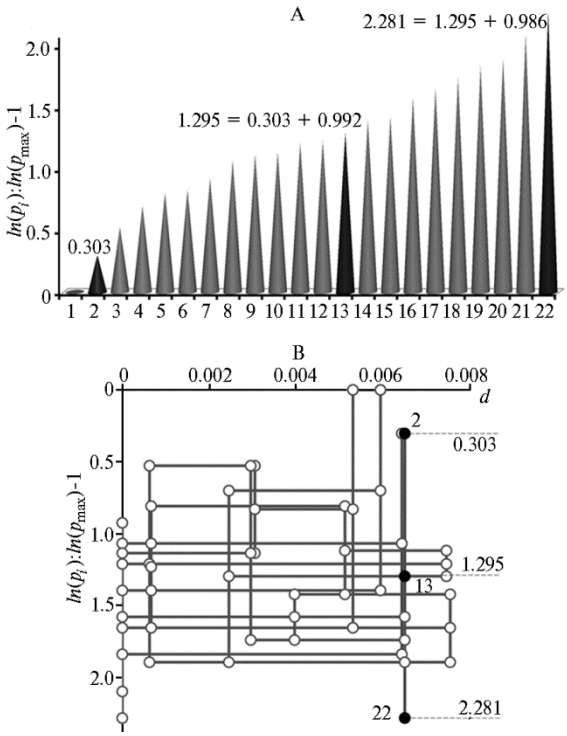
where $p_k$ is frequency of occurrence of microorganisms of the $k$ OUT which perform the destruction of organic molecules at the $k$ stage ($k$ = 0, 1, 2); $p_{\max}$ is frequency of occurrence of the microorganisms of the dominant OTU in the soil community; $a$ is a constant coefficient, a fractal index of the primary destructive set of microorganisms (PDS).

The logarithmic dependence (1) enables to isolate and identify three OTUs combined into the PDS, in which the microorganisms carry out destruction of organic molecules obeying a single rule of fragmentation. To detect several PDSs in microbial communities, it is required to try all possible combinations by three OTUs and select those which satisfy the following equations:

$$\begin{cases} \dfrac{ln(p_1)}{ln(p_{\max})} = \dfrac{ln(p_0)}{ln(p_{\max})} + a \\ \dfrac{ln(p_2)}{ln(p_{\max})} = \dfrac{ln(p_1)}{ln(p_{\max})} + a(\pm d) \end{cases}, \tag{2}$$

where $1 > p_0 > p_1 > p_2$ are frequencies of occurrence of OTUs, forming the PDS, the microorganisms of which carry out the destruction of organic molecules in three steps obeying a single rule of fragmentation; $0 \le d \le 0{,}01$ is a margin of error of the fractal PDS index to identify it, which depends on the accuracy of measuring the OTU frequency parameters.

The figure (A) shows an ordered series of normalized logarithms of frequencies of 22 OTUs from the experiment 2 (the option with the Barkon use and location of inoculated RS on the soil surface). As an example, three OTUs, the frequencies of which satisfy the equations (2), are shown. Consequently, the corresponding microorganisms were involved in the degradation of plant residues, obeying a single rule of fragmentation, and formed the PDS.




**The ordered series of the frequencies of individual taxonomic units** (OTE)**, identified by molecular genetic methods** (A)**, and the fractal taxonomic profile of the soil bacterial community with the superficial location of the rye straw inoculated with the microbial-based product Barkon** (B)**:** 1-22 — serial numbers of OTUs, *d* — the error of the fractal PDS (primary destructive set) index (2); $ln(p_{max})$, $ln(p_i)$ — natural logarithms of the OTU frequencies, respectively, with a maximum frequency of occurrence and the serial number *i* in the series of frequencies. OTU №№ 2, 13, 22 satisfy the equations (2).

The following method of constructing the fractal taxonomic profile of microbial communities was proposed to visualize all the PDSs in the community of microorganisms (see Fig., B) [37]. Every three circles connected by vertical lines on the profile represent three OTUs as part of a PDS. This is the way to display a three-stage degradation of organic molecules with corresponding microorganisms obeying one of the fragmentation rules. The greater the distance between the circles in the PDS, the higher are both the coefficient of division into fragments at the relevant stages of microbial degradation and the fractal PDS index. Horizontal lines, connecting the same OTUs, indicate microorganisms which are present in several PDSs. In addition, the horizontal axis *d*-component of each PDS indicates the error of the fractal index of the corresponding PDS (2).

In our experiment, the fractal taxonomic profile of the destructive microbial community (see Fig., B) was a complex network formation, consisting of 21 PDSs which were formed by 22 OTUs. OTU № 7 and № 22 entered the PDS only once, and all the remaining — two or more times. Consequently, some groups of microorganisms could function simultaneously in several PDSs obeying different rules of the molecular fragmentation. These microorganisms may simultaneously synthesize several enzymes that could lead to a decrease in the rate of synthesis and reduced efficiency of the entire destructive process. We therefore propose to calculate the functional efficiency index of microbial network formations (ENF) using the following formula:

$$I_{NET} = ln(N_M) : ln(3 \cdot N_F) \ , \qquad [3]$$

where $I_{NET}$ is the index of ENF; $N_M$ is a total number of OTU which formed the PDS; $N_F$ is the number of PDS in the microbial community.

The highest ENF index is 1, and all OTUs are only in one PDS in the microbial community. In other words, such a destructive microbial community operates with maximum specificity (one group of microorganisms is equal to one destructive function).

Table 2 shows the ENF indices describing the efficiency of degradation of plant residues by the soil microbial communities, initiated by microorganisms which are the ingredients of the following products: Bags, Barkon and Omug. In the control, the ENF index appeared to be lower than in other option, i.e. the

microbiological processes were disorganized and ineffective. The use of microbial-based products significantly increased the efficiency of the microbial communities, which was confirmed by increases in the ENF index values compared to the control and the experimental options without their application.

**2. The index of the effective network formation** (ENF) **of destructive microbial communities in the soil, with the rye straw** (RS) **laid at different depths, and depending on the inoculation of different biologicals**

| Option | Depth of laying RS into soil, cm | $I_{NET}$ (the ENF index) (3) |
|---|---|---|
| Control (without RS) | | 0.634 |
| Laying RS without inoculation with biologicals | 0 | 0.662 |
| | 0-3 | 0.676 |
| | 9-12 | 0.700 |
| Laying RS inoculated with Barkon | 0 | 0.746 |
| | 0-3 | 0.701 |
| | 9-12 | 0.669 |
| Laying of RS inoculated with Bags | 0 | 0.686 |
| | 0-3 | 0.689 |
| | 9-12 | 0.669 |
| Laying of RS inoculated with Omug | 0 | 0.630 |
| | 0-3 | 0.705 |
| | 9-12 | 0.753 |
| Confidence interval | | ±0.003 |

When the straw was laid in the upper layers of the soil, the highest ENF indexes were observed with the use of microbial-based products Barkon and Bags. The development of networks, initiated by these biologicals, occurred with the involvement of aerobic soil microorganisms as suppliers of energy resources [38]. In contrast, the composition of Omug included poultry intestinal microorganisms, i.e. those from the anaerobic environment. When Omug was used, the greatest value of the ENF index was observed in the setting of deep (9-12 cm) laying of the straw into the soil.

Comparing the data from the experiments 1 and 2, we can state the following. A gradual increase in the functional efficiency of the network destructive microbial formations initiated by the biological was the major reason for the negative trend in the VPH coefficient values by year in the option 3 of experiment 1. The long-term adjustment of the microbial community for the optimum destructive activity may not be only aimed at the destruction of plant residues, but also at restoring the required amount of energy resources and nutrients in the soil, as well as their leveling in the soil area.

Thus, the destruction of plant residues with the help of special microbial-based products can effectively and at lower cost solve problems of leveling the amount of energy resources and nutrients in the soil. In addition, the initiation of destructive microbiological processes contributes to restoring the required amount of energy resources and nutrient content in the soil, enhancing the stability of yields and improving the quality of the obtained plant products.

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# ABOUT THE FORMATION OF *Camellia sinensis* (L.) O. Kuntze RESISTANCE UNDER INSUFFICIENT WATER SUPPLY AT THE ROOT FERTILIZATION WITH CALCIUM CLAY

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Abstract

In the conditions of Russian Black Sea coast and in many other regions of the world (China, India) tea plant is faced with seasonal water shortages leading to a significant loss of productivity — according to different authors, up to 40-50 % (M. Mukhopadhyay et al., 2014; L.S. Malyukova, 2014). In this regard, physiological and biochemical mechanisms of tea plant resistance to water shortages as well as the effectiveness of various exogenous inducers are being researched; more drought-resistant cultivars are being searched for the breeding. Considerable interest in research is related to the study of application of exogenous calcium, which is a mediator in signaling within the cell when there is a synthesis of stress proteins, which, in turn, provide the resistance to adverse environmental factors, as well as the subsequent exit from this state (X.Y. Gao et al., 1999; M.Y. Shu et al, 2000). The papers showed calcium effect on reducing oxidative damage in various plants (including tea plant) at drought by inducing antioxidant system (X.Y. Gao et al., 1999; M. Lee et al, 2004; S.S. Medvedev, 2005; H. Upadhyaya et al., 2012; E.G. Rikhvanov et al., 2014). In Russia, it is the first time when in a field experiment we studied an effect of root fertilization with calcium on the functional state of tea plants and the mode of their nutrition at low water supply. Calcium was introduced into the soil in the form of a natural fertilizer (clay and lime matter containing 40 % of CaO) at 100 kg CaO per ha along with macronutrients ($N_{240}P_{70}K_{90}$) against solely $N_{240}P_{70}K_{90}$ in control. During summer periods of high moisture deficit (late July to August) we studied the dynamics of catalase activity in mature leaves and 3-leaf fleshes, pH of the cell sap, water supply and water loss, as well as chemical composition of plants and soil. It was found that under the influence of calcium in the stressful period there were an increase in catalase activity in mature leaves (by 10-19 ml of $O_2$/g within 3 min at different periods), a reduction of water loss (on average by 20 %), a lesser alkalescency of the cell sap (by 0.05-0.07 units), and a significant (by 27-33 %) increase in plant productivity, which indicates more stable functional state both during water stress and rehydration. Catalase activity in shoots (to a lesser extent in mature leaves) correlated with the pH of the cell sap ($r$ = 0.93 and $r$ = 0.53, respectively), which determined its important role in the formation of tea plant oxidative state. More adapted restructuring of the plants to extreme conditions and their subsequent effective recovery was due to the effect of calcium fertilizers on the cation-exchange capacity of soil absorbing complex, i.e. 1.5-3-fold enhancing the calcium exchange, while maintaining the potassium status and subsequent coordinated absorption of major biogenic nutrient elements, which provides preferential flow of potassium and calcium in plants as compared to nitrogen and phosphorus.

Keywords: tea plant, *Samellia sinensis* (L.) O. Kuntze, drought resistance, calcium, mineral fertilizers, enzyme activity, water loss, pH of the cell sap, agrochemical properties of soils, chemical composition of leaves

In the conditions of Russian Black Sea coast and in many other regions of the world (China, India) tea plant is faced with seasonal water shortages leading to a significant loss of productivity (up to 40-50 %) [1-5]. Long-term periods of insufficiency of water supply represent the most harmful factor for plants, especially for perennial plants, due to oxidative stress with production of active oxygen forms [6, 7]. Thus, research on effectiveness of various exogenous inducers in terms of perennial plants drought resistance regulation is considered to be an up-to-date line of research both worldwide [7-10] and in Russia [11-13]. Calci-

um [14-17], acting as a universal second messenger [18-21] for enhancement in synthesis of stress proteins and other compounds [22-25] ensuring resistance of plants to adverse environmental factors with subsequent reversal of this state, is considered as one of the most effective inducers. The papers showed calcium effect on reducing oxidative damage in various plants (including tea plant) at drought by inducing antioxidant system [7, 14-18]. Resistance to oxidative stress is assessed through a wide range of plants functional state indicators (antioxidant enzymes, photosynthetic pigments, low molecular weight antioxidants, stress proteins, water status, productivity as an integral indicator, etc.) [13-17, 26]. The data on a number of annual crops and some perennial crops, including tea plant, has been obtained; however, effectiveness of root application of calcium fertilizers in tea plantations of the world's most northern subtropical areas (Russian Black Sea coast), where recurrent droughts are an issue of particular concern, is still not fully understood. This gap in our knowledge can be filled with the studies presented in this work.

The purpose of this study is to assess the effect of root application of calcium on functional state of tea plant and to identify the characteristics of pool formation and biogenic elements uptake under the moisture deficit conditions in the context of nonspecific antioxidant protection.

*Techniques.* The microplot field experiment was conducted in a Colchis tea plantation (planted in 1983) (the city of Sochi, the settlement of Dagomys, 2013-2015). A natural fertilizer, i.e. a clay and lime matter containing 40 % of CaO, introduced into the surface soil at a dose of 100 kg CaO per hectare along with macronutrients $N_{240}P_{70}K_{90}$ against solely $N_{240}P_{70}K_{90}$ in control, was used as an exogenous calcium source. The plot area was 10 $m^2$, application in 3 replicates was used on acid brown forest soil. The studies were performed during the most stressful summer periods as to water supply (late July–August), characterized by absence of precipitation or intermittent precipitation. Soil (depth of 0-20 cm) and plant (3-leaf fleshes and mature 5-6 month leaves) samples were taken progressively.

The following parameters were evaluated in plant samples: catalase activity as per I.I. Gunar [27], cell sap concentration by refractometry as per L.A. Filippov [28]; pH of cell sap by potentiometry, water retention capacity by modified Arland's wilting method [27]; water loss was calculated as the ratio between water loss by leaves during a drought period and initial fresh weight of a leaf [29]. Accelerated acid digestion method as per K.E. Ginzburg et al. [30] with subsequent application of standard procedures was used for macro-element analysis of leaves. The following parameters were evaluated in soil samples: pH of $_{KCl}$ by potentiometry, $Ca^{2+}$ and $Mg^{2+}$ by trilonometry, ammonia nitrogen by spectrophotometry, labile phosphorus and potassium (as per Oniani) by spectrophotometry, field moisture by weight method [31].

AGROCHEMISTRY software for mineral fertilizers effect modeling (All-Russia Research Institute of Agrochemistry, Russia) was used for data processing by variation and descriptive statistics methods [32].

*Results.* August 2014 was characterized by precipitation deficit (13.0 mm) and high average daily air temperature, i.e. 25.4 °C with the maximum up to 33 °C. Soil moisture content amounted to 30-35 % (early August), cell sap concentration — to 8.1-8.6 % which approximated the critical value (9 %), indicating disruption of the water regime. In 2015 precipitation deficit was observed as early as in July (61.5 mm), with an increase in August (15.0 mm) due to high average daily air temperature (24.2-25.7 °C with the maximum up to 32 °C), which lead to decrease in soil moisture content up to 20 %. Under these conditions cell sap concentration amounted to 10 % as early as in July, and to 13 %

in mid-August, indicating severe disruption of tea plant water supply which lead to decrease in sprout formation. Catalase (antioxidant enzyme) activity (CA) in mature leaves of tea plant in these stressful conditions was rather high (Fig. 1) and far exceeded the value in favorable conditions [12], which is the first indication of oxidative stress, as the enzyme, along with other compounds, plays a key role in regulation of reactive oxygen intermediates content [7, 16-18, 22, 33].

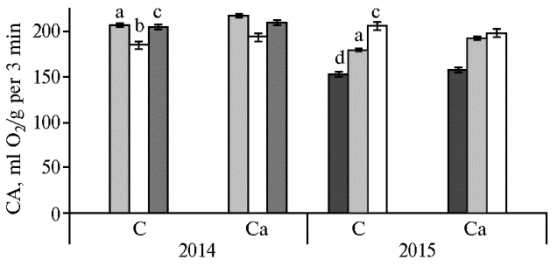


**Fig. 1. Catalase activity** (CA) **in mature leaves of Colchis tea plant [*Camellia sinensis* (L.) O. Kuntze] under root application of calcium during water stress periods:** a — August 04, b — August 19, c — August 27, d — July 27; C — control (microplot field experiment, the city of Sochi).

The use of a calcium fertilizer during all periods (except for August 19, 2015) resulted in a significant increase in catalase activity in mature leaves, indicating more effective functioning of a signaling intracellular network, where oxidative and calcium pathways are closely connected [14-17]. This is consistent with the data on reduced oxidative damage at drought in case of foliar calcium application in tea plantations due to induction of antioxidant system of a plant [3, 7].

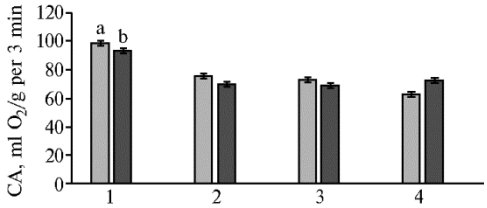


**Fig. 2. Catalase activity** (CA) **in 3-leaf fleshes of Colchis tea plant [*Camellia sinensis* (L.) O. Kuntze] under root application of calcium during water stress periods:** a — control, b — Ca; 1 — May, 2 — July, 3 — August decade I, 4 — August decade II (microplot field experiment, the city of Sochi, 2015).

Catalase activity of a tea plant fresh shoot (3-leaf fleshes) was significantly lower than that of mature leaves (Fig. 2). It was also observed by other researchers and is associated with more pronounced photostress, high degree of water loss comparing with other leaves, and with the age of a leaf [34]. During vegetation period the enzyme activity was decreasing from May (optimal conditions in terms of water supply) till August (moisture deficit) which correlated with the water loss in leaves ($r = -0.59$ for cell sap concentration). Insignificant calcium effect was observed in this part of tea plant (fresh growing shoot) only during the increased stress period (August decade II).

Cell sap concentration of fleshes (to a lesser extent in mature leaves) correlated with pH of the cell sap ($r = 0.93$ and $r = 0.53$, respectively), which determined its regulatory function in the formation of tea plant oxidative state. At that, increase in pH of the cell sap under conditions, causing accelerated transpiration (significant moisture vapor pressure deficit in air, high light intensity and leaf temperature), triggers the stomatal closure mechanism by means of modulation of abscisic acid concentration [35]. In case of calcium application this indicator was different from control (by 0.05-0.07 pH units) during the whole stressful period in 2015 (except for August 27), which confirmed the well-known role of Ca in stomatal function regulation (35, 36).

With onset of air and soil drought in August and September (air moisture of 70-71 %, soil field moisture of 20 %; for reference: in July, under relatively normal water supply conditions, these are 75-79 and 30 %, respectively) water loss under Ca application was significantly lower (88 %) than that in control

(106 %), indicating greater water retention capacity of plant cells. With regard to solids content in mature leaves (34.9 % against 36.5 % in control) and 3-leaf fleshes (22.6-25.5 % against 23.1-26.9 % in control), some increase in water supply under stressful conditions was observed as well. During several years, positive effect of Ca application was found on yield which is considered as an integral indicator of plants functioning under extreme conditions and during post-stress recovery (Fig. 3).

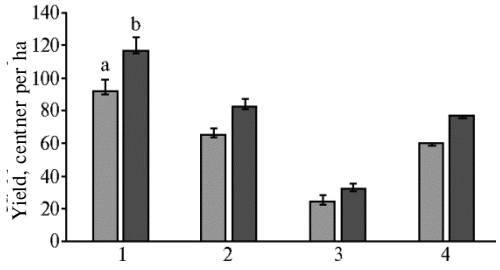


**Fig. 3. Colchis tea plantation productivity [*Camellia sinensis* (L.) O. Kuntze] by years of observation under root application of calcium during water stress periods:** a — control, b — Ca; 1, 2 and 3 — 2013, 2014 and 2015, respectively, 4 — average (microplot field experiment, the city of Sochi).

The changes in physiological functions under stress are directly related to nutrition regime. In our experiments, general reduction of ammonia nitrogen and labile phosphorus and potassium content in soil adsorption complex was observed with increase of drought (in case of soil moisture decrease from 30 to 20 %), with simultaneous increase in calcium content (Table 1). At calcium fertilizers application, an increase in calcium and magnesium ions and decrease in ammonia nitrogen and phosphate ions was observed in the soil adsorption complex structure, as compared to control.

**1. Mineral nutrients content in the tea plantation soil (Colchis tea) under root application of calcium during water stress periods** ($X \pm x$, microplot field experiment, the city of Sochi, 2015)

| Variant | Content, mg/kg | | | Content, mmol-equivalent/100 g | |
|---|---|---|---|---|---|
| | $NH_4^+$ | $P_2O_5$ | $K_2O$ | $Ca^{2+}$ | $Mg^{2+}$ |
| | | August 4 | | | |
| Control | 52±2.7 | 870±13.0 | 310±4.4 | 1.6±0.4 | 2.7±0.5 |
| Ca | 89±3.5 | 720±23.2 | 320±4.4 | 3.0±0.6 | 3.9±0.7 |
| | | August 30 | | | |
| Control | 45±1.5 | 960±10.7 | 270±8.8 | 1.4±0.2 | 2.4±0.6 |
| Ca | 64±0.9 | 590±34.6 | 320±6.5 | 4.5±0.7 | 4.2±0.8 |

When Ca application, nitrogen accumulation in leaves was predominantly observed in the beginning of stressful period, while with increase of stress calcium and potassium accumulation took place at the expense of nitrogen (Table 2). Decrease in nitrogen and magnesium content with increased calcium and potassium content in leaves indicated restructuring of cell metabolism toward reduced assimilation for more effective functioning under stress and during post-stress rehydration. The amount of $Ca^{2+}$ in cytosol induces antioxidant system of plants and regulates one of potassium channels in a cell, ensuring increase in $K^+$ concentration, which leads to increased cytoplasm viscosity and, as a result, cell stability [35-37].

**2. Chemical composition of a mature leaf** (% of dry weight) **of Colchis tea plant [*Camellia sinensis* (L.) O. Kuntze] under root application of calcium during water stress periods** ($X \pm x$, microplot field experiment, the city of Sochi, 2014-2015)

| Variant | N | $P_2O_5$ | $K_2O$ | CaO | MgO |
|---|---|---|---|---|---|
| | | August 4 | | | |
| Control | 3.37±0.01 | 0.66±0.02 | 2.29±0.02 | 0.90±0.01 | 0.72±0.02 |
| Ca | 3.44±0.02 | 0.69±0.02 | 2.05±0.01 | 0.90±0.02 | 0.60±0.01 |
| | | August 27 | | | |
| Control | 2.98±0.01 | 0.72±0.01 | 2.12±0.02 | 0.80±0.01 | 0.72±0.03 |
| Ca | 2.24±0.03 | 0.70±0.01 | 2.61±0.05 | 1.20±0.03 | 0.48±0.01 |

Thus, root application of calcium led to an increased resistance of tea plants to insufficient water supply. This was manifested by higher catalase activity of leaves (+10-19 ml $O_2$/g per 3 min), a decreased water loss (by 20 % in average) and pH of the cell sap, and an increased yield of green tea leaves (+2000-3000 kg/ha on average). These effects were due to changes in cation composition of soil adsorption complex (toward 1.5-3-fold increase in calcium content at unchanged potassium content) and increase in potassium and calcium uptake by plants as influenced by calcium fertilizers. Therefore, these fertilizers ensured resistance to oxidative stress and contributed to further post-stress plant recovery via changes in chemical composition of plants and, as a result, in intracellular concentration of elements.

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## Science Events

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# PHOTOSYNTHETIC APPARATUS OF POTATO PLANTS (*Solanum tuberosum* L.) GROWN in vitro AS INFLUENCED BY DIFFERENT SPECTRAL COMPOSITION OF LED RADIATION

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## Abstract

In vitro reproduction is an important stage in seed potato propagation. Various radiation spectra can be used to regulate in vitro the growth and morphogenesis of potato seedlings (*Solanum tuberosum* L.). We studied the effect of light emitting diodes (LEDs) light sources, which differ in the spectral composition of the radiation, on the growth processes and functional parameters of the photosynthetic apparatus in potato plants (variety Agria) grown in vitro. The red LEDs with $\lambda_m$ = 635 nm and the half-width of the emission band (HW) of 45 nm, blue LEDs with $\lambda_m$ = 463 nm (HW = 23 nm), green LEDs with $\lambda_m$ = 521 nm (HW = 38 nm), and white LEDs (400-730 nm) with an intensity of light equal to 60-65 micromol photons $\cdot m^{-2} \cdot s^{-1}$ at plant level, and white fluorescent lamps (OSRAM AG, Germany) of the same light intensity were used. On day 28 the growth parameters, $CO_2$ gas exchange rate and parameters of variable chlorophyll fluorescence were measured. The highest biomass accumulation was observed during irradiation of plants with fluorescent lamps. A smaller plant biomass accumulation was observed when plants were irradiated with white LEDs. Blue LEDs was shown to reduce the accumulation of plant biomass by 49.5 % compared to the white LEDs. Irradiation with green and red LFDs led to decrease biomass accumulation by 75.6 and 67.5 %, respectively. The observed changes in the accumulation of dry plant biomass for different spectral ranges are associated with a higher activity of the photosynthetic apparatus of the plants grown under irradiation with blue and white LEDs. The rate of photosynthesis (per unit of the leaf surface) in these plants was higher than in those grown under green or red LEDs. The effective quantum yield of PS 2 in all studied plants had small changes in the range of 0.47 to 0.53, but higher values were observed in the plants grown under fluorescent lamps and white and green LEDs. Electron transport rate (ETR) and non-photochemical quenching (NPQ) were changed in similar manner under different growth conditions. Curve analysis showed that in the conditions when the input of $CO_2$ was not limited the rate of photosynthesis decreased depending on lighters as follows: fluorescent lamps > white LEDs > red LEDs > blue LEDs > green LEDs. Maximal rate of carboxylation and greater efficiency of the reaction were observed when plants were exposed to fluorescent light and white LEDs. The irradiation of plants with blue, red and, especially, the green LEDs led to a decrease in carboxylation rate to 77.9, 67.9 and 11.1 % of the maximum values. Efficiency of carboxylation in plants grown under red and green LEDs sharply decreased to 37.5 % and 6.7 % of the maximum values. Electron transport rate when using fluorescent lamps, white, red, blue and green LEDs was equal to 100, 97.3, 75.1, 68.0 and 20.8 %, respectively. At the same time there has been a decrease in the utilization rate of triosophosphates to a value of 88.9, 48.7, 28.2 and 9.4 %, respectively, compared to 100 % under fluorescent lamps. Thus, at low levels of light intensity we did not observe significant changes in the activity of both light and dark photosynthetic reactions and the accumulation of plant dry matter. These results allow us to understand the role of separate regions of visible light on the functioning of the photosynthetic apparatus, and more effectively to use LEDs for plant cultivation.

Keywords: potato plants, *Solanum tuberosum* L., growth, photosynthesis, light-emitting diodes, LED, photoreceptors.

In vitro reproduction is an important stage in producing revitalized potato planting material. Available data indicate that the different radiation spectrum can be used to regulate in vitro the growth and morphogenesis in potato seedlings (*Solanum tuberosum* L.) [1-4]. For example, when irradiated with fluorescent lamps (red light, maximum at $\lambda$ = 660 nm), seedlings had small leaves and weak sprouts [2]. When illuminated with blue light from fluorescent lamps (maximum at $\lambda$ = 480 nm), the plants produced well-developed leaves. Red-enriched light led to an increase in leaf surface [5]. The combination of red and blue light emitting diodes (LEDs) contributed to the growth and development of potato seedlings in a laboratory assessment [6].

It should be noted that known works devoted to this subject mostly dealt with the study of a combined effect of red and blue light spectra on the growth and metabolic processes in leaves [7-9]. Certain addition of other spectral bands, in particular in the green (GSB) region, increases the effectiveness of the sources of exposure being used [7, 10]. The positive effect on the photosynthetic apparatus and production process was also shown when using additional irradiation of plants with a light at a maximum around $\lambda$ = 630 nm [11, 12]. In this case, the proportion of the active form of phytochrome B in the pool of phytochromes is changing, which may lead to increased photosynthetic activity in plants [13, 14].

Despite the identified patterns of the LED irradiation influence on the growth processes and photosynthetic apparatus [15-17], the impact of certain regions of the spectrum on the activity of light and dark reactions in photosynthesis in intact leaves still remain poorly investigated.

In this paper, we studied for the first time the association between light and dark phases of photosynthesis and in vitro growth in potato seedlings when exposed to mono- and polychromatic light.

The aim of the study was to investigate the effect of the spectral composition of light on plant development and functional characteristics of the photosynthetic apparatus during in vitro reproduction of potato plants.

*Technique.* In a laminar box, the upper parts of potato plants (Agria variety) were cut and planted in sterile test tubes containing the Murashige and Skoog medium (Murashige T., Skoog F., 1962) for rooting to produce in vitro culture. The tubes were then placed in a phytotron, where they remained under strictly controlled conditions (18-20 °C, 16-hour photoperiod). .

LEDs (OOO Focus, Russia) of red (RS, $\lambda$ = 635 nm) with a half-width of the emission band (HW) 45 nm, blue (BS, $\lambda$ = 463 nm, HW 23 nm), green (GS, $\lambda$ = 521 nm, HW 38 nm) and white light (WS, $\lambda$ = 400-730 nm), with a light intensity equal to 60-65 micromol photons $\cdot m^{-2} \cdot s^{-1}$ at plant level, were used for illumination. Some of the plants were placed under white fluorescent lamps (OSRAM AG, Germany) at the same light intensity.

The leaf area and plant height were measured at day 28. To account for the dry biomass, 6-8 plants were sampled in each test option, then the aboveground and underground parts were separated, fixed at 105 °C, dried at 70 °C, and weighed.

The $CO_2$ gas exchange rate was determined using a portable infrared gas analyzer LCPro + (ADC BioScientific Ltd, UK). The curves of $CO_2$ gas exchange in leaves depending on the light intensity and carbon dioxide concentration in the air were obtained and analyzed as described [15].

The chlorophyll fluorescence was measured on the leaves from 2-3 upper tiers, using a pulse-amplitude modulated fluorometer (JUNIOR-PAM, Heinz Walz GmbH, Germany). Fluorescence parameters were recorded after 20 minutes of dark adaptation. The effective quantum yield of the PS II (photosys-

tem II), the relative electron transport rate (ETR), and non-photochemical quenching (NPQ) were determined [18].

Statistical analysis was performed using Statistica 10 software (Statsoft Inc., USA). In all cases, P was calculated for a significance level of 0.05. The tables and diagrams provide arithmetic means with a standard error ($X \pm x$).

*Results.* One of our study objectives was to monitor the growth processes and photosynthetic rate, as well as to analyze the activity of the light and dark reactions in the photosynthetic apparatus when rooting the potato cuttings depending on the spectral composition of the light emitting diode illuminators.

**1. Dry weight (g) of organs in potato (*Solanum tuberosum* L.) Agria variety microplants in vitro at different illumination spectra ($X \pm x$)**

| Lamp type, light spectral composition | Leaf | Stem | Root | Plant |
|---|---|---|---|---|
| White fluorescent lamps (control) | 0.55±0.10 | 0.25±0.02 | 0.15±0.05 | 0.94±0.16 |
| LED lamps: | | | | |
| white (λ = 400-730 nm) | 0.52±0.03 | 0.28±0.03 | 0.06±0.02* | 0.86±0.06 |
| blue (λ = 463 nm) | 0.29±0.09* | 0.21±0.03 | 0.02±0.01* | 0.52±0.13* |
| green (λ = 521 nm) | 0.16±0.06* | 0.10±0.04* | 0.04±0.02* | 0.21±0.07* |
| red (λ = 635 nm) | 0.13±0.02* | 0.14±0.01* | 0.02±0.00* | 0.28±0.04* |

N o t e. LED — light emitting diode. These are mean values with a standard error from 6-8 plants.
* Differences vs. control are significant ($P < 0.05$).

The spectral composition of light significantly influenced the growth and developmental processes in plants (Table 1). Thus, under fluorescent lights, the growth rate, estimated by the production of the above-ground biomass, was the highest. A somewhat lesser biomass accumulation was observed with the irradiation from white LEDs. The effect of blue light was in the reduced biomass in plants by 49.5 % vs. a test where white LEDs were used. The irradiation from GS LEDs and RS LEDs resulted in the decreased accumulation of biomass by 75.6 and 67.5 %, respectively.

**2. Growth of potato (*Solanum tuberosum* L.) Agria variety microplants in vitro at different illumination spectra ($X \pm x$)**

| Lamp type, light spectral composition | Stem height, cm | Biomass (roots + stems)/leaves ratio | Number of internodes |
|---|---|---|---|
| White fluorescent lamps (control) | 16.30±1.83 | 0.72 | 8.20±0.55 |
| LED lamps: | | | |
| white (λ = 400-730 nm) | 13.78±0.99 | 0.65 | 9.00±0.37 |
| blue (λ = 463 nm) | 19.46±1.18 | 0.79 | 9.00±0.30 |
| green (λ = 521 nm) | 24.77±1.53* | 0.88 | 9.30±0.33 |
| red (λ = 635 nm) | 32.79±1.52* | 1.23 | 10.10±0.31 |

N o t e. LED — light emitting diode. These are mean values with a standard error from 6-8 plants.
* Differences vs. control are significant ($P < 0.05$).

The uneven distribution of dry biomass in plant organs depending on the type of illuminator and the spectral composition of light should also be noted (Table 2). The ratio of the weight of stems and roots to the weight of leaves showed that its high accumulation in plants under fluorescent lamps and white LEDs was due to a higher gain of dry matter in leaves vs. roots and stem. The irradiation with RS LEDs led to a greater accumulation of dry biomass in the attracting organs, i.e. the stem and the roots.

The revealed changes in the accumulation of dry matter at various spectral regimes of irradiation were associated with a higher activity of the photosynthetic apparatus when growing plants under white and blue LEDs (Table 3). The photosynthetic rate per unit of leaf area in these tests was higher than in those where green or red LEDs were used. For plants grown under fluorescent illumination as well as white LEDs, high values of stomatal conductance and transpiration rate were also observed.

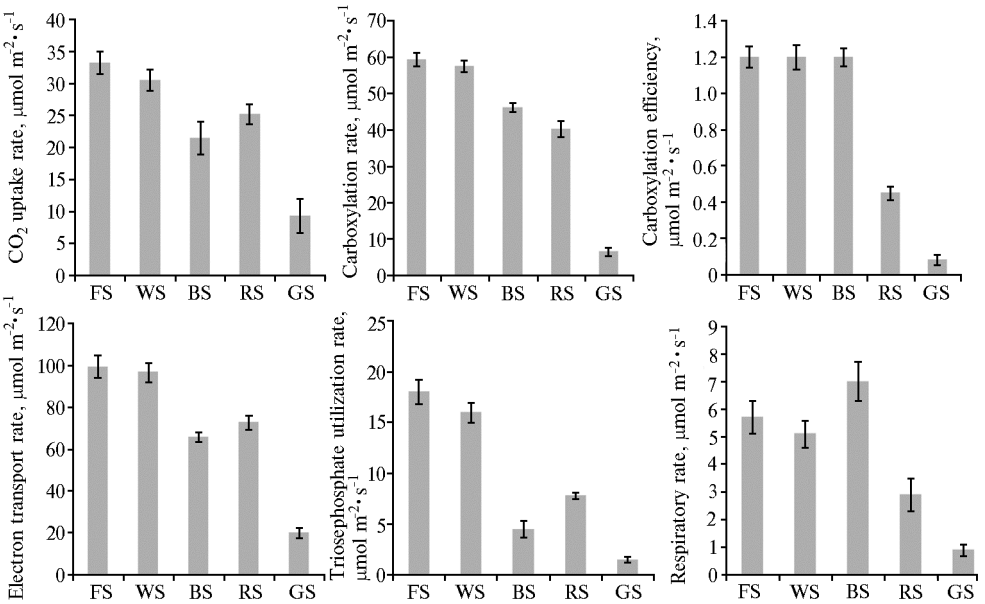
The evaluation of the photosynthetic apparatus function demonstrated

that the effective quantum yield of PS II in the studied plants varied only slightly (ranging from 0.47 to 0.53), while higher values were observed in the test with fluorescent lamps and white and green LEDs. The electron transport rate (ETR) and non-photochemical quenching (NPQ) were changed in a similar way, depending on the plant growth conditions. As a result, at low light intensity the low photosynthetic rate in case of RS LEDs and GS LEDs is primarily related to the activity of the dark reactions in photosynthesis and poor stomatal conductance.

**3. Parameters of the photosynthetic function and transpiration in potato (*Solanum tuberosum* L.) Agria variety microplants in vitro at different illumination spectra ($X \pm x$)**

| Parameter | Spectral regime | | | | |
|---|---|---|---|---|---|
| | FS | WS LED | BS LED | RS LED | GS LED |
| Photosynthetic rate, μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ | 9.6±0.8 | 9.0±0.8 | 7.8±0.7 | 5.7±0.5* | 4.7±0.5* |
| Transpiration rate, μmol $H_2O \cdot m^{-2} \cdot s^{-1}$ | 4.9±0.2 | 4.7±0.2 | 4.6±0.2 | 2.6±0.1* | 3.1±0.2* |
| Water-use efficiency, μmol $CO_2$/mmol $H_2O$ | 2.0±0.1 | 1.9±0.2 | 2.2±0.1 | 2.2±0.1 | 1.5±0.1* |
| Stomatal conductance, mol $H_2O \cdot m^{-2}$ | 0.41±0.02 | 0.38±0.02 | 0.36±0.03 | 0.14±0.02* | 0.17±0.02* |
| Y (II) | 0.51±0.03 | 0.53±0.02 | 0.49±0.03 | 0.47±0.02 | 0.53±0.02 |
| NPQ | 0.51±0.03 | 0.53±0.03 | 0.49±0.02 | 0.47±0.02 | 0.53±0.03 |
| ETR | 61.2±1.6 | 63.2±2.1 | 58.2±1.2 | 56.3±1.7 | 63.3±2.0 |

N o t e. FS — fluorescent lamps (control); LED — light-emitting diode lamps (WS LED — white light, λ = 400-730 nm; BS LED — blue light, λ = 463 nm; RS LED — red light, λ = 635 nm; GS LED — green light, λ = 521 nm); Y (II) — the effective quantum yield of photosystem II, RU; NPQ — non-photochemical quenching, RU; ETR — relative electron transport rate. These are mean values with a standard error from 6-8 plants.
* Differences vs. control are significant ($P < 0.05$).




**Rates of light and dark reactions of photosynthesis in potato (*Solanum tuberosum* L.) Agria variety microplants in vitro at different illumination spectra.** FS — fluorescent lamps, WS — white light-emitting diode lamps (LED) (λ = 400-730 nm), BS — blue LED (λ = 463 nm), RS — red LED (λ = 635 nm), GS — green LED (λ = 521 nm). A model of G.D. Farquhar et al. (1980) was used for approximation of carbon dioxide curves of $CO_2$-exchange.

To determine the potential activity of light and dark reactions of photosynthesis, carbon dioxide curves were constructed. Their analysis using a biochemical model [19] showed that when the photosynthetic rate is not limited by $CO_2$ level, the absorption of carbon dioxide by leaves decreases in a row of fluorescent lamps > white LEDs > red LEDs > blue LEDs > green LEDs (Fig.).

The maximum carboxylation rate and a greater efficiency of the reaction were observed when the plants were exposed to fluorescent light and white LED light. In the tests with BS LED, RS LED and especially GS LED, the carboxylation rate decreased down to values constituting 77.9, 67.9 and 11.1 % of that obtained under the fluorescent lights. The carboxylation efficiency sharply decreased after exposure to RS LEDs and GS LEDs, such as down to 37.5 % and 6.7 % of the maximum values, respectively. The electron transport rate under irradiation with fluorescent lights was the greatest, and it amounted to 97.3; 75.1; 68.0 and 20.8 % of this parameter in tests with WS LED, RS LED, BS LED and GS LED, respectively. There was a concurrent decrease in the rate of triose phosphate utilization, such as to 88.9; 48.7; 28.2 and 9.4 %, respectively, of the value observed under fluorescent lighting (100 %).

The results obtained indicate that different spectral compositions of light may differently affect the activity of the light and dark stages in photosynthesis. In natural $CO_2$ concentrations, under irradiation from LEDs, emitting in different spectral regions, the rate of initial photosynthetic processes varied insignificantly. At that, the plants modified functional activity of their photosynthetic apparatus. With elevated $CO_2$ concentrations, when the restriction of the photosynthetic rate due to activity of the dark processes is reduced, the maximum carboxylation rate and efficiency changed only slightly under irradiation with fluorescent lamps, white and blue LEDs, but decreased significantly with RS LEDs and GS LEDs. As a result, the data, obtained using the model, support the conclusion about the contribution of reduced stomatal conductance to decreases in the photosynthetic rate when the plants are exposed to RS LED and, especially, to GS LED, which is due to inhibition of the $CO_2$ flow to the centers of carboxylation (Table 3). Along with the rate of light and dark reactions of photosynthesis, the respiration was also affected by the spectral composition of the irradiation. The highest respiration rate was observed when plants were illuminated by BS LEDs vs. other test options. The exposure to RS LED led to a decrease in the intensity of gas exchange.

Our data on the effect of different sources of illumination on the plants during in vitro propagation, indicate that the activity of light reactions of photosynthesis does not significantly change at a low light intensity, irrespective of spectral composition and type of illuminator (fluorescent lamps or LEDs), and the differences observed in the accumulation of dry matter by plants are mediated by the processes associated with the activity of the dark phase reactions of photosynthesis (the rate and efficiency of carboxylation, the rate of triose phosphate utilization), as well as with a decrease in stomatal conductance. Moreover, there were significant differences in gas exchange, resulting in a modified relation between respiration rate and photosynthesis.

The changes in the activity of the photosynthetic apparatus are not the only plant response to different spectral composition of light [20-22]. The literature suggests an important role of photoreceptors in the regulation of plant growth and morphogenesis, as well as in the activity of the photosynthetic apparatus [23-25]. Regulatory photoreceptors in different light bandwidths play a major role in the manifestation of the light effects [26-28]. The spectral composition of light had no significant effect on the initial growth processes, however, the red light contributed to the intensive development of axial organs, probably, by means of stimulating the red light photoreceptor, i.e. phytochrome. In contrast, in case of exposure to blue light, the plant growth was inhibited, which could be due to the effect of cryptochrome. The effect of light is also manifested in the changed hormonal status of plants (the content and ratio of gibberellins and auxins) and in the regulation of the activity of physiological processes [22].

Therefore, the study of the photosynthetic function and growth in vitro in potato revealed the complexity of the changes in the activity of the individual processes of light and dark stages in photosynthesis depending on the type of illuminator and the spectral composition of light. At low intensity of the luminous flux of various spectral compositions, the changes in the dry matter accumulation by plants are related to different activity of reactions in the dark phase of photosynthesis. Our results allow to approach to the clarification of the role of individual monochromatic LED illuminators in the photosynthetic apparatus functioning, so that to specifically use LED illuminators when growing plants.

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# para-AMINOBENZOIC ACID STIMULATES SEED GERMINATION PLANT GROWTH, DEVELOPMENT, PHOTOSYNTHESIS AND NITROGEN ASSIMILATION IN THE AMARANTH (*Amaranthus* L.)

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Abstract

Currently protective, reparative, anti-mutagenic, antioxidant properties of para-aminobenzoic acid (pABA) are described. The huge number of the facts is saved up concerning its efficiency on all elements of yield structure of different plants species and household purposes. However, there is almost no information about the effect of pABA on amaranth plants in the world literature. Vegetable amaranth is a valuable food culture, promising to Central Russia. Therefore, we are especially interested in ways to facilitate its introduction, increase productivity, and nutritional value. To this end, it is possible to use pABA as environmentally friendly biologically active natural compound with a broad spectrum of action. In this work, we first investigated the effect of pABA on seed germination, and growth, development and productivity of vegetable amaranth *Amaranthus caudatus* L. variety K173 (K173) and *A. cruentus* L. variety K185 (K185) of grown plants as well as on light-dependent processes of photosynthesis and nitrogen assimilation in the leaves. To assess the characteristics and level of pABA activity the experimental results concerning influence of a synthetic citokinin 6-benzilaminopurin (6-BAP) on the same parameters are given. A dependence of amaranth seed germination on pABK $10^{-9}$ M to $10^{-4}$ M concentration used for treatment was found. The seed germination in both varieties was 23±5 % higher at $10^{-6}$ M pABK, whereas $10^{-4}$ M pABK decreased it by 22±4 %, and other concentrations had no significant effect. In K173 the 15-day seedlings exceeded the control by 10±5 % in height, by 76±6 % in weight, and by 133±17 % in root length at the latent growth stage. Parameters of K185 plants were slightly higher. The acceleration of plant development may contribute to survival and adaptation. Stimulating effect of pABA on K173 and K185 plant growth in height is maintained for the life of plants, except initiation of generative organs (days 60-80). A significant increase (30-85 %) in plant weight compared to control was identified at all stages of ontogenesis. Productivity of leaf biomass per plant in both varieties on day 115 was on average 50±11 % more than in the control. In the leaves of 45-day plants of K173 grown from treated seed, the activity of nitrate reductase increased by 37±4 % and the total protein level was 10±3 % higher when compared to control, thus improving nutritional value of the culture. The rate of photophosphorylation in chloroplasts was shown to be 27±6 % higher, and the rate of electron transport was 32±6 % higher in comparison with the control that led to an increase in leaf net photosynthesis by 22±7 %. These were no different from 6-BAP effects. The pABA influence on the seed germination and plant biometric parameters in K173 and K185 was comparable to the action of 6-BAP. pABK influenced nitrate reductase activity, protein level, chlorophyll content, and photophosphorylation rate in leaves of K173 plants much weaker, than 6-BAP, and differed from it. The pABA properties as a plant hormone are discussed, and the use is recommended for the vegetable amaranth cultivation.

Keywords: amaranth, para-aminobenzoic acid, plant hormones, seed germination, plants growth and development, latent growth, adaptation, productivity, chloroplasts, photophosphorylation, electron transport, protein content, nitrogen assimilation, nitrate reductase

The first reports on the stimulatory action of p-aminobenzoic acid (pABA) on plants were made many decades ago [1]. Up to date, its reparative [2], protective [3], anti-mutagenic [4], antioxidant [5], and phytohormone [6] properties have been established. Such a variety of effects is explained by the fact that pABA is a folate component [7] which is an essential cofactor of one-carbon unit transfer reactions [8]. Therefore, a residue of pABA is involved in many key processes, such as biosynthesis of purines, pyrimidines, pantothenate,

synthesis of S-adenosyl-1-methionine (SAM), formyl-methionine-tRNA, deoxyribonucleotides, as well as in transformation of amino acids [9]. The important role of pABA as a folate component in the regulation of the activity of DNA polymerase, DNAase and RNAase [10, 11], in transcription [12], in promoting phosphorylation, oxidative phosphorylation, photorespiration [8], etc. has been demonstrated. However, there are some new facts that do not fall into this pattern. This is true, for example, for the antioxidant [13] and some of the protective properties [14] identified in the pABA itself. It has been found recently a direct involvement of pABA in the regulation of activity of an oxidative stress-related enzyme, such as peroxidase [15]. The ability of pABA to induce the acquired systemic disease-resistance in plants was demonstrated [16]. It could well be the case that hormone-like properties of pABA are not limited just to its contribution to the folate regulation of synthesis of purines [8], while may be related to its molecule features [17].

A great body of factual evidence has been accumulated about the beneficial effects of pABA on all structural components of the yield for the plants of different species and economic designation, such as cereals (barley, oats, corn, wheat), legumes (peas, lupine), vegetables (cucumbers, tomatoes), cotton, grapes, etc. [1, 2, 5, 6, 18-20]. In addition, the pre-planting treatment of seeds was mainly applied. Despite the abundance of such information, we have found only a single report on the use of pABA in amaranth [21], where it was used «for phenotypic correction of morphological characters». The weight of seeds on the plant was increased following the spraying of the amaranth inflorescences. The most effective concentration of pABA was 0.02 % ($1.5\times10^{-3}$ M). We suggested that pABA may also have regulatory effects on amaranth plants if seed treatment would be used.

Our interest in vegetable amaranth stems from the fact that this important food crop with its high content of a well-balanced protein is very promising for the Central Russia region [22]. To facilitate its introduction, increase the productivity and nutritional value, it seems possible to use pABA, being a biologically active compound with a broad spectrum of action and an environmentally friendly substance due to its natural origin. The results of such use of pABA could replenish gap in evidence on its effects on photosynthetic and assimilatory processes.

Our aim was to investigate the action of p-aminobenzoic acid (pABA) on seeds of vegetable amaranth belonging to two cultivars and varieties and plants grown from them in order to evaluate the possibility of using this substance for the amaranth cultivation in Central Russia. The objectives were to determine the growth parameters in plants of different varieties throughout their ontogeny, as well as to assess the activity of nitrogen assimilation and photosynthetic processes based on the parameters of the light-dependent stage of photosynthesis in one of the varieties. The experiments, aimed to assess the magnitude and nature of the pABA activity, involved a comparison with the effects of a synthetic cytokinin 6-benzylaminopurine (6-BAP).

*Techniques.* In this paper, we used the seeds of vegetable amaranth, such as *Amaranthus caudatus* L., the K173 variety (K173) and *A. cruentus* L., the K185 variety (K185), obtained from the All-Russian Research Institute of Vegetable Breeding and Seed Production (Moscow Province, Russia).

To determine the effect of pABA and 6-BAP (crystalline preparative forms, Sigma-Aldrich Corp., USA) on the germination, seeds of amaranth were soaked in aqueous solutions of the preparations (from $10^{-9}$ to $10^{-4}$ M) for 24 h. Seeds were then dried in a weak stream of air at room temperature. Seeds soaked in the distilled water were used as a control. The germination was determined by a percentage of sprouted seeds from a total number of seeds after their incubation on moist filter paper in Petri dishes for 72 hours at 24 °C.

To examine the growth and biochemical parameters, the plants were derived from seeds treated with 1 μM solutions of pABA or 6-BAP (test) or with distilled water (control) as described above. The germinated seeds were planted in pots filled with sand (10 calibrated seedlings per pot, with 3 pots per each variant of the experiment), and plants were grown at 24 °C, with a 14-hour photoperiod and at the illumination of 150 $W \cdot m^{-2}$; the Knopp growth medium was used for watering. Biometric parameters were taken every 15 days up to the harvest date (Day 115), and the productivity was assessed by biomass gain. The chlorophyll content was determined in the leaves of 45-day-old plants [23]; photochemical activity of chloroplasts, isolated [24] from these leaves, was evaluated based on the electron transport rates [25] and phosphorylation [26]. The activity of nitrate reductase (NR), the amounts of nitrite [27], the content of nitrogen [28] and total protein levels [29] were measured in the leaves of plants aged from day 15 to day 45. Photosynthetic productivity (PP, $g \cdot m^{-2} \cdot day^{-1}$) was calculated over the interval between day 45 to day 55 by Nichiporovich method [30].

The article shows the results of one representative experiment out of five. Biometric parameters were determined in 30 plants. Biochemical analyzes were arranged in 3 repetitions. The tables and diagrams provide arithmetic means with a standard error. The significance of differences was assessed by the Student's *t*-test at P = 0.95.

*Results.* The germination of the amaranth seeds depended on the concentration of the pABA and 6-BAP solutions used for treatment (Fig. 1). In untreated seeds, it was 70 %. The exposure to pABA resulted in its significant increase by 23±5 % at a concentration of $10^{-6}$ M or decrease by 22±6 % at $10^{-4}$ M. The effect of 6-BAP was similar. As $10^{-6}$ M (1 μM) appeared to be the optimal concentration of the solutions for germination, it was then used in the experiments. Data on seed germination for K185 and K173 almost did not differ.

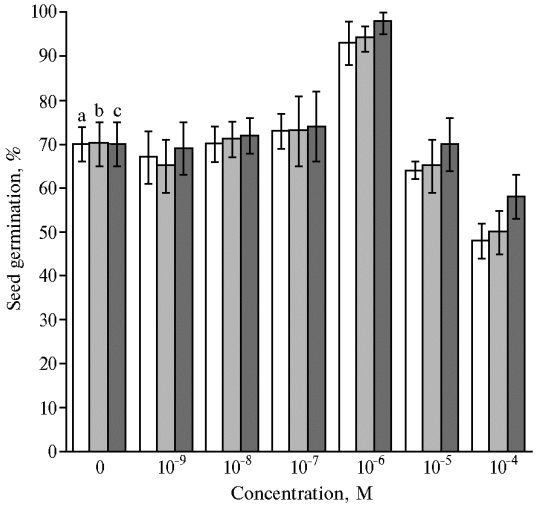


**Fig. 1. Germination of seeds in vegetable amaranth *Amaranthus caudatus* L. variety K173** (a), **and *A. cruentus* L. variety K185** (b), **treated with p-aminobenzoic acid, and in *A. caudatus* L. variety K173, treated with 6-benzylaminopurine** (c) (*X*±*x*, lab experiment).

We found that after seed pre-treatment with 1 μM pABA the 15-day-old K173 and K185 seedlings were superior to control — in height by 10±5 % (for 6-BAP by 22±4 %) and in weight by 76±6 % (for 6-BAP by 129±8 %) (Fig. 2). Usually, during this period of development, the above-ground parts stunt the growth (so-called latent phase). The presented data show that the seedlings have passed the mentioned phase under the influence of both stimulants, however, the effect of 6-BAP was stronger. It should be noted that a significant acceleration of root growth was observed concurrently. The length of the main root in control plants averaged to 1.2±0.1 cm, while after exposure to pABA it was 2.8±0.3 cm, and after 6-BAP 3.3±0.3 cm, or 233±10 % and 275±9 % respectively, as compared to control. The effect of 6-BAP on all parameters in the 15-day-old seedlings was much stronger than that of pABA. The effects from the stimulants on the parameters of the K185 seedlings were similar to those in the

K173 seedlings.

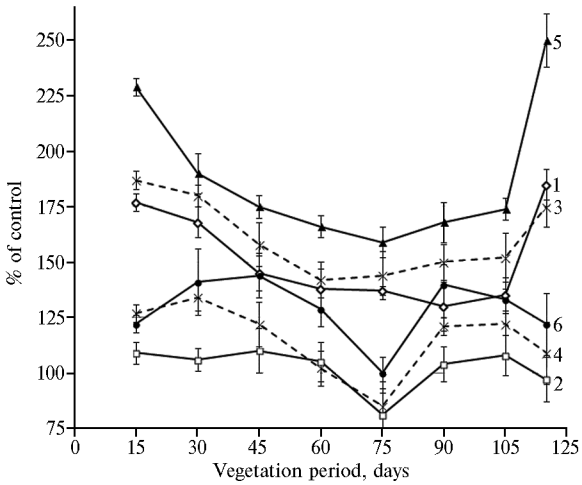


**Fig. 2. Plant growth in two varieties of vegetable amaranth after seed pre-treatment with p-aminobenzoic acid** (pABA) **and in one variety after exposure to 6-benzylaminopurine** (6-BAP): 1, 2 — *Amaranthus caudatus* L., K173; 3, 4, 5, 6 — *A. cruentus* L., K185; 1, 3, 5 — the weight, 2, 4, 6 — the height; 1, 2, 3, 4 — pABA, 5, 6 — 6-BAP (1 μM solutions) ($X \pm x$, lab experiment).

The stimulating effect of pABA on height was maintained throughout the life of plants, except for the period of the initiation of generative organs (60-80 days) (Fig. 2). A significant weight increase in the whole plants was observed at all stages of ontogenesis as compared to the control. The maximum effect was observed at harvesting (day 115), when the weight of the above-ground parts of the plants, grown from seeds treated with pABA, exceeded that in the control by 85±10 %. The nature of the pABA and 6-BAP effects on the plant height and weight in ontogeny coincided (see Fig. 2), but the degree of influence of the latter was higher. Trends in growth parameters for the second variety, K185, when using growth regulators, fully reproduced their pattern for the K173 variety, however, absolute values were slightly higher.

The main productivity indicator for the vegetable forms of amaranth is the output of the leaf biomass on the plants. On day 115, its values for the K173 variety under the influence of pABA averaged to 130±10 g in control, and to 195±14 g in the test that is 50±11 % higher as compared to control. This parameter for K185 exceeded that in control by 68±9 % (160±14 g against 95±6 g in control). The weight per 1 $m^2$ of leaves for the K173 plants, when seed pre-treated, was higher than in the control by 48±5 % (770±36 g against 520±26 g), and for the K185 plants by 40±8 %. 6-BAP increased the output of leaf biomass by 62±9 %, and the weight per 1 $m^2$ of leaves by 61±8 %. These data suggest that there was little or no significant difference between the influence of pABA and 6-benzyladenine on the leaf mass productivity in both varieties.

As the productivity of plants is largely determined by the activity of the photosynthetic apparatus, we have studied the photochemical activity of isolated chloroplasts. In the chloroplasts from leaves of the K173 45-day-old plants, we revealed an increase, compared to the control, in both electron transport rates and ATP synthesis rates if the pre-planting seed treatment with pABA and 6-BAP was used (Table 1). Meanwhile, the effect of 6-BAP on the ATP production rate was significantly stronger than that of pABA, with almost equal effects of both on the electron transport. The difference in the effect between two bioactive agents consisted in the fact that exposure to pABA resulted in the unchanged content of chlorophyll in the leaves, while under the influence of 6-BAP it greatly increased. Despite that, the values of PP in the leaves from growth day 45 to day 55 in the tests with pABA and 6-BAP did not differ significantly: PP values decreased with pABA by 22±7 % vs. control (from 2.3±0.15 to 2.8±0.19 $g \cdot m^{-2} \cdot day^{-1}$), and with 6-BAP by 30±5 % (from 2.3±0.15 to 3.0±0.15 $g \cdot m^{-2} \cdot day^{-1}$).

**1. Photosynthetic activity in isolated chloroplasts from leaves of 45-day-old amaranth plants (*Amaranthus caudatus* L., K173 variety) after seed pre-treatment with pABA and 6-BAP** ($X \pm x$, lab experiment)

| Option | Chlorophyll content | | Rates of | | | |
|---|---|---|---|---|---|---|
| | | | electron transport | | photophosphorylation | |
| | mg/g dry weight | vs. control, % | μM $K_3[Fe(CN_6)]$ | vs. control, % | μM ATP | vs. control, % |
| Control | 9.9±0.2 | 100 | 110.4±8.0 | 100 | 112.0±2.3 | 100 |
| pABA | 9.9±0.6 | 100±6 | 145.9±7.0 | 132±6 | 142.0±2.0 | 127±6 |
| 6-BAP | 14.4±2.2 | 146±15 | 148.5±15.0 | 135±14 | 209.0±15.0 | 186±13 |

N o t e. pABA — p-aminobenzoic acid, 6-BAP — 6-benzylaminopurine. The rate is calculated per 1 mg of chlorophyll per 1 hour. The concentration of pABA and 6-BAP solutions is 1 μM.

Applying both pABA and 6-BAP (1 μM) led to an increase in the NR activity, compared to the control, during the first 45 days of vegetation, providing the enhanced synthesis of nitrogen-based compounds during the active growth and their consumption for plastic processes (Table 2). However, the nature of the effects induced by these two stimulants was different. In the pABA group the NR activity consistently exceeded that in the control (by an average of 34 %), whereas in the 6-BAP group it increased gradually along with the plant growth. At that, the 6-BAP effect was significantly stronger than that of pABA.

Treatment with both pABA and 6-BAP caused elevated levels of total protein in leaves of the K173 amaranth plants from day 15 to day 45. Neverthelwss, during the growth a downward trend in the total protein levels was observed upon exposure to both bioactive agents, although with 6-BAP the total protein values remained significantly higher at day 45. Under the influence of pABA (unlike 6-BAP), nitrite accumulation by leaves was not observed. Nitrate reductase activity, the percentage of N and total protein levels in plants do not always directly correlate [31]. However, we have established significant stimulating effects of pABA on the nitrogen assimilation and transformation in amaranth plants (see Table 2).

**2. Changes in nitrogen metabolism** (vs. control, %) **in the leaves of amaranth plants (*Amaranthus caudatus* L., K173 variety) after seed pre-treatment with pABA and 6-BAP** ($X \pm x$, lab experiment)

| Option | Plant's age, days | Nitrite | Nitrate reductase activity | Total | |
|---|---|---|---|---|---|
| | | | | nitrogen | protein |
| pABA | 15 | 143±5 | 130±2 | 104.0±0.5 | 128±9 |
| | 30 | 101±2 | 134±2 | 102.0±1.1 | 121±4 |
| | 45 | 108±3 | 137±4 | 102.0±0.7 | 110±6 |
| 6-BAP | 15 | 124±4 | 147±9 | 108.0±0.4 | 142±9 |
| | 30 | 109±2 | 167±12 | 105.0±0.5 | 137±9 |
| | 45 | 122±5 | 185±18 | 106.0±1.2 | 138±14 |

N o t e. pABA — p-aminobenzoic acid, 6-BAP — 6-benzylaminopurine. The concentration of pABA and 6-BAP solutions is 1 μM.

The identified effect of pre-planting seed treatment with pABA on the commercially significant characteristics of amaranth was associated with its impact on the fundamental underlying physiological and biochemical processes. For the first time we demonstrated the promotion of the light-dependent reactions of photosynthesis and nitrogen assimilation rates in the K173 variety by the use of pABA. It should be noted that the nature and magnitude of some studied effects of pABA and 6-BAP varied, therefore, pABA does not act as a cytokinin. The high content of pABA as a folate precursor is likely to stimulate their synthesis and accumulation at the early stages of plant development from a seed, accelerating the physiological and biochemical processes throughout the ontogeny and resulting in the observed effects.

However, another mechanism may also exist. Particularly, there are some scarce evidence about the cytokine [1, 6] and auxin [17, 32] effects of pABA. Alt-

hough this area, in fact, has not been studied, there is no reason to deny that pABA does possess some properties typical for phytohormones, i.e. the ability to express a nuclear response through a system of messengers, as it is described, for example, for cytokinins [33]. There may also exist the interaction with phytohormone receptors [18]. The observed effects of pABA indicate in favor of such an assumption, considering the common features of plant hormones according to the International Plant Growth Substance Association (IPGSA). According to IPGSA, phytohormones are natural organic compounds that impact the physiological processes (growth, differentiation, development of the plant, etc.) at much lower concentrations than nutrients or vitamins [34]. The identified effects can not be determined as typical for any known class of plant hormones, and, for now, there is a lack of available factual data to claim a new class. We agree with the suggestion about hormonal activity of some small molecules [35]. Moreover, we have described the cytokinin-like properties of 4-hydroxyethylphenol alcohol, and made an assumption about its hormonal nature [36]. It was based on a precedent revealed for o-hydroxybenzic (salicylic) acid. Currently, this small molecule along with several of its derivatives are recognized as a special class of plant hormones. It may well be possible that with the buildup of data on small molecules some common characteristics may manifest themselves so that to allow combining them into a new group of compounds with specific phytohormonal properties.

Therefore, pre-planting treatment of seeds of vegetable amaranth with 1 μM aqueous solution of p-aminobenzoic acid (pABA) improves germination and enhances biometric, physiological and biochemical parameters of plants, i.e. accelerates the development of seedlings in the latent phase of growth, increases growth, improves productivity and nutritional value. The effects of pABA and a cytokinin 6-benzylaminopurine are not identical. pABA can be recommended for the use in cultivation of vegetable amaranth in the Nonchernozem belt of Russia

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# SEED ENCAPSULATION IN CHITOSAN AND ITS DERIVATIVES RESTORES LEVELS OF CHLOROPHYLL AND PHOTOSYNTHESIS IN WILT-AFFECTED COTTON (*Gossypium* L., 1753) PLANTS

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## Abstract

Chitosan-based biologicals and chemicals have been proved to possess antiviral, antibacterial and antimycotic activity, and be able to stimulate plant immunity. In field trials (Tashkent region, Uzbekistan, 2015-2016) we first evaluated an impact of seed encapsulation with UzChitan, chitosan ascorbate and $Cu^{2+}$-chelating chitosan synthesized by the authors' method in the Institute of Chemistry and Polymer Physics of the National University of Uzbekistan (NUUz, Tashkent) from waste of silkworm cocoon processing on photosynthetic activity at wilt (*Verticillium dahliae*) artificial infection and in wilt-free (healthy) crops of cotton (*Gossypium* L., 1753) variety Sultan. It was found that wilt-affected control plants (non-treated with chitosan derivatives) had higher specific leaf weight (SLW index), their chlorophyll content was reduced and rates of respiration and apparent photosynthesis were depressed in comparison with healthy control plants. Chlorophyll content in infected control plants was decreased by 27-30 % in terms of mg/g. Meanwhile, all plants from pre-treated seeds contained more chlorophyll compared to control: the pigment content (mg/g of dry matter) was 25.5 and 17.7 % higher when ascorbate and $Cu^{2+}$-chelating chitosan used. Chlorophyll content in leaves did not vary significantly in absence of the pathogen. The apparent photosynthesis rate in control under wilt was reduced by 33 % ($0.161\pm0.027$ against $0.245\pm0.028$ μmol $O_2 \cdot m^{-2} \cdot sec^{-1}$). That corresponds to toxic effect of pathogen which penetrates through roots and goes up into top parts of plants, damages their phloem system, disturbs water transport, and destroys chlorophyll and leaf tissues. Besides, some tendency to respiration rate inhibition was observed for non-treated cotton plants under wilt infection (by 22 % compared to control plants from the healthy field). Net-oxygen production (apparent photosynthesis) rates in the treated groups were higher as compared to control plants under wilt; the best results were shown by ascorbate and $Cu^{2+}$-chelating chitosan (54 and 46 % higher, respectively). Respiration rates did not differ significantly in all groups under wilt. Oxygen balance (OB) index, estimated as ratio between measured oxygen net-production and oxygen consumption rates, which reflects physiological status of plants under biogenic stress (including pathogen fungi), did not change significantly in a healthy environment when chitosan derivatives used. Under wilt, the plants from pre-treated seeds possessed higher OB indices than the corresponding control plant from the same field. Thus, the OB values with ascorbate and $Cu^{2+}$-chelating chitosan were higher compared to control by 36 and 52 %, respectively. Our findings indicate that in case the cotton seeds were pre-treated with chitosan derivatives, the oxygen production, oxygen consumption and OB indices were not significantly different in the pathogen-affected and unaffected plants. Thus we can conclude about a nonspecific resistance of cotton plants to wilt (*Verticillium dahliae*) induced by chitosan derivatives, and reduction of toxic effects of the pathogen which manifests itself in decreasing respiration and photosynthesis rates. Possibly, it is a result of non-specific elicitor activity of chitosan and its derivatives, or their ability, due to natural polycation properties, to stimulate plants to produce specific antifungal metabolites — reactive oxygen species and phytoalexins triggering subsequent defense mechanisms. Currently, using elicitors for plant immunity correction are considered promising as they contribute to formation sustainable agrophytocenosis with high adaptiveness and yield production.

Keywords: chitosan, chitosan derivatives, cotton plant, verticilliose, chlorophyll, oxygen production, respiration, oxygen balance index

Poly-(1-4)-2-amino-2-deoxy-β-D-glucan (chitosan) is a common natural mucopolysaccharide. It can be derived from deacetylation of chitin, which is a component of the cell wall of some fungi, the exoskeleton of crustaceans, insects and worms [1]. Due to the high biological activity, chitin, chitosan and their derivatives are widely used in biomedicine and agriculture [1, 2], in particular, in plant growing to protect plants against pests and diseases, for the biological control of pathogens by activating the action of antagonistic microorganisms, to enhance favorable interactions between the plant and a symbiotic microorganism, for regulating plant growth, and to increase crop yield. Preparations obtained from chitosan and its derivatives are used in the pre-planting treatment of the seeds, they are introduced into the soil, or used to spray plants or harvest [1, 3-6]. Chitosan is safe for humans, farm animals, and the environment since it naturally decomposes with associated formation of the simple monosaccharide D-glucosamine [1, 7].

It is shown that chitosan-based formulations possess antiviral, antibacterial and antimycotic activities, and are able to stimulate the immune system of the plants [1, 3, 5-8]. For example, increased disease resistance has been established in rice, tobacco, yam [2], tomato [9], soybean [10], cotton [11], wheat, barley [2, 12-15], carrots [2, 14], cucumber, and potato [8, 14]. Pre-planting seed treatment and spraying of plants lead to the oppression of grey leaf spot (*Pyricularia grisea*) [1, 16], root rot caused by *Fusarium* spp. [1, 9-11, 13, 14], *Bipolaris* spp. [13, 17], to the suppression of late blight (with *Phythophtora parasitica* and *Phytophthora infestans* being causative agents [Mont.] De Bary) [14], dark brown or net blotch (*Bipolaris sorociniana*, *Drechslera teres*), Septoria spot (*Septoria nodorum*), dusty smut (*Ustilago nuda*), leaf rust (*Puccinia recondita*), leaf mold (*Cladosporium herbarum*), Alternaria spot (*Alternaria tenuis*) [12, 13, 17, 18]. The antimicrobial effect of chitosan oligomers is related to their ability to influence the reproductive mechanism in microorganisms [7] and augment the antibiotic susceptibility. Low molecular weight chitosan is the most active against a number of bacteria, while highly deacetylated chitosans are the most effective antimicrobial agents [1, 2, 7].

Chitosan is assigned to strong elicitors, i.e. substances that induce innate immunity in plants, causing the accumulation of antipathogenic substances by increasing expression of the protective genes [1, 2, 19, 20]. It is considered that chitosan elicitor activity is based on the presence of N-acetylglucosamine residues in its molecule, capable to interact specifically with receptors on the plant cell surface, as well as on their non-specific interaction with external and internal cellular components [1, 2]. Polycationic nature of chitosan enables it to bind to negatively charged cytoplasmic membrane via electrostatic interactions, penetrate into the cytoplasm and the nucleus, interact with nucleic acids by disrupting normal DNA binding to histones and causing breaks of DNA strands [21]. Changes in DNA can become a signal to activate the repair processes and transcription of the protective genes. Chitosan, being a polycation, is also allegedly able to block viral replication by directly interacting with a negatively charged virus RNA [8]. Chitosan and its positively charged derivatives promote the production of antifungal metabolites in plants, in particular reactive oxygen species and phytoalexins [9, 20]. The antiviral effect of chitosan is also associated with the ability to increase the content of reactive oxygen species that can destroy the protein coat or the genomic RNA of the virus [8]. Non-specific action of glucosamine residues underlies the ability of chitosan to inhibit infection regardless of the pathogen [2]. The combined (specific and non-specific) effects of chitosan allow the plant to reliably capture the chitosan signal and activate defensive reactions with different mechanisms of activation and action [2].

Plant yielding capacity and biological productivity are determined by a combination of complex interactions between physiological (photosynthesis, growth and respiration) and biochemical processes, environmental conditions and agricultural methods of cultivation, while the major role in the crop formation is assigned to photosynthesis.

As one of the manifestations of verticullium vilt in plants is leaf damage, their shrinkage and abscission, it seemed interesting for us to study some of the indicators of the leaf blade state, chlorophyll content, respiration rate and apparent photosynthesis in cotton plants affected by wilt, using PlantVital_5030 device, which allows to quite promptly perform instant diagnosis. Such investigations on cotton plant were made for the first time. Here, we have assumed that the indices that characterized important physiological processes, such as photosynthesis and respiration, objectively reflected the physiological state of the plant and, consequently, the efficacy of chitosan preparations for increasing the resistance of plants to this pathogenic fungus.

The objective was to study the effects of chitosan-based products on the photosynthetic activity in wilt affected plants.

*Technique.* The field trials (Tashkent region, Uzbekistan, 2015-2016) were carried out on the Sultan variety of cotton plant. Before planting, the seeds were treated with chitosan derivatives using an encapsulation technique [11]. All the products used in the investigation were synthesized in the Institute of Chemistry and Polymer Physics of the National University of Uzbekistan (NUUz, Tashkent) from the waste of silkworm cocoon processing obtained by the proprietary methodology. The four following plant groups were investigated: group I included control plants (with no pre-planting treatment); groups II, III and IV included plants treated with UzChitan (a mixture of chitosan solution and sodium carboxymethyl cellulose), chitosan ascorbate and $Cu^{2+}$-chelating chitosan complex, respectively. In each group, one part of the plants was grown on artificially infected soil (*Verticillium dahliae*), and the other one on the wilt-free (uncontaminated) soil. In mid of the vegetation season in the active phase of the flowering (late August), top and well illuminated leaves from the 3$^{rd}$ and 4$^{th}$ internodes (counting from the top) were taken at 7:30 am for the investigation.

A conventional weight method (drying at 105 °C) was used to measure water content in the leaves [22], with further calculation of leaf mass per area (LMA) [23].

The amount of a and b chlorophylls were determined spectrophotometrically (Spectroquant NOVA400, Merck, Germany) [24, 25] after rapid homogenization of the leaf cuttings, dried at room temperature, in a porcelain mortar and the extraction using 85 % cold aqueous solution of acetone. The content of chlorophylls (on a wet weight basis, on a dry weight basis, as well as per 1 $cm^2$ of the leaf surface) was calculated by the Roebbelen method.

The rate of oxygen absorption by leaves at night and the rate of oxygen release in the light (apparent photosynthesis) were measured using a PlantVital_5030 apparatus (INNO-Concept GmbH, Germany) and a Clark-type electrochemical sensor (MF 41-INN Sensortechnik Meinsberg) at a temperature of 27 °C. An investigated sample was illuminated by a diode which operated in the red spectrum ($\lambda$ = 635-650 nm). Oxygen balance was calculated as the ratio of the apparent photosynthesis to the rate of respiration at night [26-28].

The data were processed using the Ori-ginPro 7.5 software (OriginLab Corp., USA). The figures and the table show mean values of at least 20 measurements and their standard deviations. The significance of differences between mean values was calculated by single-factor analysis of variance (ANOVA) at a significance level of P = 0.05. The paper discusses the significant differences

that meet $P < 0.05$.

*Results.* Many researchers indicate a significant reduction in the photosynthetic activity in plants affected by pathogenic fungi that may be associated with a decrease in the assimilation surface due to necrosis of the leaf tissues or proliferation of the mycelium, the destruction of chloroplasts, reduction in the chlorophyll content, and abnormal efflux of photosynthetic products because of the phloem damage [29-31]. Meanwhile, the positive effects of chitosan on plant growth and development have been described, in particular the effect on the content of photosynthetic pigments. It has been shown that the introduction of chitosan into the soil in the early stages of development of soybean, beans, tomato, rice, lettuce enabled to enhance the growth of roots and shoots, increase the leaf size and the chlorophyll content, resulting in higher yields [32, 33].

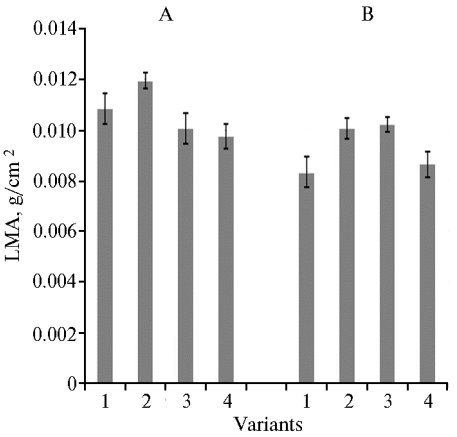


**Fig. 1. Leaf mass per area** (LMA) **in cotton** (***Gossypium*** **L., 1753) variety Sultan under the pre-planting seed treatment using chitosan-based products:** A — artificial inoculation (*Verticillium dahliae*), B — without infection; 1 — control (no treatment), 2 — UzChitan (a mixture of chitosan solution and sodium carboxymethyl cellulose), 3 — chitosan ascorbate, 4 — $Cu^{2+}$-chelating chitosan complex (field trials, Tashkent region, Uzbekistan, 2015-2016).

The ratio of the dry weight of the leaf blade to its area is an indicator, which is sensitive to many factors (lighting, level of water supply, pathogens, etc.). In our experiment, leaves affected with wilt became more dense, rough and heavy, the blade was thickened. Specific leaf mass in the control (10.8 mg/cm$^2$) increased by approximately 30 % compared to that in plants grown without infection (8.3 mg/cm$^2$). On average, there was 10.7 mg of dry matter per 1 cm$^2$ of the leaf blade in plants affected with wilt, which exceeded by 14.5 % the same parameter in uninfected plants (i.e. 9.4 mg/cm$^2$) (Fig. 1). In plants treated with chitosan ascorbate and chelating chitosan complex, this parameter was lower than in the controls with wilt. However, the water content in the leaves of the investigated plants did not differ significantly and averaged to 64.63±2.03 %.

The lower the specific leaf mass, the less photosynthetic products are spent by the plant to form the foliage and the faster the plant is growing and developing [23]. Taking into account that cotton wilt affects the vascular system of plants, violates the water exchange and, eventually, leads to leaf drying, it can be assumed that *Verticillium dahliae* causes an increase in the specific leaf mass mainly due to the slowdown of the efflux of assimilates and their accumulation in the leaf.

Of note, under the use of chitosan ascorbate, dry matter content in the leaves was almost the same in plants grown on the "pure" and infected fields (10.2 and 10.1 mg/cm$^2$, respectively).

Expectedly, the content of chlorophyll pigments in the plants not infected with *Verticillium* was higher (Fig. 2). Under inoculation the total amount of chlorophyll a and b in the control was reduced by 10 % (per cm$^2$ of the leaf surface) or 27-30 % (per gram of dry weight) as compared to control in the absence of infection. On average, the chlorophyll level in the artificially infected plants was lower than in the uninfected ones (by 13 % or 52.45±2.20 vs. 59.31±3.05 μg/cm$^2$, and 27 % or 4.90±0.46 vs. 6.35±0.35 mg/g dry weight). In wilt disease, the chlorophyll content after the pre-treatment with biopolymers

was higher than in the control: by 25.5 % and 17.7 % (per gram of dry weight) for ascorbate or chelating complex, respectively, and by 15.0 % and 16.0 % (per leaf area unit) for UzChitan or ascorbate (see Fig. 2).

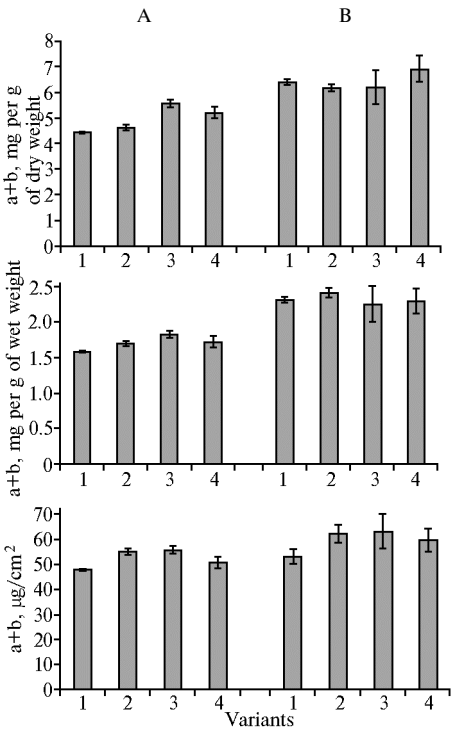


**Fig. 2. The content of chlorophyll a and b in the leaves of cotton (*Gossypium* L., 1753) variety Sultan under the pre-planting seed treatment with chitosan-based products** (calculated per weight and unit area)**:** A — artificial inoculation (*Verticillium dahliae*), B — without infection; 1 — control (no treatment), 2 — UzChitan (a mixture of chitosan solution and sodium carboxymethyl cellulose), 3 — chitosan ascorbate, 4 — $Cu^{2+}$-chelating chitosan complex (field trials, Tashkent region, Uzbekistan, 2015-2016).

Changes in the amount of chlorophyll in non-infected cotton plants were statistically not significant. This suggests that chitosan and its derivatives did not have a direct stimulating effect on the photosynthetic apparatus, and a positive effect in wilt-affected plants was associated with inhibition of the pathogen and improvements in general physiological state of the plant.

We revealed that at wilt infection the rate of photosynthesis decreased by about 33 %, i.e. down to 0.161±0.027 vs. 0.245±0.028 μmol $O_2 \cdot m^{-2} \cdot sec^{-1}$, in the control plants, which were not pretreated with chitosan-based products (see Table). This is related to a toxic effect of the pathogen that penetrates through the roots and moves through the plant vascular system upwards to leaves and the growing point, causing violations in the water exchange, degradation of pigments and leaf withering.

Statistical analysis of the mean measurements of the leaf respiration rate in the control groups showed statistically not significant differences ($P < 0.05$), however, the downward trend of this indicator should be noted at wilt infection (by 22 %) (see Table). The observed decrease in the rate of photosynthesis and respiration may also be a consequence of the closing of stomata resulted from *Verticillium* wilt.

**Absorption and release of $O_2$ by leaves of cotton (*Gossypium* L., 1753) variety Sultan affected by *Verticillium* wilt under the pre-planting seed treatment with chitosan-based products** ($X \pm \sigma$, field trials, Tashkent region, Uzbekistan, 2015-2016)

| Treatment | Rate, μmol $O_2 \cdot m^{-2} \cdot sec^{-1}$ | | Oxygen balance index |
|---|---|---|---|
| | dark respiration | apparent photosynthesis | |
| Artificial infection (*Verticillium dahliae*) | | | |
| Control (no treatment) | –0.148±0.036 | 0.161±0.027 | 1.092±0.209 |
| UzChitan | –0.156±0.061 | 0.221±0.052 | 1.539±0.373 |
| Chitosan ascorbate | –0.166±0.022 | 0.248±0.051 | 1.486±0.138 |
| Chitosan chelating complex | –0.143±0.019 | 0.235±0.032 | 1.660±0.211 |
| Without infection | | | |
| Control (no treatment) | –0.190±0.041 | 0.245±0.028 | 1.331±0.301 |
| UzChitan | –0.159±0.048 | 0.235±0.051 | 1.555±0.368 |
| Chitosan ascorbate | –0.173±0.038 | 0.232±0.077 | 1.354±0.379 |
| Chitosan chelating complex | -0.162±0.023 | 0.253±0.023 | 1.576±0.221 |

When chitosan products were applied for seed pre-planting treatment, the average rate of apparent photosynthesis (net production of oxygen) in the

cotton plants affected by *Verticillium* wilt was higher as compared to that in the untreated control (see Table). The highest results were observed in the plants grown from seeds treated with chitosan ascorbate and chitosan chelating complex, i.e. by 54 % and 46 %, respectively. The difference in the rate of dark respiration under artificial infestation with *Verticillium* in all control and test variants was statistically not significant at the 5 % significance level.

In plants grown without artificial infestation, the observed differences in the rates of apparent photosynthesis and dark respiration were statistically not significant as compared to control. Additionally, with pre-treatment with chitosan and its derivatives, the same parameters under infestation were not significantly different from those in the respective group without infection. It can be concluded that the pre-planting treatment with biopolymers contributed to enhancing the immune status of the plants, and as a result we did not reveal pathological manifestations of the *Verticillium* infection in leaves and changes in the photosynthetic activity.

Previously, it has been shown that the ratio of the photosynthesis rate to respiration rate (oxygen balance index — OBI) can be used to characterize the physiological state of an adult plant under biogenic (nutrient) stress [27, 28]. In the absence of a pathogen (see Table), we have not found statistically significant changes in OBI when compared to control. However, under wilt pathogen infestation, the plants grown from seeds pre-treated with chitosan derivatives had higher OBI values than those without treatment. In particular, the use of chitosan ascorbate and chitosan chelating complex resulted in the increased OBI values (by 36 % and 52 % compared to control, respectively; a significance level of 5 %), reaching the OBI values found in the plants without infection. This may indicate an increase in plant resistance due to treatment with chitosan or its derivatives, and a termination of negative effects of the pathogen which depressed respiration and photosynthesis.

Thus, our results show the efficacy of pre-planting treatment (encapsulation) of cotton seeds with chitosan and its derivatives (chitosan ascorbate and $Cu^{2+}$-chelating chitosan complex) to increase the immunity of cotton plants and develop resistance to *Verticillium dahliae*, the causative agent of wilt. Under wilt infection, the plants demonstrated an increased leaf mass per unit area, reduced content of chlorophyll level, decreased rates of respiration and photosynthesis. When seeds were pre-treated with tested products, the negative effect of the pathogen was not observed, and the content of chlorophyll, rates of photosynthesis and respiration did not differ significantly from those in non-infected plants. The products did not cause statistically significant changes in plants in the absence of *Verticillium* pathogen. These effects may be due to elicitor activity of chitosan and its derivatives and/or stimulation of specific antifungal metabolites — reactive oxygen species and phytoalexins. Chitosan ascorbate and $Cu^{2+}$-chelating chitosan complex demonstrated the highest efficacy.

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# PROTEINS FROM GARLIC *Allium sativum* L. WHICH ARE ACTIVE AGAINST FUNGAL PATHOGENS OF BARLEY, WHEAT AND RICE, AND CAN STIMULATE SEEDS IN GARLIC

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Abstract

Garlic (*Allium sativum* L., family *Amaryllidaceae*) plants are usually used because of bioactive compounds in their leaves and bulbs. Compounds extracted from this plant are often able to protect against some diseases. There is not enough information about infusions garlic extracts that can be used for plant pathogen control. Creating effective biologicals to protect plants against various diseases is an urgent task to improve crop yields. In this work, the bioactive compounds possessing activity against some phytopathogens were isolated from garlic plant bulbs *Allium sativum* L. A complex of lectin-allinase and peptide with molecular weight of 4392 Da was obtained from *A. sativum* bulbs. This complex described in literature before is consisted of allinase enzyme (molecular weight 54 kDa) and mannose-specific garlic lectin (ASA) with molecular weight of 6.4 kDa, and is formed when the bulb tissues are ground. Since, to our knowledge, no data on the biological action of this complex were reported before, we studied its activity against the pathogen of rice blast *Magnaporthe grisea*. This study showed that the lectin-allinaza complex had no suppressive effect on the pathogen spore germination (spore germination both in control and under treatment was at the 80-90 % level), but reduced the number of necrosis on treated leaves of rice (the percentage of uninfected leaves increased from 15 % in control to 75 %). Thus, the complex did not affect *M. grisea* directly, but protected plants upon their infection by this pathogenic fungus. These findings suggest that lectin-allinaza complex isolated from garlic bulbs is able to induce resistance to *M. grisea*, probably by activation of plant defense responses. The ability of obtained peptide not described in literature before to inhibit action of *B. sorokiniana*, the causative agent of Helminthosporium root rot and barley leaf blotch, at concentration corresponding to 10-11 mg protein/ml, was shown on wheat and barley leaves. Since the peptide 4392 Da did not inhibit the growth of *B. sorokiniana* colonies in vitro, it can be assumed that the peptide is capable of activating the protective functions of the plant during the pathogen infection. When evaluating the effect of the peptide 4392 Da on seed germination and growth stimulation in peas, cucumber, mustard, sunflower and garlic, the germination of garlic seeds increased by 13.6 % ($p < 0.01$), while 65.5 % ($p < 0.01$) increase in the length of its stems and a four-fold increase of the root length were observed, but the peptide had no effect on seeds and sprouts of the other crops tested. Thus, the resulting substances do not possess phytotoxicity, and due to its high activity at low concentrations show very low discharge at its application. Moreover, by virtue of its origin they are absolutely harmless for humans and animals and are environmentally safe technology of plant protection, which is especially important in modern agriculture.

Keywords: garlic, *Allium sativum* L., lectin, allinase, rice blast, rice, peptide, helmintospotium, growth stimulation

Leaves and bulbs of garlic *Allium sativum* L., family *Amaryllidaceae*, subfamily *Allioideae*) serve as a source of various biologically active substances for a long time [1-4]. Usually, however, studies of these parts are related to medicinal use [5-9], including issues of thrombocytosis [10] and carcinogenesis [11], while reports of use to counter phytopathogens are rare [12].

Among the means to protect plants from fungal diseases the majority are chemical. These are effective, but have a number of downsides, which include violation of biological balance, accumulation of residue chemicals in agricultural produce, progressing tolerance of pathogenic organisms, high cost of fungicides and catastrophic environmental pollution [13, 14]. Despite new chemical fungicides emerging on the market, the general situation with the protection of plants from disease and increasing productivity does not change in principle. The spread of such dangerous infections, as root rots, cucumber powdery mildew and cucumber false powdery mildew, cereal fusarium and rust, potato late blight, bacterial diseases of vegetable and fruit crops is epiphytotic and leads to a severe decrease in productivity and yield quality. Recently there is a growing interest in agricultural biologicals based on antagonist microorganisms, which increase resistance to stressors, and also to proteins and biologically active low molecular weight compounds. These biologicals are believed to better fit into integrated plant protection protocols, as they are effective, selective and relatively environment and human friendly [15, 16].

The range of preparations capable of inhibiting the development of diseases in plants, preserving or increasing productivity, is sufficiently broad nowadays. The majority of these, however, are synthetic compounds. They include derivatives of tetrahydrofuran and thiazolyl-5-carbonamide, a composition of 2-(5-phenyl-3,6-diaza-2,7-dioxaocta-3,5-diphenyl)pheniacrylamide and anilinopyrimidine [17-19].

The enzyme allinase, found in garlic, catalyzes the formation of allicin, a biologically active compound which produces the characteristic garlic smell. Allinase, containing 5.5-6.0 % of residual neutral sugars, can bond with the mannose-specific garlic lectin (ASA — *A. sativum* allinase). However, only the method of forming and conditions of existence of this complex are studied as of today [20, 21]. No data is available on its impact on living systems.

We were the first to show the biological activity (blast disease and spot blotch development suppression in cereals, stimulation of seed germination and seedling development in garlic) in the lectin-allinase complex of garlic, as well as in a new 4392 Da molecular weight peptide isolated by us from garlic bulbs.

The goal of the study was to isolate peptides from garlic and assess their antifungal and growth stimulating effect.

*Techniques.* Peeled bulbs (3 kg) of garlic (*Allium sativum* L.) Podmoskovnyi cultivar were milled into 1×1 cm pieces, put into 10 l of water-salt solution ($2.06\times10^{-2}$ M $NH_4NO_3$, $1.88\times10^{-2}$ M $KNO_3$, $3.0\times10^{-3}$ M $CaCl_2$, $1.5\times10^{-3}$ M $MgSO_4$, $1.25\times10^{-3}$ M $KH_2PO_4$) and kept in a fridge for 4-5 hours at 4 °C. The resulting extract was centrifuged (3000 g, 30 min), after which crystalline ammonium sulphate was added, with constantly stirring, to form a saturated salt solution (780 g/l) for protein precipitation [22]. The mixture was kept for 20 days at 4 °C. After centrifuging for 30 minutes at 10000 g to remove salts the residue was dialyzed at 4 °C against 0.05 M phosphate buffer (1:50 v/v), the supernatant was dialyzed at against water.

Desalted protein residue was dissolved in the minimum amount of 0.05 M phosphate buffer and fractioned by high performance gel permeation chromatography on a Bio-Sil TSK-125 300 ×7,5 mm column (Bio-Rad Laboratories, USA), using a high pressure Agilent 1200 chromatography system (Agilent Technologies, USA). The mixture was eluted for 12 min at 0.5 ml/min with 0.05 M phosphate buffer. The detection was performed at $\lambda = 280$ nm. The first fraction to exit the column was collected, because it contains proteins with the highest molecular weight. The resulting fraction was analyzed by electrophoresis in 12.5 % polyacrylamide gel with added SDS. The analysis resulted in two colored bands

in the gel, corresponding to molecular weights of 6400 and 54000 Da. Tryptic protein hydrolysis was then performed for the bands, followed by MALDI-TOF mass-spectrometry (UltraFlex 2, Bruker Daltonic GmbH, Germany). The proteins obtained were identified by the SWISS PROT and NCBI databases. The concentration of the lectin-alinnase complex (LAC) was adjusted to $10^{-11}$ mg/ml with sequential ten-fold dilutions with water, which conforms with the mean value of the range of ultralow concentrations ($10^{-8}$-$10^{-15}$ mg/ml). The resulting preparation was used in the tests.

Desalted supernatant was concentrated at 36-40 °C and fractioned by reverse phase high-performance liquid chromatography (HPLC) in a hydrophobic Kromasil C18 column (Russia) (4,6×250 mm) and a high pressure Agilent 1200 chromatography system. Elution was performed in the gradient of acetonitrile concentrations (2-96 %) in 0.1 % water solution of trifluoroacetic acid (pH 2.2) at 1 ml/min for 60 min. The detection was performed at $\lambda$ = 214 nm. The resulting HPLC fraction was analyzed by mass spectrometry with an UltraFlex 2 TOF mass-spectrometer (Bruker Daltonic GmbH, Germany) using α-cyano-4-hydroxycinnamic acid as a matrix. From the HPLC fractions, among which the 4392 Da peptide (P 4392 Da) was the main component, we picked a fraction of 1 ml with concentration peak 40 μg/ml (by peptide) for further work. The resulting peptide was adjusted to a concentration of $10^{-11}$ (by protein) with sequential ten-fold dilutions with water.

The rice blast agent *Magnaporthe grisea* (strain H5-3, race 007 with virulence genes *Av-k*$^{s+}$, *Av-a*$^{+}$, *Av-i*$^{+}$) and wheat and barley net blotch agent *Bipolaris sorokiniana* (strain Tul-12-1-3) were obtained from the State Collection of Phytopathogenic Microorganisms of the All-Russian Research Institute of Phytopathology. *M. grisea* was grown on agar carrot medium for 7-10 days at 24 °C, after which a washout of spores (conidia) was prepared in a laminar box (40-50 ml of sterile distilled water — SDW, 2-4 Petri dishes). The suspension was concentrated in a centrifuge (10 min, 8000 rpm), its density was counted in a Goryaev's chamber and diluted to $10^5$/ml when necessary. *B. sorokiniana* was grown on potato-glucose agar (PGA) for 10-12 days at 24 °C. In a laminary box 10-15 ml SDW was added to the dishes with the agent, spores were brought down from aerial mycelium with a microbiological loop. The spore suspension was filtered through a sterile cotton filter and diluted with SDW to $10^3$ and $10^5$ spore/ml density.

The impact of LAC on rice blast development was assessed in pot test with susceptible rice (*Oryza sative* L.) cultivar Sha-tiao-tsao (contains complete resistance genes *Pi-k*$^{s}$). The P 4392 Da activity in wheat and barley net blotch was assessed in a biotest with Lada cultivar wheat plants and Zazerskiy 85 cultivar barley plants. The growth stimulating effect of P 4392 Da was studied on cucumber (Fenix cultivar), pea (Sakharnyi cultivar), sunflower (Master cultivar) and garlic (Podmoskivnyi cultivar).

In the study of lectin-allynase complex (LAC) activity against *M. grisea* 45 μl of SDW or LAC and 5 μl of $10^5$ spores/ml suspension was placed in wells of a Cellstar 96 well cell culture plate (Greiner Bio-One, Germany) for tissue cultures. The samples were incubated for 24 hours in the dark at 23 °C. Germination rate (%) was calculated in samples of 100 spores. The mean value for the test was determined. The significance of differences was evaluated by Student's *t*-test. The results were recorded using a Leitz Diavert inverted microscope (Leica Microsystems GmbH, Germany).

In pot test of LAC impact on the development of rice blast 2-3-day-old seedlings were planted into plastic containers (0.5 l) with Sadovaya zemlya non-sterile universal nutrient soil and grown in a greenhouse for 21-24 days with 18 hours of daylight, at 22/30 °C (night/day) and good humidification. At leaf 3

stage the plants were sprayed with LAC ($10^{-11}$ mg/ml by protein) or distilled water (control) at 0.5 ml per container and left in the same conditions. For each variant we used 3 containers, with 8 plants in each. Seven days after the spraying, when the leaf 4 appeared, its middle part (6 cm in length) was placed in an inoculation chamber. A suspension of *M. grisea* spores ($10^5$/ml, three 20 μl drops) was put on the leaf with a drop holder [23]. The plants were kept for 22-24 hours in the dark at 23 °C in a thermostat, then the drop holders and inoculation chambers were removed and the plants were put back in the greenhouse. Seven days after the inoculation the symptoms were assessed visually and the leaves were scanned with an Epson Perfection 3200 Photo tablet scanner (EPSON, Japan). The plant response was evaluated by the 5-point F.M. Laterell's scale [24]: lack of spots or weak necrosis — 0, brown spots — 1, brown spots 0.5-1 mm in diameter with rough edges — 2, rhomb-shaped spots 2-3 mm in diameter with brown center — 3, big rhomb-shaped grey-brown spots with sporulation in the center — 4, toxic effect spread over the whole plant — 5. The percentage (%) of inoculum drops, which formed compatible (4 points, susceptibility) and incompatible infection spots (1-3 points, resistance) or those, which had caused no symptoms (0 points, resistance) was calculated [25].

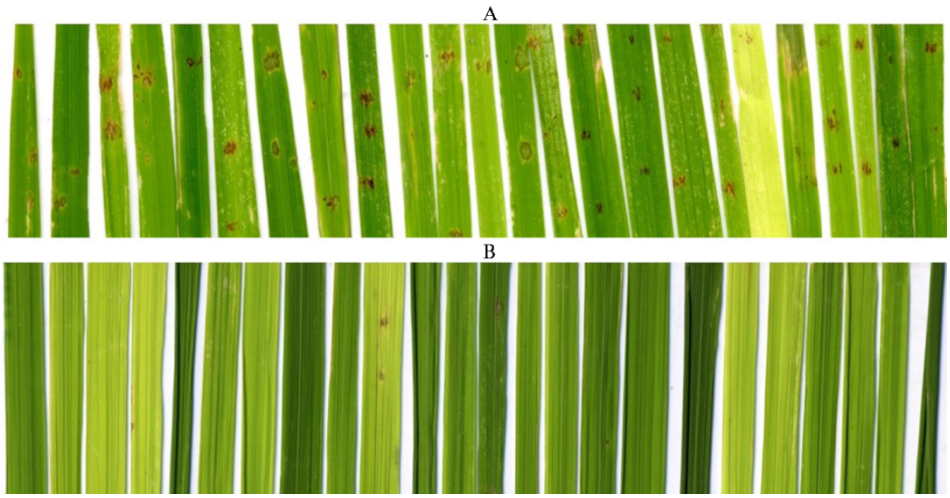
The impact of P 4392 Da peptide on *B. sorokiniana* was studied by diffusion into agar [26]. Spore suspension ($10^3$/ml) aliquots of 100 μl were spread onto potato-glucose agar (PGA) in Petri dishes. Three wells of 8 mm in diameter were made in agar, in which 100 μl of peptide solution ($10^{-11}$ mg/ml), or 100 μl SDW in control, were put and incubated in a thermostat at 26 °C. At day 3 the diameter of growth delay areas was measured. The test was performed twice in three-fold repetitions. The development of net blotch infection after treatment with P 4392 Da peptide was assessed in a biotest using a modified method [26, 27]. Wheat and barley first fully unfolded leaves (segments of 5-6 cm in length, 8-10 pcs per Petri dish, 3 dishes for test and control each) were put on 1 % agar with benzimidazole. For each leaf segment, one half was treated with 10 μl of *B. sorokiniana* spore suspension in P 4392 Da solution (protein concentration $10^{-11}$ mg/ml, resulting spore suspension density $10^5$/ml), and other half was treated with 10 μl of water spores suspension of the same density (control). The results were assessed 7 days after the inoculation by the number of typical spots.

In the study of the impact of P 4392 peptide ($10^{-11}$ mg/ml) on seed germination and seedling growth stimulation the sample for each culture consisted of four samples of 100 seeds (or 50 for big seeds). Sterilized filtering paper (2-3 layers soaked with peptide solution) in Petri dishes was a substrate. The seeds were organized in rows, with no less than 0.5-1.5 cm between them. The dishes were put into a thermostat and incubated at 25 °C for 7-10 days. The percentage of germinated seeds was calculated, the length of roots and stems of seeding was measured.

The significance of differences was assessed by the Student's *t*-test (Statistica 6.0, StatSoft. Inc., USA).

*Results.* Extraction of lectin-allynase complex was performed at a low temperature (4 °C) which allowed to prevent non-specific proteolysis of proteins. The tryptic hydrolysis of the 6400 Da and 54000 Da fractions, obtained after electrophoretic separation of the first chromatography peak using denaturing conditions, and the following spectrometry showed the 6400 Da protein to be homologous to the A-chain of the mannose specific agglutinin (lectin) of garlic, and the 54000 Da protein appeared to be the enzyme allynase. Therefore, we managed to isolate the lectin-allynase complex.

When studying whether or not this complex has a direct impact on a pathogen, we revealed that the viability of *M. grisea* spores did not differ in the control and LAC treatment groups (90-90 % of spores germinated).



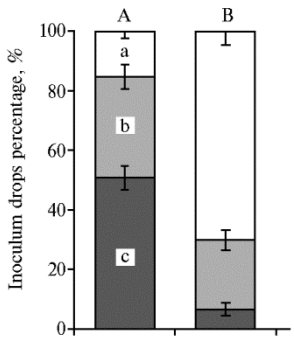




**Fig. 1. Necrosis on the leaf segments in rice (*Oryza sativa* L.) Shatiao-tsao cultivar, susceptible to rice blast, after inoculation with *Magnaporthe grisea* in the presence of water** (A) **and lectin-allynase complex from of garlic (*Allium sativum* L.) cultivar Podmoskovnyi** (B)**:** a — drops which caused no symptoms (0 points, resistance), b — drops which caused incompatible infection spots (1-3 point, resistance), c — drops, which caused compatible infection spots (4 points, susceptibility) (all differences from control are significant at $p < 0.01$).

When studying possibility to develop systemic resistance to rice blast using LAC, it was showed that treatment of the leaf 3 reduced the development of the disease on the leaf 4, which appeared later, that was manifested in a lesser number of necrosis compared to the control (Fig. 1).

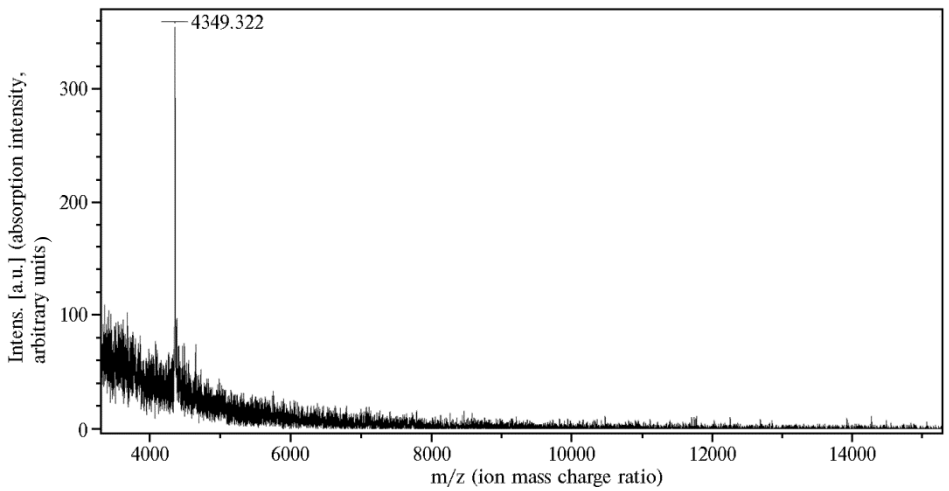



**Fig. 2. The mass spectrum of the P 4392 Da peptide isolated from garlic (*Allium sativum* L.) cultivar Podmoskovnyi** (Matrix-assisted laser desorption/ionization—Time of flight mass spectrometry — MALDI-TOF, Time-of-flight mass-spectrometer UltraFlex2, Bruker Daltonic GmbH, Germany, matrix — α-cyano-4-hydroxycinnamic acid).

At purification, large proteins are first to sediment from water-salt extract, while peptides remained in the solution [18]. Using reverse phase HPLC the main peptide of 4392 Da (P 4392 Da) was isolated from the supernatant (Fig. 2). We did not find colony growth inhibition when studying the impact of

this peptide at concentration of $10^{-11}$ mg/ml on *B. sorokiniana*. However, the biotest on wheat and barley leaves showed the P 4392 Da capability to completely suppress or significantly decrease manifestation of symptoms 7 days after the *B. sorokiniana* inoculation (Fig. 3).

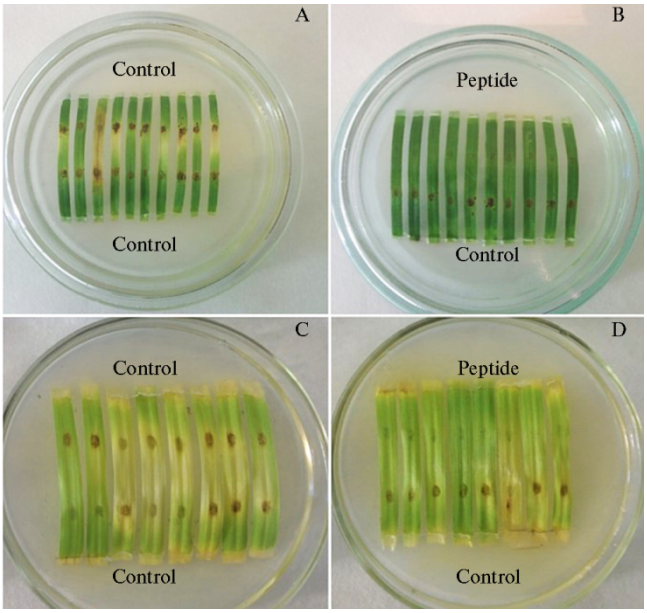



**Fig. 3. The development of dark-brown net blotch on leaves of wheat cultivar Lada** (upper row) **and spring barley cultivar Zazerskiy 85** (lower row) **in response to inoculation with *Bipolaris sorokiniana* in the presence of the P 4392 Da peptide from garlic (*Allium sativum* L.) cultivar Podmoskovnyi:** A, C — control (water spore suspension), B, D — joint application of spores and P 4392 Da (day 7 after inoculation).

P 4392 Da peptide had no phytotoxicity against wheat and barley, and also peas, cucumber, mustard, sunflower and garlic (data not shown). The peptide also had no effect on seed germination in pea, sunflower, mustard and cucumber, and further development of seedling of these crops in which an increase in stem and root length was not observed. At the same time, adding peptide in concentration of $10^{-11}$ mg/ml had a reliable positive effect on garlic seed germination (an increase by 13.6 %, $p < 0.05$). It should be noted, that with the peptide the stem length in garlic seedlings increased by 65.5 %, and the root length was 4 times as much as that in the control (Fig. 4).

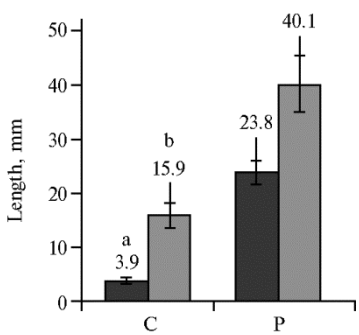



**Fig. 4. Root** (a) **and stem** (b) **length in garlic (*Allium sativum* L.) cultivar Podmoskovnyi 10-days seedlings in the presence of P 4392 Da peptide isolated from garlic:** C — control (water), P — peptide solution.

To date, various plant peptides with protective, antimicrobial and antifungal properties have been reported [28-31]. These are mostly cationic peptides, rich in cysteine, which have a broad range of effect against phytopatogens and pests. Most of the known antifungal peptides are effective in micromolar quantities. The P 4392 peptide that we have isolated from garlic differs from other known peptides due to significantly lower effective concentration and the apparent capability of activating the plant's own defensive mechanisms at infection.

Therefore, bioactive substances of protein nature, effective against a range

of phytopathogens, have been isolated from bulbs of garlic (*Allium sativum* L.). In our findings, the lectin-allynase complex (LAC), previously described by other researchers, has no direct effect against *Magnaporthe grisea* (the rice blast agent) in the concentration of $10^{-11}$ mg/ml, but decreases the lesion of rice leaves infected with the pathogen. This can be considered as an evidence for LAC capability of inducing the plant's defensive response, leading to systemic resistance. In addition, we have isolated a 4392 Da peptide which effectively countered the development of net blotch infection (*Bipolaris sorokiniana* agent) in the $10^{-11}$ mg/ml concentration, and significantly stimulate seed germination and seedling growth in garlic.

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# THE APPEARANCE OF THE INDUCED DORMANCY IN SEEDS OF SOME *Umbelliferae* VEGETABLE CROPS UNDER THE EFFECT OF ALLELOPATHIC SUBSTANCES

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Abstract

Allelopathic effects in plants are due to a combination of many adaptive and environmental factors having evolutionary and economic importance. Currently the investigations on the effects of allelopathic substances on seed germination in order to develop new environmentally friendly approach to plant protection against weeds are in progress. The main goal of our experiments was to examine the peculiarities of the seed dormancy induced under the influence of an extract from seeds of dill (*Anethum graveolens* L.) variety Kentavr and specific overcoming of the dormancy in various vegetable crops — carrot (*Daucus carrota* L.) variety Rogneda, root parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill.) variety Lyubasha, celery root (*Apium graveolens* L.) variety Kupidon, lavage (*Levisticum officinale* W.D.J. Koch) variety Don Zhuan, coriander (*Coriandrum sativum* L.) variety Yantar', and parsnip (*Pastinaca sativa* L.) variety Kulinar. The seeds of tested crops were treated with 15 % aqueous extract from dill seeds at 23 °C for 0 (control), 5 and 20 days in the dark. A germination of the treated seed germination was studied in dynamics at different temperature conditions — 20 °C (control); 3 °C; and 3 °C (8 hours)/20 °C (16 hours). We calculated $T_{ip}$ (initial period, i.e. the time prior to seed germination starts), $T_{wagt}$ (the weighted average germination period, i.e. the time to reach maximum germination rate) and $T_{50}$ (the time to reach 50 % germination), measured the embryo length and calculated the temperature coefficient (Q10). It was shown the extract noticeably inhibited the embryo growth and seed germination in the studied crops. After exposure to allelopathic factors for 5 days the seeds of carrots, celery root, parsnip and coriander did not germinate at a standard temperature. The $T_{wagt}$ values exceeded the control by 229-328 %, and the $T_{50}$ values were 310-379 % higher. In dill, parsley root and lovage the germinated seeds accounted for 42, 52 and 23 %, respectively. After a 20 day exposure to the allelopathic factor the seeds of celery root, carrots, parsnip and coriander did not germinate whereas in dill and lovage seeds germination delayed by 21 and 22 days. Increasing exposure time resulted in a reduced temperature coefficient ($Q_{10}$) for embryo growth at different temperatures. Low temperature including constant 3 °C and variable (3/20 °C) contributed to a partial recovery of these processes in the seeds pre-exposed to allelopathic factor. In this, the $T_{wagt}$ and $T_{50}$ increased by 11.7-35.3 days and 11.7-43.1 days, respectively, compared to control. Analysis of a combined effect of the allelopathic factor and temperature evidenced that the inhibition of seed germination influenced by the substances contained in the extract of dill seeds is due to the dormancy phenomenon.

Keywords: allelopathy, temperature, seed dormancy, seed germination, growth rate of the embryo, the temperature coefficient ($Q_{10}$)

Allelopathy, based on the chemical interaction, is one of the oldest communications between biological objects, different organs, tissues and cells. Allelopathic interference of plants is caused by a combination of numerous permanent and transient biogenic factors [1], and has an adaptive, ecological, evolutionary and economic importance [2, 3]. Current studies show that allelopathy may be a part of a network of chemical communication [4], and perform a variety of functions, including protective ones [5, 6]. Both stimulatory and inhibitory effects of extracts from fruits, seeds and other plant organs have been established [7].

There are numerous reports on the effect of various substances on seed germination [8, 9]. A study of allelopathic agents will allow to develop novel

eco-friendly methods for weed control ([10, 11]. For example, an extract from seeds of *Myrica gale* L. exhibits an inhibitory effect, and a substance called Myrigalone A affects the gibberellin biosynthesis and signaling systems of germinating seeds [12, 13]. Metabolites interrupt the germination of seeds and have a negative effect on growth, respiration and protein synthesis [14, 15].

A group of substances (strigolactones) discovered in the root exudates is capable of stimulating germination of parasitic weed species, such as *Orobanche* and *Striga*. These substances are considered a new class of hormones [16, 17] that affect the balance of abscisic acid and gibberellin which are responsible for seed dormancy [18].

In our previous studies, we described the effect of a 15 % extract of dill seeds on the dormancy induction in tester seeds of mustard greens and Japanese mustard (Mizuna) [19].

In this paper, for the first time we demonstrated that the effect of the dill seed extract led to a delay (up to complete inhibition) in the germination of seeds of vegetable crops in the *Umbelliferae* family. The induced dormancy is maintained for a long time even after seeds are transferred into standard conditions, however, it does not cause their death.

The aim of our study was to examine the peculiarities of seed dormancy induced by an extract from dill seeds, and the overcoming of the dormancy state in the *Umbelliferae* vegetable family

*Techniques.* We studied the seeds of dill (*Anethum graveolens* L.) variety Kentavr, carrot (*Daucus carrota* L.) variety Rogneda, root parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill.) variety Lyubasha, celery (*Apium graveolens* L.) variety Kupidon, lovage (*Levisticum officinale* W.D.J Koch) variety Don Zhuan, coriander (*Coriandrum sativum* L.) variety Yantar', and parsnip (*Pastinaca sativa* L.) variety Kulinar.

To prepare an aqueous extract, 15 g of dill seeds were pounded in a mortar with pestle, followed by addition of distilled water (100 ml), then infused for 1 h, and then filtered through a paper filter. The seeds of the tested cultures were incubated in the extract at 23 °C in Petri dishes on filter paper with no exposure to light for 0 (control), 5 and 20 days. We repeated the experiment 3 times (using 1,000 seeds per each replication). After incubation the seeds were washed in water and germinated on filter paper with no exposure to light.

Seed germination was studied at 20 °C (standard mode), 3 °C, and 3 °C (8 h)/20 °C (16 h) temperature conditions. The following parameters were calculated: $T_{ip}$ — the time from seed layout up to the germination; $T_{wagt}$ — the weighted average germination period which can be considered as the number of days until the maximum germination rate [20, 21]; $T_{50}$ — the time to reach the germination of 50 % seeds [22-24], as well as the proportion of germinated seeds. The completion of seed germination was evaluated by their protrusion [25]. We repeated the experiment 3 times (using 100 seeds per each replication).

The length of the embryo was measured under a Levenhuk 670T (Levenhuk, USA) microscope equipped with the digital microscope eyepiece camera DCM 300 MD (Microscope Digital, China), at ×40, using the Scope Photo software (Image Software v. 3.1.386). The temperature coefficient ($Q_{10}$) of the embryo growth rate was calculated using the Van 't Hoff equation.

Statistical analysis was performed using the programming language R (i386 v.3.3.0).

*Results.* Within 20 days of incubation, the seeds of all studied crops did not germinate in the dill seed extract (15 %). In carrots and root parsley, after 18-20 day exposition, autolysis of a part of the seeds (20-40 %) occurred. The morphometric analysis showed no growth of the embryo.

After being incubated for 5 days and then transferred to standard conditions (20 °C), the seeds of celery, parsnip, carrots and coriander did not germinate. The growth processes in the seeds of all the remaining crops gradually resumed, however, all parameters, characterizing the intensity of the growth processes, were higher in them than in control plants, indicating a slowdown in seed germination. Accordingly, $T_{ip}$ in dill, root parsley and lovage was 15-17 days which was 10-14 days longer than in control. The $T_{wagt}$ value exceeded control by 229-328 % and the $T_{50}$ value by 310-379 %. Seeds of carrots, root parsley and parsnip did not germinate at 3 °C (see Table).

Low temperature (3 °C) promoted (albeit with a delay) the germination of seeds of dill, lovage, celery and coriander. Seeds which suffered from the effects of the allelopathic factor for 5 days needed more time to germinate at a reduced temperature (3 °C). There was an increase by 11.7-35.3 days for $T_{wagt}$ and by 11.7-43.1 days for $T_{50}$, as compared to control, and by 9.8 and 2.8 days, respectively, when compared to standard conditions. The difference between $T_{wagt}$ and $T_{50}$ values increased concurrently up to 5.8 to 9.2 days.

**The indicators characterizing the rate of seed germination in the *Umbelliferae* vegetable crops affected by the allelopathic stress (incubation in the 15% extract of dill seeds) and specific germination conditions** (lab experiments)

| Crops | Duration of incubation, days | $T_{wagt}$, days | | | $T_{50}$, days | | |
|---|---|---|---|---|---|---|---|
| | | 20 °C | 3 °C | 3/20 °C | 20 °C | 3 °C | 3/20 °C |
| Carrot, *Daucus* | 0 (control) | 7.5±0.5 | 16.7±0.6 | 10.2±0.5 | 8.5±0.6 | 18.5±0.6 | 11.9±1.0 |
| *carrota* L., Rogneda var. | 5 | – | – | 35.6±1.2 | – | – | 44.3±1.8 |
| | 20 | – | – | 38.7±2.0 | – | – | 49.1±2.7 |
| *Anethum* | 0 (control) | 7.9±0.7 | 14.3±1.1 | 10.3±1.0 | 90±0.4 | 20.1±2.3 | 10.0±1.0 |
| *graveolens* L. dill, Kentavr | 5 | 25.9±1.6 | 26.0±0.7 | 27.7±1.7 | 34.1±3.8 | 31.8±2.0 | 51.2±7.9 |
| var. | 20 | 37.5±2.0 | 44.7±1.2 | 33.8±1.0 | 45.3±4.1 | 56.2±2.6 | 50.4±7.0 |
| *Apium graveolens* L. celery, | 0 (control) | 11.0±0.6 | 23.5±1.0 | 11.2±0.7 | 14.0±0.3 | 26.9±1.0 | 12.2±0.5 |
| Kupidon var. | 5 | – | 58.8±1.3 | 48.0±0.8 | – | 70.0±4.5 | 60.1±4.9 |
| | 20 | – | 68.2±2.0 | 53.3±3.1 | – | 89.8±5.3 | 75.1±5.4 |
| Root parsley, | 0 (control) | 10.4±0.6 | 20.0±1.0 | 90±0.4 | 12.5±1.6 | 23.1±1.2 | 10.5±0.6 |
| *Petroselinum crispum* (Mill.) | 5 | 21.9±0.5 | – | 20.3±1.4 | 27.3±2.6 | – | 28.9±2.9 |
| Nyman ex A.W. Hill., Lyubasha var. | 20 | 29.1±1.1 | – | 27.2±1.0 | 40.1±3.0 | – | 35.1±2.0 |
| Parsnip, *Pastinaca* | 0 (control) | 16.0±1.0 | 26.7±0.5 | 13.3±0.7 | 21.5±2.0 | 30.8±1.0 | 15.3±2.5 |
| *sativa* L., Kulinar var. | 5 | – | – | 43.8±2.9 | – | – | 55.0±7.1 |
| | 20 | – | – | 52.4±0.6 | – | – | 64.2±1.4 |
| Lovage, *Levisticum officinale* | 0 (control) | 9.1±0.7 | 12.7±0.8 | 11.0±0.2 | 11.9±2.0 | 16.9±1.1 | 15.0±0.4 |
| W.D.J. Koch, | 5 | 20.8±1.4 | 30.6±1.0 | 20.5±2.0 | 37.0±7.1 | 39.8±4.0 | 27.6±4.4 |
| Don Zhuan var. | 20 | 29.5±1.0 | 42.2±2.0 | 42.4±2.7 | 60.5±11.9 | 57.4±6.5 | 60.1±4.0 |
| Coriander, *Coriandrum sa-* | 0 (control) | 9.7±0.3 | 21.8±1.2 | 15.0±0.7 | 13.0±1.0 | 28.2±1.1 | 21.7±1.4 |
| *tivum* L., Yantar' var. | 5 | – | 46.0±1.2 | 44.2±4.3 | – | 62.1±3.7 | 62.8±6.4 |
| | 20 | – | 71.9±0.8 | 54.0±3.0 | – | 95.0±7.0 | 67.2±2.7 |

N o t e. $T_{wagt}$ — the weighted average germination period; $T_{50}$ — the time to reach the germination of 50 % seeds. Dashes indicate there was no germination.

Variable temperature profile had a positive effect as compared to the standard mode, promoting the germination of all the studied crops. It reduced the negative effect of allelopathic factors. However, the $T_{wagt}$ and $T_{50}$ values, affected by the 5-day incubation, increased by329-429 % and 372-492 %, respectively, as compared to the control.

After suffering exposure to the allelopathic factor for 20 days, the seeds germinated even more slowly. At a temperature of 20 °C, the seeds of celery, carrot, parsnip and coriander did not germinate. The germination of seeds of dill and lovage started with a delay by 21 and 22 days compared to control. $T_{wagt}$ increased up to 37.5 and 29.5 days, and $T_{50}$ up to 45.3 and 60.5 days, which were, respectively, 20.4-29.6 and 36.3-48.6 days higher than in the control.

Seeds of dill and lovage germinated less actively at 3 °C (after incubation for 20 days) when compared to the standard conditions. The seeds of celery and

coriander still began to germinate, although with a considerable delay (55 and 58 days), however, the $T_{wagt}$ value was 68.2 and 71.9 days, respectively, and $T_{50}$ reached 70.0 and 95.0 days. The seeds of parsnip and carrot were the only ones not germinated.

Under variable temperatures, seeds of all crops, exposed to the allelopathic factor for 20 days, germinated, however, the $T_{wagt}$ and $T_{50}$ values increased 4.0-4.2 times as compared to control.

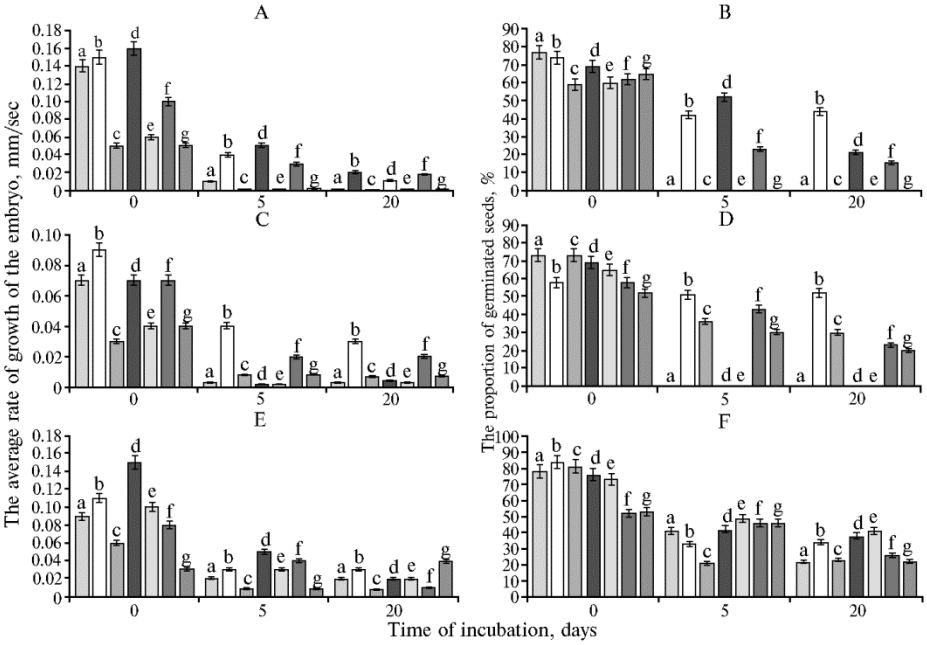


**Fig. 1. The average rate of growth of the embryo** (left) **and the proportion of germinated seeds** (right) **in carrot (*Daucus carrota* L.) Rogneda var.** (a)**; dill (*Anethum graveolens* L.) Kentavr var.** (b)**; celery (*Apium graveolens* L.) Kupidon var.** (c)**; root parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill Lyubasha var.** (d)**; parsnip (*Pastinaca sativa* L. ) Kulinar var.** (e)**, lovage (*Levisticum officinale* W.D.J. Koch, Don Zhuan var.** (f)**, coriander (*Coriandrum sativum* L.), Yantar' var.** (g) **depending on temperature and time of incubation in the 15 % extract of dill seeds:** A, B — 20 °C (standard mode), C, D — 3 °C, E, F — 3/20 °C (lab experiments).

When seeds were germinated post incubation at the constant positive temperature, the embryo growth rate steadily decreased with increasing stress period (Fig. 1). In control, the embryo in the seeds of dill, carrot, root parsley grew at a rate of 0.14-0.16 mm/day. After 5 days of incubation, the embryo growth rate reduced to 0.01-0.05 mm/day, and after 20 days down to 0.02 mm/day. The coriander embryo, which grew in the control at a rate of 0.15 mm/day, virtually ceased to grow with increasing incubation time.

One of the key endogenous factors affecting seed germination in crops of the *Umbelliferae* family is a morphological immaturity of the embryo [26, 27]. The immaturity occurs irrespective of time the seeds maturates on the mother plant. After seed separation, the full development of the embryo completes under adequate moisture and favorable temperatures [28]. This underlies special requirements when dealing with the seeds of the *Umbelliferae* family plants. To evaluate any changes occurring during their germination, it is insufficient to determine their dimensions and dry weight. Only the analysis of the continuous growth of seed morphological elements, primarily of the embryo, may reveal significant patterns. In celery and parsnip, the growth rate of the embryo was 0.05 and 0.06 mm/day in the control, while after even 5 day-exposure to the extract it decreased sharply down to 0.0007 mm/day.

At 3 °C, the average rate of the post incubation embryo growth in all the crops (except for parsley) was 30 to 60 % higher than under the standard temperature conditions. However, the growth rate of the embryo linear size remained lower by 43-93 % as compared to control.

Under variable temperature conditions, an increase in the embryo growth rate by 20-92 % was observed in all crops as compared to the standard conditions, and only in carrot, coriander and parsley (by 11-96 %) if compared to the constantly low temperature. However, it was impossible to completely overcome the negative effects of allelopathic stress with the variable temperature. The growth rate of the embryo was 300-330 % lower than in control.

The temperature is known to be one of the most important abiotic factors that ensure exiting dormancy [29-31]. Exposure to low temperature activates the mechanisms promoting the onset of the gibberellin synthesis [32]. At low temperatures, the breakdown of storage compounds in the endosperm is activated and the synthesis in the embryo is stimulated in immature seeds, which are typical for the *Umbelliferae* vegetable family [33].

The inhibition of germination under the influence of the extract of dill seeds, which was observed in our experiments, appeared to result from the induced dormancy.

The proportion of germinated seeds for different crops varied depending on a combination of the studied factors. After exposure to the allelopathic agent for 5 days, no germinated seeds were observed at the standard temperatures in the carrots, celery, parsnip and coriander. The proportion in dill, root parsley and lovage was 42, 52 and 23 %, which was lower, respectively, by 32, 17 and 39 % than in control (see Fig. 1).

At low temperatures (3 °C), the germination of seeds, in addition to dill, celery and lovage, was observed in coriander, and at varying temperatures the seeds of all the studied crops did germinate. With increasing time of incubation, the proportion of germinated seeds was usually reduced by 12-100 %, which was especially noticeable in celery, lovage and coriander.

The increased time of seed incubation in the extract resulted in a reduced temperature coefficient ($Q_{10}$) for the embryo growth rate at different temperature settings for germination (Fig. 2).

Correlation and regression analyses showed that the temperature coefficient was strongly negatively correlated with the incubation time ($r$ = –0,700) which can be described using the following regression equation y = 1.37 – 0.04x.

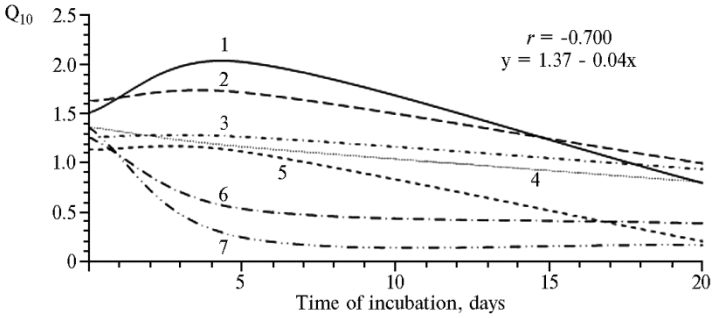


**Fig. 2. Changes in the temperature coefficient ($Q_{10}$) for the embryo growth rate in the *Umbelliferae* vegetable family depending on time of incubation in the 15 % extract of dill seeds:** 1 — carrot (*Daucus carota* L.) Rogneda var.; 2 — root parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill. Lyubasha var.; 3 — lovage (*Levisticum officinale* W.D.J. Koch Don Zhuan var.; 4 — dill (*Anethum graveolens* L.) Kentavr var.; 5 — coriander (*Coriandrum sativum* L.) Yantar' var.; 6 — parsnip (*Pastinaca sativa* L.) Kulinar var.; 7 — celery *Apium graveolens* L.) Kupidon var. (lab experiments).

Dill was characterized by gradual reduction of the temperature coeffi-

cient when the time of stress exposure was increased: $Q_{10}$ was 1.37 in the control and gradually decreased to 0.81 after exposure to high temperatures within 5 and 20 days. In root parsley and carrots, an increase in $Q_{10}$ occurred (1.63-1.51 in control, and 1.72-2.03 when affected by incubation), although further influence of the allelopathic stress resulted in a decrease of the temperature coefficient. In celery, coriander and parsnip, a prolonged stress resulted in drastically reduced temperature coefficient. The temperature coefficient for the embryo growth rate was least affected by the incubation time in lovage, although some reduction of the $Q_{10}$ value was observed.

Therefore, we have established the apparent manifestation of allelopathic activity of the dill seed extract which may have a significant effect on seed germination of the *Umbelliferae* family crops and changes of the embryo linear dimensions. Seed exposure to low temperature post incubation contributes to the restoration of these processes. Analysis of the effects caused by allelopathic factor and temperature evidenced that the inhibition of seed germination under the influence of dill seed extract was due to the dormancy phenomenon.

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# GENETIC STRUCTURE OF REGIONAL POPULATIONS OF *Mycosphaerella graminicola* (*Septoria tritici*), THE SEPTORIA LEAF BLOTCH AGENT OF WHEAT

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Abstract

*Mycosphaerella graminicola* (anamorph *Septoria tritici*), the causal agent of septoria tritici blotch (STB) of wheat, is dominating species in *Septoria/Stagonospora* complex on crops in the main grain-producing areas of Russia. Resistance to STB may be either quantitative (horizomtal) or isolate-specific (vertical). At present 17 genes for resistance have been identified (*Stb1-Stb17*). The gen-for-gen interaction in the «wheat—*M. graminicola*» pathosystem has been demonstrated by genetic analysis; therefore, the aviability of resistance genes in the host proposes the existence of specific virulence genes in the pathogen. The relative frequency of virulence genes within a geographic region may be calculated as a fraction of the isolates expressing this virulence genes from the overall number of isolates used in the study. The purpose of the present study was to estimate the virulence genes in populations of *M. graminicola* from different geographic regions of Russia on the basis of a gen-for-gen relationship, using the cultivars with known resistance genes, i.e. Bulgaria 88 (*Stb1*), Oasis (*Stb1*), Veranopolis (*Stb2*), Israel 493 (*Stb3*), Tadinia (*Stb4*), CS/Synthetic 7D (*Stb5*), Flame (*Stb6*), Estanzuela Federal (*Stb7*), W7984 (*Stb8*). A total of 47 isolates from the North-Caucasian region, 66 isolates from the Central-Chernozem region, 29 isolates from the Volga region, 64 isolates from the Central region, and 34 isolates from the North-West region were tested under greenhouse and grows chamber conditions**.** The virulence was estimated on seedlings at two-leaf stage, using two parameters, the infection degree of plants and sporulation of fungus in vivo. The effectiveness of *Stb*-genes to each regional population of *M. graminicola* was revealed on the basis of the frequency of virulence genes. The regional populations of *M. graminicola* differed in virulence genotype, spectrum and frequency of virulence genes. The populations from south zone (the North-Caucasian, the Central-Chernozem and the Volga regions) are more virulent in comparison with the central and the north-west populations. For example, 19.2 % of isolates from the north-caucasian population and 6.0 % of isolates from the central-chernozem population have no virulence genes, while in the central and north-west populations — 42.2 % and 44.1 %, respectively. Isolates from the Volga population of *M. graminicola* had most various combinations of virulence genes. High frequency of virulence to genes *Stb1*, *Stb5* and *Stb7* was revealed in all populations. The genes *Stb2*, *Stb3*, *Stb4* have considerable effectiveness to the central, the central-chernozem and the north-west populations of *M. graminicola*, however it distinctly reduced concerning isolates from the North-Caucasian and the Volga regions. The genes *Stb6* and *Stb8* were highly effective (*Stb8* — absolutely effective) to all investigated Russian populations of *M. graminicola* and may be recommended for using in selection as sources of resistance to STB.

Keywords: *Mycosphaerella graminicola,* population, isolate, virulence genes, frequency, effectiveness of *Stb*-genes

Septoria tritici blotch (STB) agent, *Mycosphaerella graminicola* (anamorph of *Septoria tritici*), is dominating species in *Septoria/Stagonospora* complex on crops in the main grain-producing areas of Russia (North Caucasian and Central Chernozem economic regions). Furthermore, the pathogen is common in central Russia and in southern Volga region, reaching 40-50 % of species composition.

The species also presents in North-West region, Kaliningrad region, as well as Western and Eastern Siberia.

Knowledge of genetic structure of pathogen populations, virulence gene frequency, spatial distribution and dynamics, as well as the efficiency of resistance genes are considered obligatory conditions of successful breeding for disease resistance. As compared to other diseases (rust, mildew, etc.), genetic basis of resistance against Septoria blight is less well understood. Furthermore, there are two types of interaction in wheat—*M. graminicola* system, i.e. resistance to STB may be either quantitative (horizontal) or isolate-specific (vertical). Both types are important for pathosystem.

Specificity of interaction between *M. graminicola* and wheat was firstly proved by Z. Eyal et al. [1, 2]. They analyzed many individual combinations «cultivar × isolate» and suggested that there are 28 complementary genes. Existence of interacting gene pairs was confirmed by the results of other studies using 80 isolates and 47 cultivars [3]. The gene-for-gene interaction was finally proved by molecular genetic studies of host and pathogen. This fact precluded all doubts against the interaction regarding to at least some known resistance genes [4-7].

In recent years, 17 main resistance genes against *M. graminicola* (*Stb1-Stb17*) were identified according to the interactions between wheat cultivars and fungus isolates. Experiments all over the world determined chromosomal localization and molecular markers of these genes which are proposed to be used in marker selection [8-14].

According to the gene-for-gene relationship, the existence of resistance genes in the host means the existence of specific virulence genes in the pathogen. Virulence gene frequency in the region can be calculated as a percentage of isolates expressing such genes against total number of the isolates found [2]. It also allows determining efficiency of *Stb* genes which is of great importance for breeding, as introduction of certain *Stb* gene will not cause effective resistance if the pathogen population is virulent to the cultivar with this gene. More specifically, French studies of 11 cultivars with known resistance genes and monopycnidial isolates from 5 regions of the country revealed that many *Stb* genes was ineffective against most French *M. graminicola* isolates [15]. Nevertheless, efficiency of some *Stb* genes is confirmed by the world practice. The gene *Stb1* introduced in winter wheat cultivars Oasis and Sullivan retained its effectiveness in Indiana and neighboring states for more than 25 years [8]. Spring wheat cultivar Tadinia has one dominant resistant gene *Stb4* used to control *S. tritici* in California for 30 years [9]. The gene *Stb6* is used as a source of resistance against STB worldwide [16]. However, there is lack of information about *Stb* gene effectiveness against Russian pathogen populations. There are only data obtained by Yu.V. Zeleneva [17] who studied isolated wheat leaves and found that the genes *Stb1*, *Stb4*, *Stb5* were most effective and the genes *Stb2*, *Stb3* were less effective against *M. graminicola* isolates from Central-Chernozem region.

Heretofore, there is no consistent methodology of assessment and differentiation between resistance and susceptibility. It is known that there is no immunity (complete resistance) against *M. graminicola* because necroses and/or pycnidia always present [18, 19]. Genetic base of STB resistance can manifest itself as a decreased lesion area and reduced fungus fertility. These parameters are controlled by different genes and they are both important for disease assessment [3]. Fungus fertility is usually evaluated by pycnidia number. Plant response is classified from almost immune (little necrosis without pycnidia) to very susceptible (large confluent spots and numerous pycnidia) [5, 20-22]. However, visual assessment of pycnidia size, especially using a point scale, is very subjective. And then, number of spores per pycnidium in susceptible cultivars was stated to be

2.0-2.5 times higher than in resistant ones [23]. Due to this fact, spore counting in Goryaev chamber is proposed to determine sporulation of fungus in vivo [24].

The purpose of our study was to estimate the virulence genes in populations of *Mycosphaerella graminicola* from different regions of Russia on the basis of a gene-for-gene relationship, using the cultivars with known resistance genes. This information will allow determining *Stb* gene efficiency, their functional activity in the territory of Russia and feasibility of their use in breeding programs as potential sources of STB resistance.

*Technique.* Infected plants were collected during crop examination in 2009-2015 vegetation period according to the standard method [25]. Pure culture of monosporous isolates of *Mycosphaerella graminicola* were obtained using streak technique [26]. Pieces of infected tissue with fungal pycnidia were rinsed under running water, then in several portions of sterile distilled water and then placed in sterile Petri plate onto specimen glass in drop of sterile water. Few minutes after, obtained suspension was transferred on potato glucose nutrient agar by inoculation loop. After 7-8 days of incubation at 20-25 °C, single colonies from the same conidium were separated to other plates. Isolate stability was monitored during three successive reinoculations of 10-day colonies on fresh nutrient medium. Cultural and morphologic characteristics of isolates were evaluated on day 30 after inoculation. Structural characteristics, diameter and color of the colonies were registered. Isolates from different morphologic groups with stable cultural and morphologic characteristics were chosen from a viewpoint of more complete covering of intraspecific variation in populations.

Monogenic cultivars with known resistance genes, the Bulgaria 88 (*Stb1*), Oasis (*Stb1*), Veranopolis (*Stb2*), Israel 493 (*Stb3*), Tadinia (*Stb4*), CS/Synthetic 7D (*Stb5*), Flame (*Stb6*), Estanzuela Federal (*Stb7*), and W7984 (*Stb8*), were used for tests. Studies were performed in greenhouse and growth chamber. The plants were grown in 400 $cm^3$ pots (10 plants per pot) up to fully unwrapped leaf 2. Fungal inoculum was cultivated on plates with potato glucose agar for 4-5 days at room temperature without additional lighting. Plants were infected with individual isolates by spraying spore suspension ($1\times10^7$ spores/ml, 100 ml/$m^2$). A drop of Tween 20 detergent was added to suspension before application. After inoculation, the plants were placed into wet chamber for 48 hours at the temperature of 20-25 °C and then grown in growth chamber or box at 18-20 °C/22-24 °C (night/day), relative air humidity of 70-80 %, 16-hour photoperiod, and illumination of about 15000 lx.

Plant damage was evaluated 20 days after the inoculation by the method developed in All-Russian Research Institute of Phytopathology, using degree of plant infection and fungus in vivo sporulation as main parameters [24, 27]. Plant infection was determined visually by percentage of affected area of the leaves 1 and 2. In order to determine the sporulation, relevant leaves were cut off and placed for 3-4 hours in laboratory glasses with accurately measured amount of water. Then number of spores in suspension was counted using Goryaev chamber and the number of spores per relevant leaf (N) was calculated as $N = 2500 MV/n$, where M is the number of spores in 100 large squares of Goryaev chamber; V is amount of water in the glass, ml; n is the number of leaves in the sample; 2500 is the coefficient derived experimentally. The degree of plant infection was differentiated as low (up to 20 % of leaf area affected in average), medium (21-50 %), or high (more than 50 %). By sporulation intensity, isolates were differentiated as low-sporulating (up to 100 thousand spores/leaf), medium-sporulating (100-200 thousand spores/leaf) or high-sporulating (more than 200 thousand spores/leaf). By combination of these two parameters, isolates were differentiated into three groups: group I — low-virulent, group II — medium-virulent, group

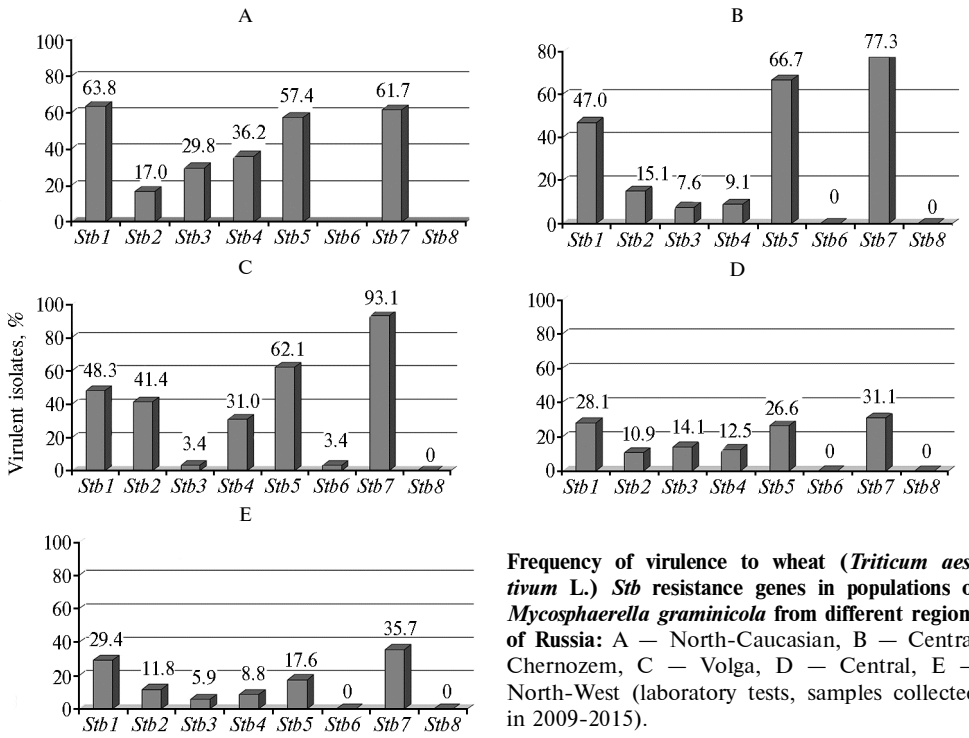
III — high-virulent.

The frequency and a set of virulence genes in the population, along with *Stb* gene effectiveness were assessed by percentage of virulent isolates among total tested ones. *Stb* genes were differentiated as effective if cultivars were susceptible to 0-20 % isolates, medium-effective when susceptible to 21-50 % isolates, and non-effective when susceptible to more than 50 % isolates.

*Results.* The following combinations of sporulation intensity and the degree of plant infection indicated virulence groups (Table 1). Isolates of group I was considered avirulent while isolates of groups II and III were considered to have the virulence gene.

**1. Parameters to differentiate *Mycosphaerella graminicola* isolates into virulence groups with due regard to sporulation and infection development in the wheat plants (*Triticum aestivum* L.) in laboratory tests**

| Mean degree of plant infection, % | Sporulation intensity, thousand spores per leaf | | |
|---|---|---|---|
| | low (up to 100) | medium (100-200) | high (more than 200) |
| Low (up to 20) | I | I | II |
| Medium (21-50) | I | II | III |
| High (51-100) | II | III | III |




**Frequency of virulence to wheat (*Triticum aestivum* L.) *Stb* resistance genes in populations of *Mycosphaerella graminicola* from different regions of Russia:** A — North-Caucasian, B — Central Chernozem, C — Volga, D — Central, E — North-West (laboratory tests, samples collected in 2009-2015).

A total of 47 isolates from the North-Caucasian region (14 from North Ossetia, 19 from Stavropol Territory, 13 from Krasnodar Territory, 1 from Chechnya), 66 isolates from the Central-Chernozem region (26 grom Voronezh region, 10 from Tambov region, 10 from Lipetsk region, 12 from Kursk region, 8 from Belgorod region), 29 isolates from the Volga region (9 from Saratov region and 20 from Volgograd region), 64 isolates from the Central region (59 from Moscow region, 3 from Tula region, 1 from Bryansk region, 1 from Ryazan region), and 34 isolates from the North-West region (6 from Leningrad region, 9 from Pskov region, 19 from Novgorod region) were tested in order to determine genetic structure of *M. graminicola* populations. All isolates were tested on eight cultivars with monogenic resistance except those from north-cau-

casian population which were tested on six cultivars because we have not the cultivars with genes *Stb6* and *Stb8* at that time.

We have revealed the virulence to resistance genes *Stb1*, *Stb2*, *Stb3*, *Stb4*, *Stb5* and *Stb7* with the frequency which varied significantly in all the populations of *M. graminicola* (Fig.).

North-caucasian population demonstrated different virulence genotypes, i.e. isolates had different combinations of relevant genes. A total of 55.3 % of the isolates had wide virulence spectrum (3-6 monogenic cultivars affected), 25.5 % isolates had 1-2 virulence genes, and 19.2 % isolates had no virulence genes. The cultivars with resistance genes *Stb1*, *Stb5*, and *Stb7* were affected most commonly (by 57.4-63.8 % isolates). The cultivars with the genes *Stb2*, *Stb3*, and *Stb4* were affected more rarely, being susceptible to 17.0-36.2 % isolates.

A total of 50 % isolates from central-chernozem population had only 1-2 virulence genes, in 43.9 % isolates the virulence was wider, and 6.0 % isolates had no virulence genes. The cultivars with resistance genes *Stb1*, *Stb5*, and *Stb7* were affected most commonly (47.0-77.3 % of virulent isolates). The cultivars with the genes *Stb2*, *Stb3*, and *Stb4* were resistant against most isolates (virulence frequency was 7.6-15.1 %). There were no virulence to the resistance genes *Stb6* and *Stb8*.

Isolates from the Volga population of *M. graminicola* had most various combinations of virulence genes. Only one isolate was virulent to all the cultivars (3.4 %), 13.8 % of isolates had one virulence gene. Most isolates had 3-6 virulence genes (65.5 %), the vast majority (93.1 %) was virulent against the cultivar with the gene *Stb7*. The frequency of virulence to *Stb5* was 62.1 %, while that to *Stb1*, *Stb2* and *Stb4* was 31.0-48.3 %. There was low virulence (3.4 %) against *Stb3* and *Stb6*. The cultivar with the gene *Stb8* was resistant against all isolates from this population.

Among isolates from central population, 42.2 % had no virulence genes. Remaining one had mostly 1-2 genes (40.6 %), rarely 3-5 genes (17.2 %). In general, frequency of virulence against *Stb* genes was low, reaching 26.6-31.1 % against *Stb1*, *Stb5*, and *Stb7*, half as much (10.9-14.1 %) against *Stb2*, *Stb3* and *Stb4*. No virulence (0 %) was found against *Stb6* and *Stb8*.

North-west population of *M. graminicola* showed the poorest diversity on virulence genotypes. Most isolates had no virulence genes (44.1 %) or only one virulence genes (32.3 %). The portion of isolates with 3-6 genes was significantly less (14.7 %). Virulence to the resistance genes *Stb1* and *Stb7* was more common (29.4-35.7 %), while that to the genes *Stb2*, *Stb3*, *Stb4*, and *Stb5* was significantly less (5.9-17.6 %). There were no isolates virulent to the genes *Stb6* and *Stb8*.

Therefore, regional populations of *M. graminicola* differed on virulence genotypes, gene sets and frequency. The populations from south zone (the North-Caucasian, the Central-Chernozem and the Volga regions) are more virulent as compared to the central and the north-west populations with avirulent isolates. Furthermore, the populations from south zone demonstrated variety of virulence genotypes and higher number of isolates with wide virulence spectrum (3-6 genes). Such geographic distribution of virulence of *M. graminicola* in Russia indicates that south populations are more aggressive. It can be a reasons of domination of *M. graminicola* in *Septoria/Stagonospora* complex on crops in southern Russia.

In all *M. graminicola* populations, higher frequency of virulence to the cultivars with the resistance genes *Stb1*, *Stb5*, and *Stb7* was observed indicating these *Stb* genes to be worst effective (Table 2). Plant response was usually manifested as necrotic spots on leaves with large affected area and pycnidia with

medium and high sporulation. The genes *Stb2*, *Stb3*, *Stb4* demonstrated considerable effectiveness against central, central-chernozem and north-west *M. graminicola* populations, being, however, distinctly less effective against isolates from the North-Caucasian and the Volga regions. The gene *Stb6* was highly effective against all five populations of *M. graminicola*. Only Volga population demonstrated virulence to the cultivar with this gene (at a low frequency of 3.4 %). The gene *Stb8* was absolutely effective against all tested isolates. The plants rarely had seeable signs of infection, and sporulation was absent or much reduced.

**2. Effectiveness of wheat (*Triticum aestivum* L.) *Stb* genes against regional Russian populations of *Mycosphaerella graminicola*** (laboratory tests, samples collected in 2009-2015)

| Region | Stb gene effectiveness (by percentage of virulent isolates) | | |
|---|---|---|---|
| | effective (< 20 %) | medium-effective (20-50 %) | non-effective (> 50 %) |
| North-Caucasian | *Stb2* | *Stb3, Stb4* | *Stb1, Stb5, Stb7* |
| Central-Chernozem | *Stb2, Stb3, Stb4, Stb6, Stb8* | *Stb1* | *Stb5, Stb7* |
| Volga | *Stb3, Stb6, Stb8* | *Stb1, Stb2, Stb4* | *Stb5, Stb7* |
| Central | *Stb2, Stb3, Stb4, Stb6, Stb8* | *Stb1, Stb5, Stb7* | Absent |
| North-West | *Stb2, Stb3, Stb4, Stb5, Stb6, Stb8* | *Stb1, Stb7* | Absent |

Thus, our study showed that 5 of 8 known wheat resistance genes (*Stb1-Stb5*) were functionally limited in natural populations of *Mycosphaerella graminicola* in Russia, and efficiency of the gene *Stb7* was not confirmed in all tested populations. Cultivars with the genes *Stb6* and *Stb8* were identified as potential sources of resistance to Septoria tritici blotch. They may be recommended for using in breeding and genetic programs focused on creation of wheat with resistance against *M. graminicola* in the Russian Federation.

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## Science Events

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**(3-6 May 2017, Heidelberg, Germany)**

**Organization:** EMBO/EMBL

The principal objective of this conference series is to provide an international forum for cutting edge research in chromatin and epigenetics. This conference provides «the focal hub» for people to present their research and exchange ideas in a European venue. The organising team assembles top speakers covering the latest advances in the field, incorporating diversity in every sense (gender, geography etc). The overarching goal of this conference series is to cover chromatin regulation, chromatin dynamics, signalling to chromatin, nuclear architecture and dynamics, developmental epigenetics, epigenomics, epigenetics and human diseases, genome stability, environmental epigenetics, and transgenerational inheritance. We also include talks on latest approaches for studying these biological questions, including functional genomics, system biology and super resolution microscopy.

**Topics:**

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- Nuclear architecture and dynamics
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# ALLELOCHEMICS: AN INTERACTION BETWEEN PHYTOPHAGES AND *Pseudomonas syringae* pv. *tomato* ON TOMATO *Solanum lycopersicum* PLANTS

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Abstract

Until recently, induced resistance to pathogens and phytophages considered separately and only in recent years the attention are being paid to the possibility of an induced cross-resistance. The aim of this work was to study the nature of the chemical interaction between plants and phytopathogenic microorganisms and arthropods phytophages, inhabiting the same ecological niche. The possibility of mutually-modifying effects of phytophagous and pathogens on quantitative and qualitative indicators of the defense response of tomato plants were shown. As the first order consumers the Western flower thrips *Frankliniella occidentalis* and whitefly *Trialeurodes vaporariorum* were chosen as most dangerous herbivores of greenhouse crops, and *Pseudomonas syringae* pv. *tomato*, a bacterial pathogen of tomato mottle, was used. The evaluation criteria were the changes in behavioral responses of herbivores and their demographic parameteres, and for pathogen the degree of infection development was expressed in points. Under the insects' free choice, the tomato plants previously infected with *P. syringae*, were more preferable by thrips, while remained not more attractive for whitefly. Attraction of thrips to infected plants may be due to the appearance and increasing content of volatile substances such as 2-methylbutanoic acid and dodecane, which are components of the thrips pheromone and allomons. Under the primary damage of plants by thrips and whitefly the further pathogen development differed (e.g., the thrips suppressed the disease, while the whitefly served as promoters for its development). Inhibition of the pathogen on plants damaged by thrips, may be due to an increase in the content of these chemical compounds, such as, for example, (E)-β-ocimene and α-humulene that are part of many essential oils and plant extracts with antimicrobial activity. The content of these same substances was increased in plants in response to inoculation with the pathogen. The results indicate both the differences and similarities of some signaling pathways and mechanisms of defense reaction in plants in response to induction phytophagous or phytopathogens. It identified the induced resistance and partial antagonism (down to completely opposite effect — the reduction of plant resistance) with respect to a group of consumers. A thorough assessment of the nature of these responses, its biochemical and molecular genetic basis will contribute to the strategy of environment-friendly plant protection.

Keywords: western flower thrips, greenhouse whitefly, induced defense, *Pseudomonas syringae*

One of generally acknowledged approaches to introduction of environmental science into plant growing is decreasing pesticide load on agrobiocenosis. This is associated with more thorough research of plants' adaptive potential including resistance to main biotic and abiotic stressors. In order to choose appropriate plant variety, it is necessary to enhance plant defense response, particularly to induce a non-specific resistance. There is good evidence of general defense mechanisms which are known as acquired immunity providing resistance under repeated infection. Induced resistance can be both local and systemic. It can manifest itself in plant parts and organs which are distant from the primary infection site. This phenomenon, deeply investigated in plant—phytopathogen system, includes activation of protection genes and metabolic changes which are detrimental to pathogen. A systemic cross-resistance against fungal, bacterial, and viral pathogens is shown to occur after preceding or concurrent contact with related

and unrelated agents [1, 2].

Analogous increase in resistance against phytophages after damage caused by conspecific or heterospecific arthropods species was observed well after [3]. Currently, there is massive evidence in favor of involvement of different protective mechanisms in these processes [4-6]. Induction of volatile compounds is important fast reaction in plants to damage caused by phytophages. These compounds (e.g. repellents, deterrents, etc.) play important role in complex interactions between different trophic levels [7, 8]. Some substances that provide induced plant resistance were chemically analyzed and identified. Such substances are not directly toxic to injurious object and render protective effect through regulatory mechanisms [9-12].

Until recently, induced resistance to pathogens and phytophages considered separately, and only in recent years the attention is being paid to the possibility of an induced cross-resistance. There is contradictory information about mutual influence of changes induced by phytophages or phytopathogens to organisms of the same biotope [13-16].

For the fist time, we studied a nature of plant response to simultaneous impact of multiple damaging agents with different damage localization and different damaging mechanisms.

The aim of this work was to study chemical interaction of plants with phytopathogenic microorganisms and arthropods phytophages as different level consumers in the same ecological niche.

*Techniques.* Tomato (*Solanum lycopersicum* L.) Belyi Naliv cultivar (sensitive) plants were individually grown in plastic cups at 24-26 °C with regular watering and complex fertilizing. Western flower thrips *Frankliniella occidentalis* and whitefly *Trialeurodes vaporariorum* were chosen as most dangerous herbivores of greenhouse crops, and *Pseudomonas syringae* pv. *tomato* (bacterial agent of tomato mottle, obtained from Russian State Agrarian University—K.A. Timiryazev Moscow Agricultural Academy) was a phytopathogen. The same objects were used as inducers.

In order to evaluate the response to phytopathogen, the tomato plants at a stage of 3-4 true leaves were inoculated with *P. syringe* ($10^6$ cells/ml, two lower true leaves were scratched with sand and then treated with pathogen suspension). Control plants were sprayed by water after damaging. Later, the plants with 5-6 true leaves and damage of 2.2-2.5 points (using 5-point scale) were used. Before the experiment, 2 lower damaged leaves were removed in all infected and intact plants. The plants prepared in such a manner were placed into glass cylinders (5 L) pairwise (test+control). Female Western flower thrips or whitefly imagos were put in these cylinders (30 insects per plant). Distribution of insects among the plants was counted 2 days after. After imago removal, the tomato plants were placed into isolated boxes to prevent repeated attack. Next generation thrips were counted after emergence of larvae, whiteflies were counted after appearance of second instar larvae. Response effectiveness was evaluated on the basis of number of phytophage offspring.

To examine the influence of the Western flower thrips or whitefly on a course of the disease, the plants were individually placed into glass cylinders with adult insects (30 insects per plant). One day after (two days for the whitefly), imagos were removed and the plants were left for 5 or 7 days to allow larvae of the Western flower thrips and the whitefly, respectively, to inflict damage (the load was $13.8 \pm 1.47$ and $25.4 \pm 4.59$ larvae per plant for the Western flower thrips and the whitefly, respectively). After larvae counting (in case of thrips the larvae were further removed), *P. syringe* ($10^6$ cells/ml) was applied to damaged and intact plants. In control plants, upper leaves were pre-scratched with sand. Leaf

damage was evaluated 7 days after the inoculation.

Volatile substances were extracted from upper tomato leaves. The leaves were milled, and samples (1 g) were placed into 50 ml a conical flask to which hexane was added up to upper edge of plant material. Extraction was performed by sonication in ultrasound bath Grad 13-35 (OOO Grad-Technology, Russia) for 15 minutes. After decantation the extracts were filtered through anhydrous sodium sulfate. Hexane was distilled in vacuum at 35 °C, and extract was concentrated to 500 μl under nitrogen and stored at –18 °C.

Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) using a GCMS-QP5000 complex (Shimadzu Corp., Japan). Components were identified by comparison of retention times in their mass spectra to reference values. Specifications of gas chromatographic separation: 250 °C as solvent temperature; sample injection without flow separation (0.2 min); initial temperature of column (DB-5) 40 °C, temperature increase rate 10 °C /min, final temperature of column 270 °C, exposure at final temperature 15 мин; carrier gas helium with flow velocity 1 $mm^3$/min; interface and detector temperature 280 °C. Specifications of mass spectrometry: ionizing electron energy 70 eV, interface and ion source temperature 280 °C, TIC (total ion current) identification.

Tests were performed in 10 replicates. Each test data were analyzed using ANOVA. Differences were considered significant at $P \leq 0.05$.

*Results.* Evaluation criteria for phytophage were behavioral changes and demographic parameters (as the insects can refuse to eat or lay eggs on the plant due to plant defense reaction). For pathogen, the infection development was used as a parameter.

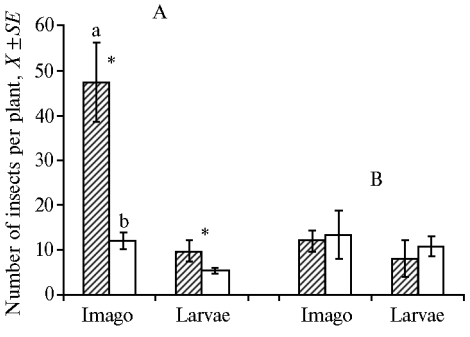


**Fig. 1. Behavior of the Western flower thrips *Frankliniella occidentalis* Pergande** (A) **and the whitefly *Trialeurodes vaporariorum* Westwood** (B) **on plants of tomato (*Solanum lycopersicum* L.) sensitive cultivar Belyi Naliv infected by *Pseudomonas syringae*:** a — test group, b — control group. Total sample number — 600, number of replications — 10. The data significant at $P \leq 0.05$ are marked with asterisk (laboratory tests).

Tests of defense response in tomato plants to phytopathogen revealed that female Western flower thrips preferentially ate and laid eggs on phytopathogen-infected plants, when free choice. Number of adult thrips on infected plants was 3.0 times higher than that in control group. Such distribution of female insects resulted in offspring size (1.8 times higher than that in control group). However, in whiteflies, there were no significant differences in attractiveness to imago or in offspring size (Fig. 1).

*P. syringae* infection resulted in production of 2,2-dimethylheptane, 2-methylbutanoic acid, 3-methyloctane, dodecane, stearic acid. Content of 1,4-dimethylcyclohexane, (E)-β-ocimene and α-humulene increased 2.0-fold, butyl isobutyl phthalate and dibutyl phthalate content increased 2.6-fold and 8.7-fold, respectively, as compared to control, linalool, palmitine acid and linolenoic acid were 1.3 times higher.

Study of influence of primary damage of the tomato plants by Western flower thrips larvae and imago on disease development revealed systemic induced resistance. Average weighed score infection for upper leaves on the plants damaged by phytopphages was 3.0 times lower than that in control plants. On the contrary, the whitefly did not act as resistance inducer in tomato: test group showed significant 1.8-fold increase in damage score (Fig. 2).

Damage by the thrips resulted in 2.0-fold β-ocimene and α-humulene level, 1.7-fold α-terpinene level, 1.2-fold sabinene level, more than 5-fold diethyl phthalate and diisooctyl phthalate levels, 2.0-fold 9-octadecenol level, and 1.2-fold diisobutyl phthalate, dibuthyl sebacinate, dibutyl phthalate levels as compared to control. Damage by the whitefly resulted in production of cymene, myrcene, methyl salycilate and an increased content of α-pinene.

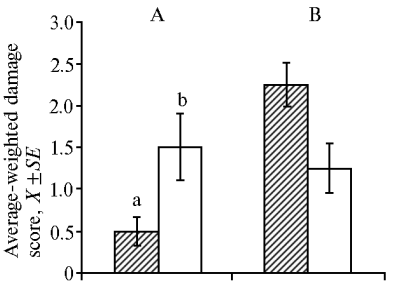


**Fig. 2.** ***Pseudomonas syringae*** **infection in the plants of tomato (*Solanum lycopersicum* L.) sensitive cultivar Belyi Naliv damaged by the Western flower thrips *Frankliniella occidentalis* Pergande** (A) **and he whitefly *Trialeurodes vaporariorum* Westwood** (B)**:** a — test group, b — control group. Total sample number — 600, number of replications — 10. The data significant at $P \leq 0.05$ are marked with asterisk (laboratory tests).

It should be noted that the plants face different external factors during vegetation. These factors induce wide range of both synergetic and anatagonistic responces. Due to this fact, studying the nature of such response under interaction with several groups of heterotrophic consumers (arthropods phytophages and pathogenic microorganisms) in the same trophic niche is of great interest. Moreover, sequential or concurrent attacks of phytophages and infectious agents are commonly observed. This causes the necessity to study mutual effects of these agents.

Analysis of publications indicates possible mutual modifying effects of phytophages and phytopathogens on quantitative and qualitative characteristics of defense response in plants. It is reported that damage by thrips and aphid prevents plants from development of fungus *Colletotrichum orbiculare* [17], and green dock leaf beetle *Gastrophysa viridula* can induce plant resistance to phytopatogens *Ramularia rubella* and *Venturia rumicis* [14]. In *Arabidopsis thaliana* damaged by larvae *Pieris rapae*, a significantly higher resistance was found to bacterial pathogens *P. syringae* pv. *tomato* and *Xanthomonas campestris* pv. *armoraciae*, but not to fungal pathogen *Alternaria brassicicola* [18]. In tomato, larvae *Helicoverpa zea* negatively affect both larvae (in case of repeated attack) and *P. syringae* pv. *tomat*o. Moreover, in the same tomato plants the inoculation with *P. syringae* causes systemic induced resistance against both conspecific pathogen species and moth species *H. zea* and *Spodoptera exigua*. Unlike, tomato mosaic disease promoted body weight gain in larvae *S. exigua*, though inhibited proliferation of aphid *Myzus persicae*, therefore, its effects on the phytophages with different feeding habits were opposite [16, 19].

Pathogen inhibiting on plants damaged by thrips, may be due to an increase in the content of certain chemical compounds. (E)-β-ocimene and α-humulene are components of many essential oils and plant extracts with antimicrobial activity [20- 22]. Terpenoid α-terpinene significantly inhibits growth of fungi *Sporothrix* and *Ceratocystis minor* [23]. Sabinene possesses antimicrobial activity [24, 25]. Diethyl phthalate inhibits growth of *Rhizobium vitis* and *Bacillus subtilis* [26]. Other ethers of phthalic acid demonstrate significant antibacterial and antifungal activity against three gram-positive and two gram-negative bacteria and three pathogenic fungi [27].

It is more difficult to interpret the results of chemical analysis for the plants damaged by whitefly, where *P. syringae* infection score is significantly higher than that in control group. Probably, the amount of secreted substances is of importance. Despite antimicrobial activity of monoterpens, 50 and 150 ppm o-cymene promotes growth of certain oomycetes [28].

Accumulated data indicate that biological inductors can significantly change plant metabolism resulting in increasing production of secondary me-

tabolites secreted by intact plants or in de novo synthesis of protective substances (volatile or extractable compounds) which were not previously produced. Probably, each pathogen or phytophage species triggers different defense mechanisms and induce certain chemical reactions in plant.

In our experiments, the plants previously infected by *P. syringae*, were more preferable for thrips than intact ones, though both plant groups were equally attractive for whitefly. Under the primary damage of plants by thrips and whitefly the further pathogen development differed (the thrips suppressed the disease, while the whitefly could promote its development). Probably, it is associated with specific effects of the inductors (phytophages and phytopathogens) on target tissues that causes distinct responses manifested in unequal accumulation of previously secreted and newly synthesized substances.

Attraction of thrips to infected plants may be due to the synthesis and increasing content of some volatile substances. In fact, 2-methylbutanoic acid and dodecane are components of the thrips pheromone and allomons [29, 30]. Western flower thrips showed a significant electro-antennographic response to α-humulen [31], and (E)-β-ocimene was determined as minor component with synergetic activity in composition of aggregated pheromone of this pest [32]. Thrips *Cycadothrips chadwicki* (*Thysanoptera*: *Aeolothripidae*) is attracted by (E)-β-ocim-ene [33]. Linalool and linoleic acid are found in chrysanthemums, the food plants for the Western flower thrips, and linalool is said to be the substance that attracts the insect by its flavor [34, 35]. Dibutyl phthalate is characterized by slight fruit flavor that can attract insects, and butyl isobutyl phthalate is found in some flowering plants [36].

Summarizing data on chemical composition of the tomato plants allows us to reveal common pattern of damage caused by pathogen and the Western flower thrips. In both cases, there was significant increase in content of (E)-β-ocimene, α-humulene and phthalates.

Thus, our results indicate both the differences and similarities of some signaling pathways and mechanisms of plant defense response to induction by phytophages or phytopathogens. We found both induced resistance and partial antagonism against a group of consumers, down to completely opposite effect of reducing plant resistance. Probably, plant response depends on taxonomic position of acting biotic agents and can be directed toward both conspecific and heterospecific species. A thorough study of induced defense reactions will give the opportunity to determine their ecological role and biochemical and molecular genetic mechanisms. It will contribute to the strategy of development of synthetic elicitors with different selectivity against certain biotic agent groups.

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# STUDYING THE ACTIVITY OF *Chenopodium album* SEED EXTRACTS AND *Fusarium sambucinum* CULTURE LIQUID AGAINST SEVERAL PLANT PATHOGENIC FUNGI

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## Abstract

Plant extracts and microbial culture liquids contain a great number of bioactive substances, including potential biofungicides, which are effective against plant deseases. Screening of extracts and cultural filtrates for ability to suppress several pathogenic fungi that damage various agricultural plants can result in discovery of new compounds with a broad range of fungicidal activity. This work is the first report on in vitro testing the activity of plant extracts, which obtained by serial extractions of *Chenopodium album* seeds with hexane followed by ethyl acetate and then with ethanol, and also *Fusarium sambucinum* culture liquid filtrate (CLF), against six widespread fungi (*Alternaria alternata*, *A. dauci*, *A. radicina*, *Bipolaris sorokiniana*, *Septoria tritici*, *Stagonospora nodorum*) that are strongly pathogenic for several economically important crops. These fungi were found to demonstrate different sensitivity to CLF and seed extracts. Thus, CLFs with the activity level that was shown earlier for *Stagonospora nodorum* and *A. radicina* (effective dilutions up to 1:200 и 1:5, respectively) fully inhibited spore germination of all pathogens except *B. sorokiniana*, saving their antifugal activity to dilutions 1:5 (for *A. dauci*) or 1:50 (for *Septoria tritici*). Seed extracts possessed no toxicity against *Stagonospora nodorum*, *Septoria tritici*, *A. dauci* and *A. radicina*, but some of them reduced *A. alternata* and *B. sorokiniana* spore germination. For instance, after seed extraction with hexane followed by ethyl acetate, the number of germinated *B. sorokiniana* spores decreased by 66 % as compared to control. Hyphae of spores germinated in diluted CLF were morphologically defective and much shorter than control ones. Besides, a significant growth retardation of *Stagonospora nodorum*, *Septoria tritici* and *B. sorokiniana* mycelia was observed on agar media supplemented with CLF (100 μg/ml). Collectively, these results suggest that further research of CFL and the seed extracts can result in identification of anifungal metabolites, which could be promising as biofungicides against leaf and glume blotches of wheat, spot blotch or common root rot of other cereals, and Alternaria diseases of carrot.

Keywords: *Chenopodium album*, *Fusarium sambucinum*, fungitoxicity, biofungicides, glume and leaf blotches, spot blotch, common root rot, Alternaria diseases

Plants and microorganisms produce many biologically active compounds, which functions are the subject of intense research. In particular, one of the modern trends in agricultural science is seeking for active ingredients to create biofungicides and other biopesticides [1-3] as an alternative or an adjunct to available chemical pesticides [4-8]. These promising compounds of microbial or plant origin include antimicrobial peptides and other secondary metabolites [9-11]. Their most accessible sources are extracts of wild (often weed) plants, as well as the culture liquid (CL) of fungi and bacteria grown in vitro [12-17].

Moreover, plant extracts and culture filtrates with pesticidal activity may be used to treat plants without additional (sometimes cost and complicated) purification [18-21]. For example, it was shown that the methanol extracts of lamb's quarters (*Chenopodium album*) inhibit accumulation of *Macrophomina phaseolina* fungus biomass in submerged culture [22], and a non-phytotoxic CL

of the FS-94 isolate of *Fusarium sambucinum* Fuckel effectively inhibits in vitro germination of spores in causal agents of Stagonospora glume blotch and black rot of carrots [16, 17).

It is obvious that a particular interest for crop protection is in searching for extracts and filtrates, able to simultaneously suppress the development of several fungi pathogenic to various crops.

In view of this, we for the first time investigated the activity of the extracts of *Chenopodium album* seeds and CL filtrates of a non-pathogenic *Fusarium sambucinum* against six common plant pathogenic fungi (*Alternaria alternata*, *A. dauci*, *A. radicina*, *Bipolaris sorokiniana*, *Septoria tritici*, *Stagonospora nodorum*), which cause serious damage to many economically important crops.

*Techniques.* To prepare plant extracts, 30 g of lamb's quarters (*C. album*) seeds were powdered and extracted sequentially with hexane, ethyl acetate and ethanol (a ratio for each solvent 1:8 w/v) under vigorous stirring for 1 h at room temperature. After each extraction step, the homogenate was centrifuged at 10,000 g and at 4 °C for 10 min, the supernatant was filtered through a paper filter and evaporated to dryness in a rotary evaporator. The precipitate was stored at –80 °C and dissolved immediately prior to experiments in a minimal volume of 5 % dimethyl sulfoxide (DMSO).

The culture liquid filtrate (CLF), obtained by submerged cultivation of the FS-94 isolate of *F. sambucinum* (16), was purified from culture medium components and low molecular weight exometabolites, not assimilated by the fungus, by means of ultrafiltration fractionation [23], while washing the CLF concentrate with sterile distilled water ($sdH_2O$). Preparations of the concentrates transferred into aqueous medium were adjusted with $sdH_2O$ up to a volume of the initial CLF, sterilized by filtration through a 0.22 μm membrane (GPWP type, MilliporeSigma, USA), frozen and stored until use at –20 °C.

*Alternaria alternata* (Fr.) Keissl; *Bipolaris sorokiniana* (Sacc.), Shoemaker; *Septoria tritici* Desm. and *Stagonospora nodorum* Berk., Castellani & E.G. Germane isolates were obtained from the National Collection of Phytopathogenic Microorganisms in the All-Russian Research Institute of Phytopathology, and *A. dauci* (J.G. Kuhn), J.W. Groves & Skolko and *A. radicina* Meier, Drechsler & Eddy isolates were from the Institute for Breeding Research on Horticultural Crops, Germany. To prepare suspensions, on the day of the experiment fungal spores, using $sdH_2O$, were washed off the surface of the colonies grown for 14 days on PDA at 25 °C (*A. alternata*, *B. sorokiniana*, *Stagonospora nodorum*, *Septoria tritici*) or on vegetable broth agar medium at 22-23 °C (*A. dauci*, *A. radicina*). Spores were separated from mycelial fragments by filtering through cotton and nylon filters, then pelleted by centrifugation at 3,000 g for 20 min and resuspended in a minimal volume of $sdH_2O$.

To evaluate the plant extracts and CLF for fungitoxicity, a method of spore germination in a thin layer of agar was used. To do this, equal volumes of the initial suspension and the test samples were mixed so that the density of a final suspension was $10^4$/ml for *A. alternata, A. dauci*, *A. radicina* and *B. sorokiniana* or $\mathbf{3}\times10^3$/ml for *Stagonospora nodorum* and *Septoria tritici*. The suspensions were incubated at a constant slow stir for 25-30 minutes at room temperature and then applied onto slides coated with 1 % aqueous agar (200 μl per slide, 3 slides per each test option), which were placed in a moist chamber. The spores were germinated in the dark at 22 °C for 16-18 (*A. dauci*, *A. radicina*, *Stagonospora nodorum*, *Septoria tritici*) or 5-6 hours (*A. alternata* и *B. sorokiniana*). As a control we used spore suspension in $sdH_2O$ in the experiments with CLF, and in sterile 2.5 % DMSO for the analysis of the *C. album* extracts. The effects in-

duced by CLF and extracts on germination of plant pathogens were evaluated by microscope, scanning at least 600 spores in each treated option and the control. Germination frequency was expressed as a percentage relative to either a number of spores germinated in the control or a total number of spores, scanned in the control and in the experimental options.

To evaluate the effect of CLF on the radial growth of colonies of *Stagonospora nodorum*, *Septoria tritici* и *B. sorokiniana*, they were grown for 14-16 days in the dark at 25 °C on the PDA containing the filtrate (100 μl CLF/ml culture medium). The mycelium fragments were used as the inoculum to be spiked to the center of Petri dishes (9 cm in diameter). Control cultures were grown under the same conditions on media without the addition of CLF. As cultures grew, the diameter of the colonies (2 measurements per each colony in 2 different directions, 3 dishes per test option) was measured and the average diameter of the colonies for each of the pathogens was calculated.

Tests on the spore germination and the study of the growth trends in the plant pathogens were repeated at least 3 times. Statistical processing of the results was performed using Statistica 6.0 software (SoftStat Inc., USA). The significance of differences vs. control ($p \leq 0.05$) was determined using *t*-test for independent variables.

*Results.* As we have previously found that the CLF was capable of inhibiting the germination of *Stagonospora nodorum* and *A. radicina* spores [16, 17], in this study we used these plant pathogens as the reference test objects for monitoring the activity of the resulting filtrates when evaluating their fungitoxicity against *Septoria tritici*, *A. dauci*, *A. alternata* and *B. sorokiniana*. These species are causative agents of dangerous diseases of important agricultural crops, such as leaf and glume blotches of wheat (*Septoria tritici*), barley leaf spot and common root rots of the grasses (*B. sorokiniana*), carrot leaf blight (*A. dauci*), dark mildew of wheat ears, as well as blackspots of a variety of other plants (*A. alternata*) [24-27].

**1. Comparative fungicidal activity of the culture liquid filtrate** (CLF) **of *Fusarium sambucinum* against *Septoria*, *Helminthosporium* and *Alternaria* pathogens**

| Pathogen | Germination of spores in the CLF, % of control | The CLF titer |
|---|---|---|
| *Stagonospora nodorum* | 0 | 1:200 ($p = 0.02$) |
| *Septoria tritici* | 0 | 1:50 ($p = 0.01$) |
| *Bipolaris sorokiniana* | 98.3±1.3 | No dilution |
| ***Alternaria** radicina* | 0 | 1:5 ($p = 0.03$) |
| *A. alternata* | 5.0±0.80 | 1:5 ($p = 0.02$) |
| *A. dauci* | 2.0±0.04 | 1:5 ($p = 0.04$) |

N o t e. The table presents the average values for 600 spores per each test option and a standard error (mean±SE) and the maximum CLF dilutions, with which there was still significant ($p = 0.05$) inhibition of spore germination vs. control.

The tests have shown that the CLF with a typical fungicidal activity against the reference test objects (the absence of germinated spores in undiluted filtrates and the expected titers of 1:200 and 1:5, respectively, for *Stagonospora nodorum* and *A. radicina*) had a similar activity against *Septoria tritici*, *A. alternata* and *A. dauci*, and the inhibitory action of the CLF against *S. tritici* was more effective, although this species was 4 times less sensitive than the other causative agent of septoria wheat, i.e. *Stagonospora nodorum* (Table 1). As in the case with *A. radicina*, a statistically significant inhibition of germination of *A. alternata* and *A. dauci* spores was observed up to 5-fold dilution of the CLF, and in the initial filtrates the germination was inhibited almost completely. The fungus *B. sorokiniana* appeared to be resistant to the effect of CLF.

Note that all the investigated plant pathogens, including *B. sorokiniana*,

hyphae of spores, which retained the ability to germinate after exposure to CLF, had morphological defects, particularly marked thickenings, and were significantly shorter than in control (Fig. 1 ).

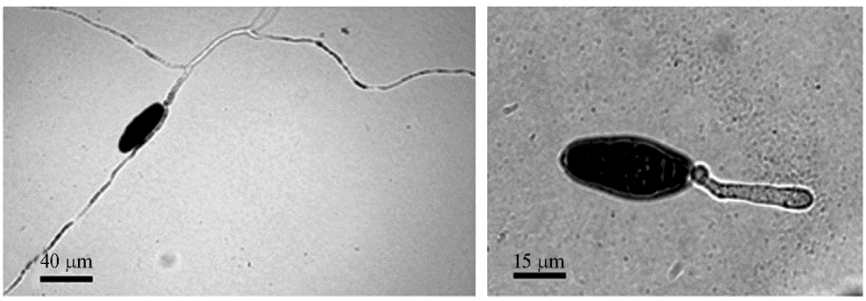


**Fig. 1.** ***Bipolaris sorokiniana*** **spores, germinated in sterile distilled water** (left) **or in the culture liquid filtrate of** ***Fusarium sambucinum*** (right).

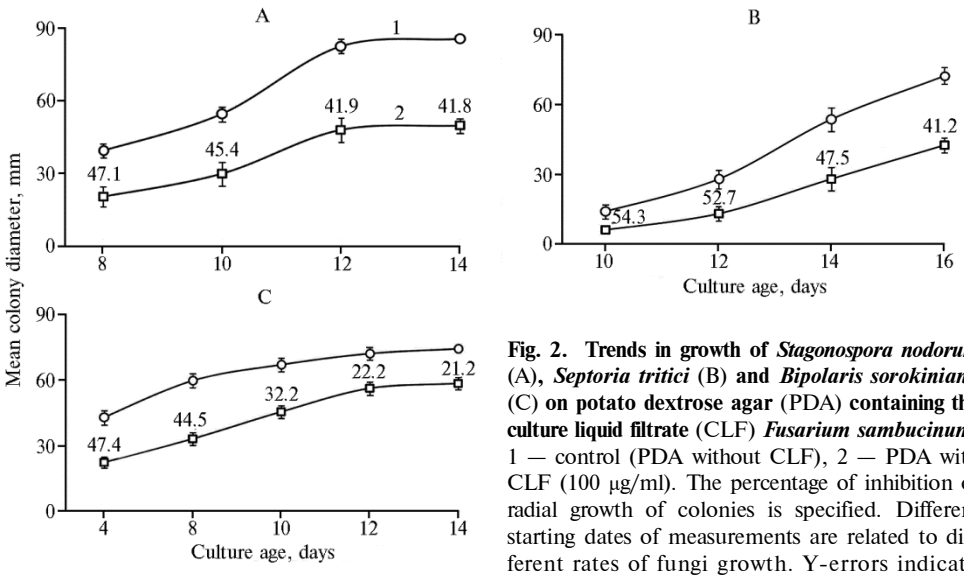


**Fig. 2. Trends in growth of** ***Stagonospora nodorum*** (A), ***Septoria tritici*** (B) **and** ***Bipolaris sorokiniana*** (C) **on potato dextrose agar** (PDA) **containing the culture liquid filtrate** (CLF) ***Fusarium sambucinum***: 1 — control (PDA without CLF), 2 — PDA with CLF (100 μg/ml). The percentage of inhibition of radial growth of colonies is specified. Different starting dates of measurements are related to different rates of fungi growth. Y-errors indicate mean deviations for two experiments (6 replicates per test option per each experiment).

An analysis of the growth trends for the *Stagonospora nodorum*, *Septoria tritici* and *B. sorokiniana* mycelium in the presence of CLF revealed that, when cultured in media containing the filtrate, these plant pathogens markedly decreased their colony size (Fig. 2). The observed fungistatic effect on the growth of *Stagonospora nodorum* and *Septoria tritici* was stable and changed only slightly by the end of cultivation. In contrast, the inhibitory effect of the CLF on the growth of *B. sorokiniana* mycelium gradually weakened, however, the differences vs. control remained until the completion of observations (see Fig. 2).

Testing of preparations obtained by sequential extraction of *C. album* seeds using hexane, ethyl acetate and ethanol demonstrated that none of them possessed fungitoxicity against *Stagonospora nodorum*, *Septoria tritici*, *A. dauci* and *A. radicina*. Meanwhile, some of these plant extracts were able to inhibit to different extents the germination of the *A. alternata* and *B. sorokiniana* spores (Fig. 3). Thus, incubation of the *A. alternata* spores in the extracts obtained using hexane resulted in significant reduction in the number of germinated spores (by 16 %), and further decreased it in the ethanol-based extracts by 14 % more.

The number of the *B. sorokiniana* spores, which germinated after incubation in extracts using hexane and ethyl acetate, was reduced by 34 and 32 %, respectively. Therefore, combined inhibition of germination using *C. album* ex-

tracts for these plant pathogens reached 30 % (*A. alternata*) and 66 % (*B. sorokiniana*). Interestingly, that if *B. sorokiniana* concerned, the fungitoxicity effect, observed in two of the three experiments on the concurrent use of the CLF and preparations after extraction with hexane or ethyl acetate (Table 2), according to Limpel's formula ($E_0 < E_{real}$), had the synergistic action [28]. Furthermore, the estimated excess of the additive inhibitory effect in case of a combined use of the filtrate and both of the sequentially produced extracts amounted to 27-28 %.

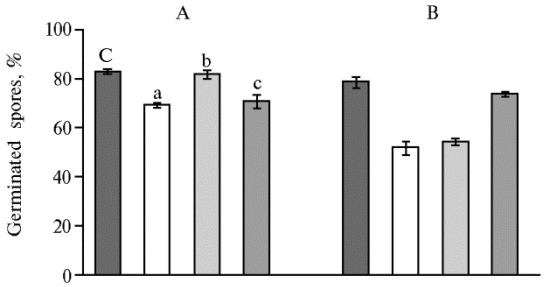


**Fig. 3. Germination of the *Alternaria al*ternata (A) and *Bipolaris sorokiniana* (B) spores when incubated with the preparations, obtained from the seeds of lamb's quarters (*Chenopodium album*) by sequential extraction with hexane** (a), **ethyl acetate** (b) **and ethanol** (c). The extracts were dissolved in 2.5 % dimethyl sulfoxide (DMSO); C — control (2.5 % DMSO). The spores were placed in the studied extracts. To calculate the percentage of germinated spores (mean of 3 experiments), at least 600 spores per test option in each experiment were scanned after 5-6 hours. Standard errors of mean are given.

**2. Inhibition of spore germination** (% of total) **in *Bipolaris sorokiniana* in a combined use of the *Fusarium sambucinum* culture liquid and *Chenopodium album* seed extracts**

| CLF | EH | EEA | CLF + EH | | CLF + EEA | |
|---|---|---|---|---|---|---|
| | | | $E_0$ | $E_{real}$ | $E_0$ | $E_{real}$ |
| 0.8 | 36.3 | 31.9 | 37.1 | 49.6 | 32.7 | 47.2 |
| 1.3 | 34.7 | 29.6 | 36.0 | 48.5 | 30.9 | 46.7 |
| 0.6 | 31.0 | 34.2 | 31.4[a] | 31.9[a] | 34.8[b] | 35.2[b] |

Note. EH, EEA — extraction withhexane or ethyl acetate, respectively; titer of culture liquid filtrate (CLF) 1:2. $E_0$ — expected additive effect, %; $E_0 = (X + Y) - XY/100$, where X — CLF, Y — EH or EEA. $E_{real}$ — Effect (%) obtained in the experiment. The differences between E0 vs. Ereal are significant at $p = 0.05$, except for the values designated with the same Latin letters. If E0 < Ereal (28), the inhibitory effect is synergistic.

Thus, our results on evaluating the influence of the *Fusarium sambucinum* culture liquid and *Chenopodium album* seed extracts on in vitro development of six pathogenic fungi suggest a promising outlook of further research devoted to the composition of these products aimed at identifying the metabolites responsible for the antifungal activity, as well as to assume the possibility of creating biopesticides based on them against leaf and glume blotches of wheat, Alternaria diseases of carrots and spot blotch of the grasses.

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# K.S. MERESCHKOWSKY AND THE ORIGIN OF THE EUKARYOTIC CELL: 111 YEARS OF SYMBIOGENESIS THEORY

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Abstract

Theory of symbiogenesis proposed 111 years ago by K.S. Mereschkowsky, postulated the emergence of plants through the integration of phototrophic microbes into heterotrophic host cells. To date, it has become apparent that this theory can be relevant to describe an extremely wide range of evolutionary processes induced in the systems of cooperative adaptation. We have proposed a new definition of symbiogenesis as of a multi-stage process converting the symbiotic system into the entire organism (holobiont), based on the formation of an integral partners' system of heredity. This system emerges in the course of transition of partners from facultative to obligatory symbiosis and evolves from the functional integrity, based on the signaling partners' interactions (symbiogenome) to the structural integrity, based on the exchange of partners' genes (hologenome). Trade-off between the proposed approach with the symbiogenesis theory of K.S. Mereschkowsky is shown using the material of paper «The nature and origin of chromatophores in the plant kingdom» (C. Mereschkowsky 1905. Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Biologisches Centralblatt 25: 593-604). We analyzed the relationship of traditional argumentation of symbiogenesis (genetic continuity of the cellular organelles based on their transmission in the host generations) with its current argumentation, used by the Theory of Serial Endosymbioses (TSE) proposed by L. Margulis: a) the presence of rudimental organelle genomes; b) phylogenetic kinship of organelles with the free-living and symbiotic microorganisms; c) identification of the transitional cellular forms bridging the free-living bacteria and organelles. Modern versions of TSE suggest that the introduction of aerobic α-proteobacteria into anaerobic archaea gave rise to eukaryotes, which further evolved through the recruiting into their cellular structures of additional endosymbionts, including phototrophic cyanobacteria and viruses. The forms of archaea, close to the common ancestor of eukaryotes, are represented by the newly discovered chemotrophic *Lokiarchaeota* which cells are characterized by a number of eukaryotic features, including the actin cytoskeleton and the ability for endocytosis. Convincing evidence in favor of TSE was obtained in the study of cyanelles (phototrophic symbionts of protozoa, combining the properties of free-living cyanobacteria and plastids), as well as insects' endocytobionts with the deeply reduced genome (less than 200 kb), which, in contrast to mitochondria and plastids, retained the ability to implement autonomously the basic template processes — replication, transcription, translation. One of the intriguing destinations of modern TSE is the analysis of the emergence of the nucleus and chromosomes, which may be associated with the introduction of highly organized «giant» DNA-viruses into ancestral cellular forms having RNA genomes (the hypothesis of viral eukaryogenesis).

Keywords: symbiogenesis theory, evolution of bacterial genome, plastids and mitochondria, origin of eukaryotic cell, holobiont, hologenome and symbiogenome, theory of serial endosymbiosis

Development of the theory of symbiogenesis is the milestone achievement of 20th century biology. It was conceived at the turn of 19th century based on a hypothesis on symbiotic origin of chloroplasts proposed by A.S. Famintsyn and K.S. Mereschkowsky. To date, the theory of symbiogenesis has developed into a general biological concept, which ascribes evolution of major cell organization forms to their symbiotic relations, represented by the formula: bacteria + archaea = eukaryotes. At the same time, the correlation between the classical theory of

symbiogenesis and its present-day versions, considering the formation of cell organelles as further evolution of symbiotic bacteria, representing various levels of their integration with host organisms, is still poorly understood.

Our purpose was to perform a hindsight analysis of symbiogenesis concept, based on comparison of its classical version, presented by K.S. Mereschkowsky in 1905-1910, with the theory of serial endosymbiosis, developed in 1960-1980, as well as with present-day hypotheses on origin of the main components of an eukaryotic cell, including the nucleus and chromosomes, founded on this theory. This approach allows us to give a new definition of symbiogenesis, as well as to demonstrate the continuity of various stages of integrative evolution, during which genetically independent organisms form supraspecific complexes with shared systems of heredity.

In the authorized Russian translation of the first work of K.S. Mereschkowsky, which focused on symbiogenesis concept justification [1] and was published in German, several out-of-date taxonomic names are replaced by modern equivalents (doi: 10.15389/agrobiology.2016.5.746rus). The addendum published by K.S. Mereschkowsky two months after the main paper had its own reference list, and we took the liberty of combining it with the main reference list; the annotated strict English translation may be recommended for clarifications (2).

Notes in the text will be useful for comparison of out-of-date and modern terms and definitions.

Symbiogenesis as a key strategy of evolutionary process (*notes to translation*). The theory of symbiogenesis proposed by K.S. Mereschkowsky 111 years ago [1] currently represents one of the best developed divisions of evolutionary biology. Development of this theory was preceded by works of A.S. Famintsyn and O.V. Baranetsky [3], who isolated the free-living green algae *Trebuoxia* apart from lichens which were previously considered as unitary organisms. Based on these results, A.S. Famintsyn [4] attempted to prove that phototrophic components (chromatophores) extracted from another lower plant, i.e. algae *Vaucheria*, are able to exist independently; however, these attempts were fruitless.

Nevertheless, the works of A.S. Famintsyn formed the basis for development of symbiology [5], which was closely related to evolutionism from the very start. Anton de Bary [6] defined symbiosis as «the living together of differently named organisms», where mutualistic and antagonistic (parasitic) interactions are connected by frequent evolutionary transitions [7]. Considering symbiosis as an analog of hybridization (in Greek symbiosis — living together, irregular marriage), A. de Bary preempted the development of symbiogenetics, analyzing supraspecific systems of heredity, which control the development of deeply integrated superorganismic complexes (symbiotic, biocenotic, etc.) [8].

**Properties of two plasmas — major cellular forms recognized by K.S. Mereschkowsky [9, 10]**

| Mycoplasma (bacteria, cyanophyceae[a], fungi) | Amoeboplasma (animals, protozoa) |
|---|---|
| Has the ability of protein formation from inorganic substances | Has no ability of protein formation from inorganic substances |
| Tolerates the temperatures up to 90 °C and higher | Does not tolerate the temperatures above 50 °C |
| Consume hydrocyanic acid, strychnine, morphine. High tolerance | Hydrocyanic acid, strychnine, morphine are strong poisons. Low tolerance |
| No amoeboid movement, no formation of pulsating vacuoles | Amoeboid movement, formation of pulsating vacuoles |
| Rich in nuclein | Does not contain nuclein |
| Can exist without oxygen | Can not exist without oxygen |

N o t e. a — cyanobacteria.

The symbiogenesis concept was formulated by K.S. Mereschkowsky in the context of the theory of two plasmas [9, 10], providing for existence of cell

organization forms with independent origin, which, however, are able to combine into novel organisms (see table). According to K.S. Mereschkowsky, the plant living form appeared as a result of invasion of phototrophic representatives of mycoplasma (cyanobacteria) into heterotrophic amoeboplasma; the former give birth to cell organelles (chromatophores). K.S. Mereschkowsky considered genetic continuity of these cell components (binary fission and inability to differentiate from unstructured protoplasm), related to vertical transmission of organelles during reproduction of hosts, as the main criterion of symbiogenic origin of these cell components [1, 9].

Creation of the theory of symbiogenesis by K.S. Mereschkowsky became the culminating point of a 40-year long development of concepts of close cooperation between phototrophic and heterotrophic organisms. Based on the data on binary fission of chloroplasts provided by C. Nageli [11], as well as on the data on their development from colorless proplastids provided by A.F.W. Schimper [12, 13], K.S. Mereschkowsky [1, 9] came to a conclusion that chloroplasts are independent organisms, rather than plant cell organs, developing during its protoplasm differentiation. The author's reference to genetic concepts, which was quite novel in the early 19th century, played an important role in substantiation of symbiotic origin of chloroplasts.

Novelty of K.S. Mereschkowsky's approach was not associated with the hypothesis on symbiotic origin of chloroplasts proposed by him, as it was earlier formulated by A. Schimper [12, 13] and F. Schmidt (14); it was attributable to identification of possible ancestors of chloroplasts, i.e. cyanobacteria and zoochlorellas (unicellular algae, photobionts of invertebrates). This opened up the possibility of verification of the theory of symbiogenesis. The conclusions on symbiogenic origin of differences between plant and animal living forms, observed at the cellular, organismic and ecological levels, were also a breakthrough. The assumption that the presence of photosynthesizing symbionts can make an aggressive lion behave like a peaceful palm tree is a good example of the power of K.S. Mereschkowsky's scientific foresight. The hypothesis on direct origination of plants from animals by obtaining chloroplasts was not confirmed, but several cases of the presence of chloroplasts were revealed in animals, changing over to phototrophy. A shellfish named *Elysia*, which is capable of long-term preservation of chloroplasts obtained with vegetable food (kleptoplasts), is a well-known example. At that, *Elysia* not only assimilates the products of photosynthesis taking place in chloroplasts and completely refuses from heterotrophic nutrition, but also obtains some chloroplast genes: the animal transforms into a functional and genetic equivalent of plants [15].

Numerous provisions of K.S. Mereschkowsky's theory [1, 9, 10] which was well in advance of the experimental biology level of the early 20th century were subjected to severe criticism. For example, A.S. Famintsyn, whose works had formed the basis for development of the theory of symbiogenesis, did not accept the origin of chloroplasts from both cyanobacteria and zoochlorellas [as per 16]. Later both objections turned out to be inadequate: both primary plastids (originating from cyanobacteria), surrounded by 2 membranes, and secondary plastids (originating from green or red algae), surrounded by 3-4 membranes, were discovered [17]. However, the heuristic potential of the symbiogenesis concept was highly appreciated by some contemporaries of K.S. Mereschkowsky. Based on it, I.E. Wallin [18] proposed the hypothesis on symbiotic origin of mitochondria, which later proved to be relevant. E.B. Wilson [19], referred to by K.S. Mereschkowsky as his major opponent, admitted that the symbiogenesis concept, despite its speculative nature, may «require detailed consideration» in future.

In 1920-1950 the theory of symbiogenesis was hardly ever mentioned in

literature; however, accumulation of facts intended to prove it took place. Among them, the key role belonged to identification of fundamental differences in cell organization of prokaryotes and eukaryotes [20], as well as cytoplasmatic genes of eukaryotes, which turned out to be functionally related to plastids and mitochondria [21]. These works have set the background for rebirth of the symbiogenesis concept, strongly associated with the works of L. Margulis-Sagan [22, 23]. She was guided by the data obtained in 1950-1960 on the presence of nucleus-independent genomes and translation systems in plastids and mitochondrias, similar to those of bacteria (genome in the form of a circular DNA molecule, protein synthesis on 70S ribosomes).

The new version of the symbiogenesis concept is known as the theory of serial endosymbiosis; this name reflected the multiple stages of symbiogenic evolution in eukaryotes. Introduction of aerobic α-proteobacteria, which gave birth to mitochondria, into anaerobic archaea cells, which used endosymbionts as sources of ATP, forming during oxidative phosphorylation, is usually considered as its first stage [23]. When justifying the theory of serial endosymbiosis, L. Margulis referred to the theory of symbiogenesis, developed by K.S. Mereschkowsky [9, 10], as well as to the concepts of symbiogenesis as a universal evolutionary strategy, supported by natural selection, proposed by B.M. Kozo-Polyanski [24]. According to L. Margulis, most, if not all, eukaryotic cell components are of symbiotic origin irrespective of presence of their own genomes.

In fact, it is currently evident that identification of semi-independent genomes is not a mandatory criterion for symbiotic origin of organelles: genome-free mitochondria (mitosomes, hydrogenosomes) and plastids have been discovered, which have preserved their cell structure and ability of binary fission, as well as the significant part of ancestor microorganisms proteome, coded by nuclear genes, which were obtained by the hosts from their endosymbionts [17, 25]. At the same time, attempts to apply the symbiogenesis concept to ciliums and flagellums of eukaryotes (locomotive structures, which are the most ancient cell organelles, according to L. Margulis), as well as to peroxisomes, have found no confirmation: neither own genomes, nor proteins of bacterial origin were discovered in these organelles [26, 27].

Identification of transitional forms, connecting nonsymbiotic bacteria with cell organelles, provided a convincing proof of the theory of symbiogenesis. They include cyanellas of protozoa *Paulinella chromatophora* (amoeba) and *Cyanophora paradoxa* (Glaucophyta). Cyanellas of *P. chromatophora* have preserved a genome of significant size (about 1,000 kbp, which amounts to 30 % of the genome of *Synechococcus* — nonsymbiotic equivalents of these cyanellas), containing a complete set of genes for basic template processes, i.e. replication, transcription and translation [28]. Cyanellas of *C. paradoxa* are characterized with the size of genome similar to that of green algae plastids (less than 200 kbp); however, these cyanellas have preserved cytological traits of nonsymbiotic ancestors, i.e. murein cell wall and intracellular thylakoids with phycobilisomes [29]. It is important to note the absence of nitrogen fixation genes in both types of cyanellas, which are present in their nonsymbiotic relatives, presumably due to incompatibility of nitrogenase reaction with metabolic processes taking place in the host cell cytoplasm.

Intracellular symbionts (endocytobionts) of insects can also be considered as an example of transitional forms between nonsymbiotic bacteria and organelles [30]. They provide their hosts with essential nutrients (amino acids, cofactors), and the size of their genome is similar to that of permanent cell organelles: 140 kbp for *Tremblaya* (endocytobiont of scale insects), 160 kbp for *Car-*

*sonella* (endocytobiont of psyllids). Interestingly, these bacteria are taxonomically unrelated to plastids and mitochondrias (*Tremblaya* is a β-proteobacterium, *Carsonella*is a γ-proteobacterium) which demonstrates the ability of representatives of all main bacteria groups to transform to cell organelles.

Discovery of *Lokiarchaeota*, a group of anaerobic chemotrophic archaea with cytological organization similar to that of eukaryotes, was a triumph of the theory of symbiogenesis [31]. Unlike the other prokaryotes, *Lokiarchaeota* have actin cytoskeleton, are capable of intracellular membrane components formation and endocytosis, which probably ensured obtaining of symbiotic bacteria - ancestors of organelles. Genome of *Lokiarchaeota* contains more than 5300 protein-coding genes; only 26 % and 29 % of them show homology to known genes of archaea and bacteria, respectively, while more than 30 % code previously unknown proteins. According to molecular phylogeny methods, *Lokiarchaeota* and eukaryotes had a common ancestor, which confirms the hypothesis on origin of eukaryotes from anaerobic organisms, which obtained mitochondrias as a result of endosymbiosis with aerobic α-proteobacteria [32].

L. Margulis emphasized in one of final studies that symbiogenesis does not come down to exchange of trophic or signal factors, but the combination is based on assimilation of microsymbiont genomes by a host, leading to formation of a supraspecific system of heredity [33]. Later it was called hologenome and was considered as a set of genes of closely interacting symbiotic partners [34]. Developing this approach we have proposed the term “symbiogenome” [8], defining it as a product of initial stages of partners coevolution, i.e. functional integration of their inherited material as a result of development of inter-organismic signal interaction systems [35].

Thus, based on the present-day data, symbiogenesis can be defined as a multiple stage process of formation of a new functionally integrated individual with a fused system of heredity formed by the interacting partners. The partners become more dependent on symbiosis in the course of evolution (facultative→ecologically obligate→genetically obligate→absolute) and integrity of their genetic material (functional→structural) [35].

Summarizing the brief review of the symbiogenesis concept, it should be highlighted that its classical version, proposed by K.S. Mereschkowsky 111 years ago [1], has still preserved the huge heuristic potential. For example, the assumption on symbiotic origin of the nucleus and chromosomes was recently developed within the theory of viral eukariogenesis [36, 37]. The classification of cell forms by mycoplasma and amoeboplasma, proposed by K.S. Mereschkowsky [10, 11], was not confirmed, but the presence of two different prokaryote types, i.e. bacteria and archaea, combination of which gave birth to eukaryotes, can be considered proved [23]. The hypothesis on independent origin of various cell organization forms, proposed by K.S. Mereschkowsky, was not confirmed as well: multiple data indicate that bacteria and archaea had a common ancestor, presumably an anaerobic organism [38] with RNA genome [36]. However, the basic idea of K.S. Mereschkowsky with regard to symbiogenic origin of eukaryotic cell organelles have stood the test of time and determined the main direction of development in biology for decades to come.

It should be noted that the first work of K.S. Mereschkowsky, which focused on symbiogenesis concept justification, evoke wide response among biologists of various specializations. This work can be rightly considered as a masterpiece of scientific literature: it combines in-depth theoretical analysis with an easily accessible form, and novel approach to the problems of evolutionary biology with extremely correct references to works of predecessors and opponents.

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