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Address: build. 16/1, office 36, pr. Polesskii, Moscow, 125367 Russia
Tel: + 7 (916) 027-09-12
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Crotolaria juncea L., A NEW LEGUME CROP FOR CULTIVATION IN RUSSIA: CHARACTERIZATION AND PROSPECTS

(review)

J.V. PUKHALSKY[1, 2, 3] ✉, N.I. VOROBYOV[1], S.I. LOSKUTOV[2, 3], R.I. GLUSHAKOV[4, 5], Yu.V. KOSULNIKOV[1], A.I. YAKUBOVSKAYA[6], G.V. NIKITICHEVA[2], L.A. GORODNOVA[2], A.P. KOZHEMYAKOV[1], Yu.V. LAKTIONOV[1]

[1]*All-Russian Research Institute for Agricultural Microbiology*, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail puhalskyyan@gmail.com (✉ corresponding author), kullavayn@gmail.com, kojemyakov@rambler.ru, laktionov@list.ru;
[2]*Pushkin Leningrad State University*, 10, Petersburgskoe sh., St. Petersburg, Pushkin, 196605 Russia, e-mail lislosk@mail.ru, gorodnovalarisa@gmail.com, uchhoz@mail.lengu.ru;
[3]*All-Russian Research Institute for food additives, Branch of the Gorbatova Federal Scientific Center for Food Systems RAS,* 55, Liteiny prosp., St. Petersburg, 191014 Russia;
[4]*Kirov Military Medical Academy,* 6, ul. Academika Lebedeva, St. Petersburg, 194044 Russia, e-mail: glushakoffruslan@yandex.ru;
[5]*Saint Petersburg State Pediatric Medical University,* 2/litera B, ul. Litovskaya, St. Petersburg, 194100 Russia;
[6]*Research Institute of Agriculture of Crimea,* 150, ul. Kievskaya, Simferopol, 295043 Russia, e-mail yakubovskaya_alla@mail.ru
ORCID:
Pukhalky J.V. orcid.org/0000-0001-5233-3497
Vorobyov N.I. orcid.org/0000-0001-8300-2287
Loskutov S.I. orcid.org/0000-0002-8102-2900
Glushakov R.I. orcid.org/0000-0002-0161-5977
Kosulnikov Yu.V. orcid.org/0000-0003-1134-3503
Yakubovskaya A.I. orcid.org/0009-0001-8434-2689
Nikiticheva G.V. orcid.org/0009-0003-0229-3994
Gorodnova L.A. orcid.org/0009-0002-3767-4562
Kozhemyakov A.P. orcid.org/0000-0002-9657-2454
Laktionov Yu.V. orcid.org/0000-0001-6241-0273

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Abstract

Sunn hemp (*Crotalaria juncea* L.) is a multi-purpose annual legume plant. This is the oldest bast crop grown in tropical regions for fiber (H.R. Bhandari et al., 2016; 2022). In 1791, the plant was brought to Europe where it is cultivated as an alternative green manure crop. Crotalaria has been shown to produce sufficient dry matter to cover and protect the soil from potential erosion, as well as providing nitrogen in amounts useful for subsequent harvests of crops in a diversified crop rotation (D. Scott et al., 2022, A.P. Barros et al., 2022). In the United States, the plant occupies one of the leading places in the list of intermediate cover crops. Dry green biomass contains from 18 to 22 % protein, but can only be used in limited quantities for livestock feed (< 10 % of the silage produced), since sunn hemp during flowering accumulates the toxic alkaloid monocrotaline. The sunn hemp seeds are up to 35-40 % protein, and also contain a small amount (up to 0.1 %) of toxic dehydropyrrolizidine alkaloids (trichodesmine, junsein, apigenin-7-4'-0-diglucoside, apigenin-7-glucuronide, lectin, senecionine and seneciphylline) and amino acids (alpha-amino-beta-oxylaminopropionic, alpha-aminoxylaminobutyric and/or alpha,gamma-diaminobutyric) (V.B. Malashetty et al., 2015; F. Prada et al., 2020). Their use in animal feeding requires special attantion and is, if possible, undesirable. The main non-toxic variety currently used in the United States is Tropic Sun. In other varieties, the accumulation of toxic concentrations of alkaloids in the biomass occurs at flowering, so plant biomass for silage should be harvested 60 days after sowing (J.E. Garzon et al., 2021; J.B. Morris et al., 2015). It was noted that pruning shoots to 30 cm 60-100 days after sowing and re-growing for 70 days increases the nitrogen content in the biomass (A.S. Abdul-Baki et al., 2001). In Russia, crotalaria can be an unconventional cover crop in biological farming to reinforce the soil, improve fertility and for reclamation. The area for sunn hemp cultivation may be southern regions, in particular the Krasnodar Territory, the Republic of Adygea and Crimea. Polysaccharides (galactomannans) from the sunn hemp seeds are bioactive growth stimulant for other plants (R.P. Zakirova et al., 2020). These metabolites, obtained as a result of the refining (degumming) process of vegetable oil extracted from seeds, can be comparable in quality to seed extracts from guar (*Cyamopsis tetragonolaba* (L.) Taub) – another currently in demand

annual legume crop (E.A Dzyubenko et al., 2023). Secondary metabolites extracted from Crotalaria leaves are a rich source of carbohydrates, steroids, triterpenes, phenols, flavonoids, alkaloids, amino acids, saponins, glycosides, tannins and volatile oils (S.K. Dinakaran et al., 2011). Thus, *C. juncea* has hypolipidemic, antioxidant, antibacterial, antifungal, antidiarrheal, anti-inflammatory, hepatoprotective and many other pharmacological effects. Another practical application of *C. juncea* is the production of cost-effective biofuels (S. Sadhukhan et al., 2016).

Keywords: *Crotalaria juncea,* biological farming, recultivation, galactomannans, natural gum, pharmacology

Crotalaria is one of the largest genus in the family *Fabaceae*, the subfamily *Papilionoideae* [1]. The genus is currently assigned to the tribe *Crotalarieae* with another 15 genera divided into three groups (Fig. 1).

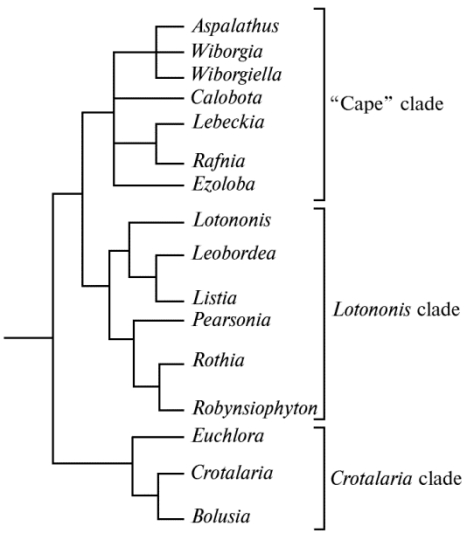

Fig. 1. Phylogenetic tree of the tribe *Crotalarieae* based on a combination of morphological and molecular genetic synapomorphies [4, 9**].**

The *Crotalaria* group consists of three genera, the *Euchlora*, *Bolusia* and *Crotalaria*. The *Crotalaria* genus comprises more than 713 species of annual and perennial plants growing throughout the world [2]. Africa and India are the main centers of *Crotalaria* biodiversity (approximately 540 species), as well as Madagascar [3-5]. In India, the habitat of 73 species is limited to the peninsular territory (Karnataka, Andhra Pradesh, Kerala, and Tamil Nadu), with most of them occurring in the western states (Maharashtra, Tamil Nadu, Karnataka, Kerala) [6]. Approximately 15 representatives of Indian species are endemics listed in the Red Data Book of Indian Plants [7, 8].

In 1753, Carl Linnaeus was the first to describe 12 species of the genus *Crotalaria*, i.e., *C. perfoliata*, *C. sagitalis*, *C. juncea*, *C. triflora*, *C. villosa*, *C. verrucosa*, *C. lotifolia*, *C. lunaris*, *C. laburnifolia*, *C. micans*, *C. alba* and *C. quinquefolia* [9]. In 1786, Jean-Baptiste Lamarck was the first to propose a general classification of species in the genus. The existing sectional classification of *Crotalaria* based on morphological charaacters is not fully consistent with its molecular phylogeny. Some members of the genus *Crotalaria*, particularly *C. cornetii*, *C. peschiana*, *C. prolongata*, and *C. variegata* are indicators of the copper and cobalt ions in the environment, capable of their hyperaccumulation (≥ 50 μg Cu/g DM and ≥ 5 μg Co/g DM) [10]. It is known that the metallophytes *C. cobalticola* and *C. peschiana* do not require high concentrations of copper during early ontogenesis and can grow on an uncontaminated substrate for a relatively long time [11].

The purpose of this review was to summarize the data available in scientific literature on the cultivation of the leguminous crop *Crotalaria juncea* which is atypical for the Russian Federation.

Botanical description and distribution of the species *Crotalaria juncea*. *C. juncea* plants have been grown as a bast crop in India since 600 BC. In the 1960s, *C. juncea* was the main source of income for the country's economy. The plant name is due to its resemblance to rush grass (*Spartium junceaum* L.), a Spanish Mediterranean shrub with green shoots and sparse yellow leaves [12].

C. juncea is an erect, shrubby annual plant, typically 1 to 4 m tall [13].

Pubescent stems are up to 2 cm in diameter. The leaves are alternate, simple, linear-elliptic to oblong-lanceolate in shape, up to 15 cm long and 3 cm wide, bright green in color, usually with an acute tip. Stipules are 1-2 mm long, slender. The petiole is approximately 3-5 cm in length. The inflorescence is a leaf-shaped, opposite raceme 10-50 cm long, loose, consisting of 6-20 flowers. Spectacular butterfly-shaped flowers are bisexual, zygomorphic, 5-membered. If cross-pollination is absent, self-pollination occurs due to stimulation of the stigmatic surface by insects or wind [12].

The main insect pollinators of the crop are three species of bees, the *Xylocopa fenistroides*, *X. latipies* and *Megachile lanata* [14, 15]. The flowers are also visited by *Apis florea* and *A. indica*, but they are not effective pollinators because of their smaller bodyweight. The calyx is five-lobed, 1.5-2.0 cm long, covered with short brown hairs. The blades are 3-4 times longer than the tube. The corolla is bright yellow, elliptical, with a reddish tint. Ten stamens are free almost to the base. Bracts are elliptical, up to 3-5 mm in length. The fruit is a cylindrical bean, from 3.0 to 5.5 cm long, velvety, with 6-12 heart-shaped seeds 4-6 mm in diameter, dark brown to almost black in color. Depending on the variety and environmental conditions, the number of seeds varies greatly from 18,000 to 30,000 per kg of crop [16]. The Hawaiian cultivar Tropic Sun produces 30,000 to 35,000 seeds/kg.

The plant traditionally grows in Asian countries, especially in its tropical part (Bangladesh, Bhutan, India), but is widely cultivated in drier areas of the tropics, subtropics, and in areas with a temperate climate and hot summers. *C. juncea* grows in many countries of the African continent from the Atlantic coast to the Red Sea, from Tunisia to South Africa and on the islands of the Indian Ocean (Fig. 2).

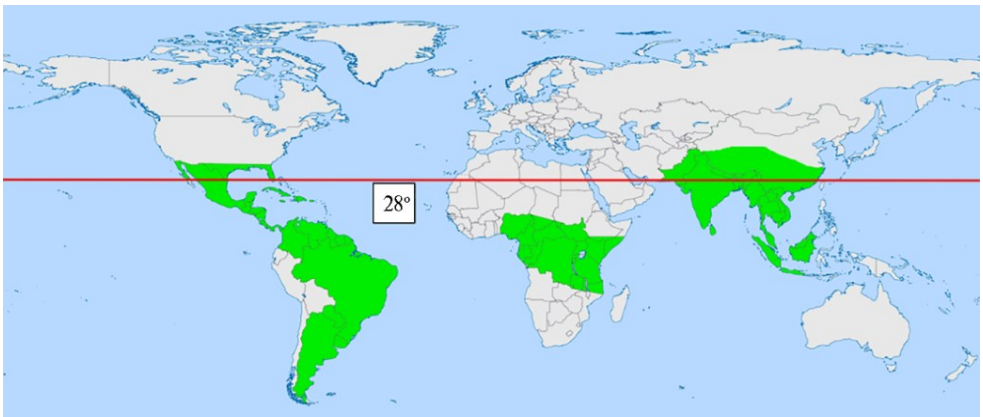

Fig. 2. Distribution of *Crotalaria juncea* L. (area highlighted in green).

The species was first introduced to Europe in 1791 as a cover and bast crop [6, 17]. The BECOOL project (https://www.becoolproject.eu/) has now been launched to assess the potential for growing non-conventional lignocellulosic crops in diversified crop rotations across Europe [18, 19].

The *C. juncea* came to the USA from the Hawaiian Islands where its large-scale research has been carried out since the 1930s. Now *C. juncea* occupies one of the leading places in the list of intermediate green manures in the USA southeast (e.g., Florida, Texas, Alabama, Oklahoma, Georgia) [20]. The plant is also grown in the north (Washington), but not intensively.

Features of growing *Crotalaria juncea*. The culture has a C3 type of metabolism. It is a light-loving, short-day crop [21] which reproduces only by seeds. The optimal air temperature for growth is 28-32 °C. Optimal soil conditions

are good drainage, pH 5.0-7.5 and 170-200 mm precipitation during the growing season [16].

Crotalaria can grow either as a monoculture or in a legume-cereal mixture [22], e.g., with millet *Pennisetum glaucum* (L.) R.Br., corn *Zea mays* L., or sorghum/Sudangrass *Sorghum* × *drummondii* (Nees ex Steud.) Millsp. & Chase (Fig. 3). Less commonly, the crop grows together with other legumes, thetephrosia *Tephrosia vogelii* Hook. f. and sesbania *Sesbania sesban* (L.) Merr.), American vetch *Aeschynomene americana* L., Chinese cowpea *Vigna unguiculata* (L.) Walp., hairy indigo *Indigofera hirsuta* L., and thin-leaved rattle *Crotalaria ochroleuca* G. Don [23].

Fig. 3. ***Crotalaria juncea*** **L.** (1) **and sorghum** ***Sorghum*** **×** ***drummondii*** **(Nees ex Steud.) Millsp. & Chase** (2) (photo courtesy of Stacy Swartz and Daniel Calzadilla) **grown in a mixture; 5 weeks after sowing** (left)**, and mature plant roots** (right) **[26].**

Sowing crotalaria immediately after harvesting corn and soybeans remains the land fallow for less time thus minimizing soil degradation. This crop increased nitrogen content in a 0-5 cm soil layer compared to fallow [24, 25]. In crop rotation, corn can follow crotalaria. The residual effect of planting *C. juncea* provided higher corn yield, freshness of spikelets with straw, and greater productivity of marketable cobs compared to the control [26].

Crotalaria is not particularly demanding on soil fertility, but on poor soils, it will not produce the same biomass as on rich soils. It was noted that the crop might not form seeds north of the 28° parallel. Nevertheless, in the conditions of the Russian Federation, crotalaria is a candidate crop for use in a modern biological farming system. As a complementary non-traditional legume plant, it will improve the state of soil ecosystems [27]. The area for crotalaria cultivation in our country could be the southern regions with a warm temperate climate, in particular the Krasnodar Territory, the Republic of Adygea or Crimea.

We did not find scientific publications on the cultivation and use of crotalaria on the territory of the Russian Federation. It is known that at the Vavilov Federal Research Center the All-Russian Institute of Plant Genetic Resources (VIR), there is a collection of crotalaria lines. The latest seed adaptability assessment of the *C. juncea* performed in Kuban dates back to 1978-1984. However, the research was not continued.

In crotalaria monoculture, early sowing dates, from June 15 to July 15 are preferable [28, 29]. In the Republic of Uzbekistan, this is early April-May [30]. Sowing dates vary depending on location, but sufficient soil moisture and frost-free, warm weather will ensure rapid emergence and high yields. Late sowings leads to abundant branching. The seed rate for commercial sowing is ~ 17.0-34.0 kg/ha [31], sowing is continuous, in double rows. For fiber production in Brazil and India,

seeding rates are higher, up to 60.0-100.0 kg/ha. With a decrease in crotalaria planting density, the weed population also decreases [32]. For better germination, the seeding depth should be 2.5 cm, with 10 cm distance between plants in a row and row spacing no more than 10-20 cm. The recommended crop dencity is 48-100 plants per 1 m^2 [33, 34]. For paper production where fine fibers are required, the distance between rows can be up to 30-36 cm [21]. Increasing the distance between rows leads to a decrease in fiber diameter, making plants susceptible to lodging, which affects seed production. Planting density generally does not affect plant height, but unthinned plants reach greater height and average biomass, which may be due to increased competition for light energy. However, dry shoot biomass was greater at higher planting densities [35]. Low sowing density helps to increase the diameter of the stem and the number of formed lateral shoots. Due to less competition, there are more branches per plant.

Reports on the rate of application of mineral fertilizers vary. After germination, N at 30 kg/ha and K_2O at 40 mg/kg are recommended [22, 36]. The phosphorus P_2O_5 dosage is 20 kg/kg though the soils with low levels of this element require a higher dose [37, 38]. In a recent study, for typical gray soils in the Tashkent region, the optimal rate (kg/ha) was $N_{120}P_{160}K_{120}$ [39]. With sufficient humidity, temperature and soil fertility, the plant growth rate is 14.0-30.0 cm per week, or 2.0-4.3 cm per day.

Treatment of crotalaria seeds with native inoculants for Chinese cowpea (*Bradyrhizobium japonicum*, *Rhizobium leguminosarum*) forms a legume-rhizobil symbiosystem, enhancing the atmospheric nitrogen fixation in crops and its accumulation in the soi [40, 41]. For crotalaria inoculation, highly specific rhizobia of the genus *Methylobacterium* have been isolated [42], namely, *M. nodulans* strain ORS2060 [43] and the closely related strain CMCJ317 [44]. Additional application of organic fertilizers to the soil induces the development of the *R. leguminosarum* population [45]. A better dosage of organic fertilizers is 3-5 t/ha. Increasing the content of humic and fulvic acids in soil organic matter provides stimulating and stabilizing effects, since these compounds significantly improve growth and increase the titer of rhizobia.

Keeping seeds in hot water (70 °C) for 8 h before sowing provides surface sterilization and stimulates germination [46]. An example of another *Crotalaria* species, the *C. verrucosa,* shows that aggressive chemical compounds can also serve as sterilizing reagents, e,g., sulfuric (H_2SO_4) and hydrochloric (HCl) acids [47, 48] provide a higher elimination of microorganisms from seeds. Additional ultraviolet radiation (UV-B) increases the activity of enzymes (peroxidase, polyphenol oxidase, superoxide dismutase and phenylalanine ammonialyase) and the production of reactive oxygen species, e.g., superoxide anion, hydroxide ion, and hydrogen peroxide. These forms are extremely active and cytotoxic [49]. Thus, growth activation under irradiation may be an adaptive mechanism of plant tolerance to stress [50].

The optimal harvest time ensures the highest quality crotalaria fiber. Although opinions vary, the general recommendation is that it should be done at the mature fruit stage when the plant has 40, 60, 80, and 100% dry yellow beans that produce a characteristic cracking sound when shaken [51]. Usually this is the 133-155 days after the flower has fully opened (anthesis). Flowering occurs 60-71 days after sowing. Sometimes the minimum period from sowing to flowering is 20-25 days. The full growing season is 153-226 days [52]. If crotalaria is grown for too long, the bast fibers can become lignified which will complicate their further processing. Cutting shoots to 30 cm 60-100 days after sowing [53, 54], and then 70-day re-growing the plant increases the nitrogen content of the biomass [54]. The optimal time for cultivation is 170 days. The yield is harvested mechanically with

a combine or manually.

To date, the productivity of crotalaria in the humid subtropics has been poorly studied [25], and information on cultivation in temperate climates is lacking [55]. According to some reports [56], the production of *C. juncea* green biomass during the pre-monsoon period in India was 22-27 t/ha. Moreover, depending on soil conditions, the fiber yield was 0.12-0.60 t/ha, and the seed yield was up to 10-22 t/ha.

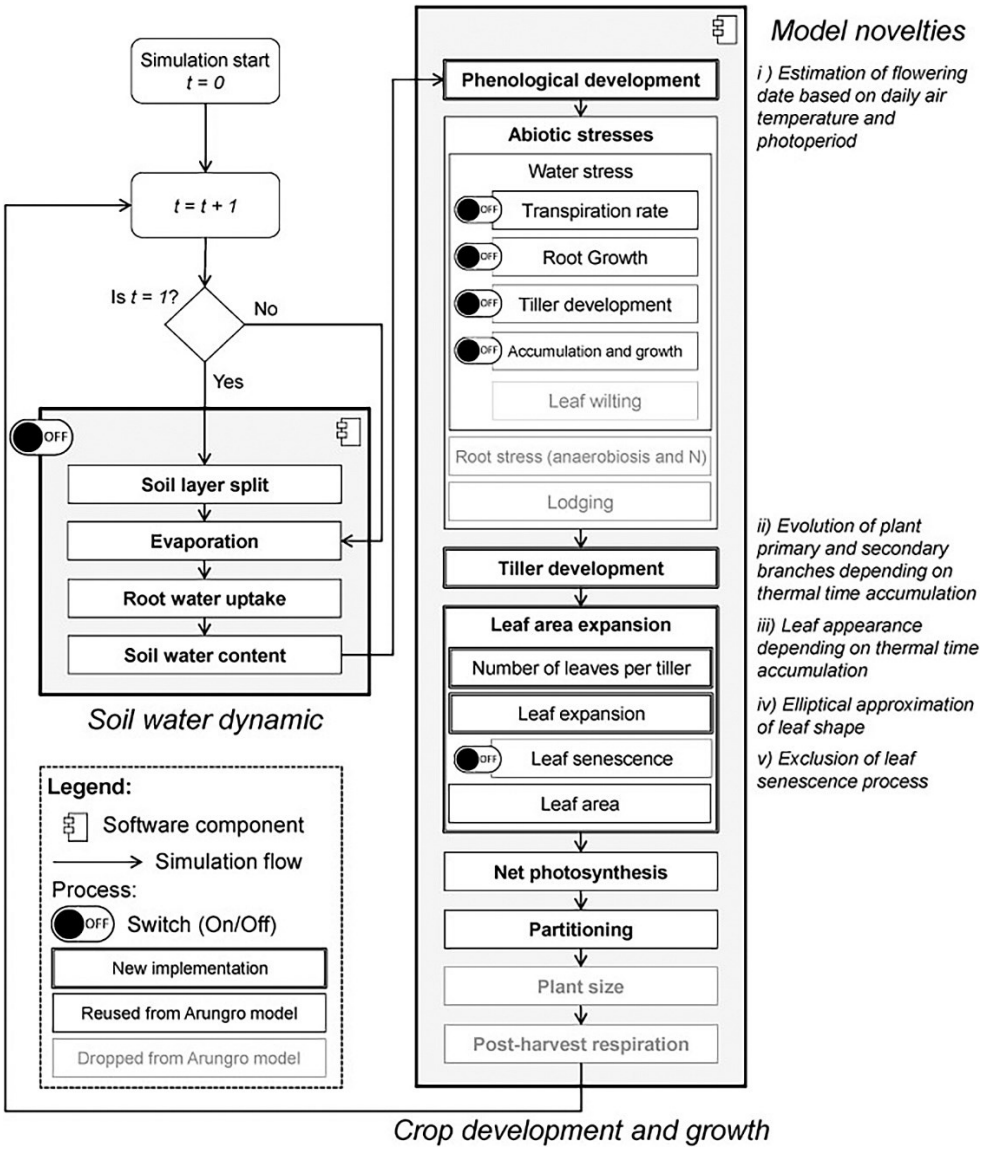

Fig. 4. Flowchart showing the main *Crotalaria juncea* L. yield modeling processes incorporated in the SunnGro program. Boxes with a light gray border indicate processes that were excluded from the original implementation of the Arungro model developed for sugarcane [57].

Recently, an international team from Italy, Spain and Greece developed a simulation model called SunnGro which showed high accuracy in predicting crotalaria productivity in different soil and climatic conditions using biophysical modeling methods [57]. The simulator was based on finding empirical relationships between production/biometric traits and harvest time. The previously created Arungro model for assessing the growth dynamics of giant reed plants (*Arundo donax* L.) was taken as the basis for new algorithms. T. Stella et al. [58] presented

an algorithmic description of Arungro. Figure 4 shows the processes implemented in the adapted SunnGro module. To calculate and reproduce data on crop productivity with regard to the external limiting factors, the authors used data from long-term experiments (1999-2018) performed at different times and densities of crop sowing in three different locations (one for each country) [57].

Diseases and pests of *Crotalaria juncea*. Of the serious diseases reported in the United States, crotalaria is susceptible to anthracnose caused by the fungus *Colletotrichum acutatum*. Plants are also susceptible to powdery mildew (*Microsphaera diffusa*) and root and stem rot (*Sclerotium rolfsii*) [59]. Treating seeds with fungicides and crop rotation are the most common practiced measures to combat the pathologies.

The main insect pests of *C. juncea* in the United States are the pulse pod borer moth (*Etiella zinckenella* Treitschke, 1832) and the bella moth (*Utetheisa bella* L.) [60]. In Florida, these insect pests have been reported to attack beans with little or no seed production. In India, the main insect pests of crotalaria are the crimson-speckled moth (*Utethesia pulchella* L.) which feeds on leaves and seed pods, and the codling moth (*Cydia pomonella* Linnaeus, 1758) which damages apical shoots by feeding there and causing excessive branching and cessation of apical growth [61]. Additional insect pests reported to periodically attack the crop are caterpillars of crotalaria pod borer *Argina astrea* (Drury, 1773) and *Argina syringa* (Cramer, 1775) fed plamt leaves, and the southern green stink bug *Nezara viridula* L. [60]. As in the case of disease control, the main protective measures include treating crops with insecticides. To avoid outbreaks of pests and the spread of diseases, the crop should be returned to crop rotation no earlier than in 3 years.

Economic importance and use of the species *Crotalaria juncea*. *C. juncea* is a multi-purpose crop. Of all the species of the genus, only *Crotalaria ruminata* is grown for its fiber the harvest of which accounts for about 8% of the stem dry biomass [6, 17]. The fiber is 10.0% moisture, 67.8% cellulose, 16.6% hemicellulose, 3.5% lignin, 0.3% pectin, 1.4% water-soluble substances and 0.4% fat and wax. Nanocellulose of various morphologies is extracted from biomass using acid hydrolysis. The highest yield (94.83%) was obtained when using 32% H_2SO_4 solution, the lowest yield (12.03%) when using 72% H_2SO_4 solution. However, in the latter case, the product had the highest thermal stability among other nanocellulose morphologies [62].

Crotalaria fiber is classified as soft and is used mainly in the production of cigarette paper, fishing nets, bags, and ropes [21, 56, 61]. In terms of strength, it is superior to jute, but inferior to flax, agave fiber (sisal) and textile banana (Manila hemp, or abaca) [63]. Basal shoot diameter and plant height were found to significantly correlate with fiber yield. The thickest fiber is usually found in the middle of the stem [64, 65]. Research conducted at commercial greenhouses in Texas evaluated the feasibility of producing and using shorter core fibers when growing plants in soilless potting. The fiber can exceed kenaf (*Hibiscus cannabinus* L.) in terms of bast length and width. Additionally, unlike *H. cannabinus*, *C. juncea* can grow in soils infested with root-knot nematodes [6]. Moreover, the species can be a predecessor of crops (e.g., potatoes, tobacco, soybeans, etc.) prone to damage by these parasites [66]. The culture exhibits nematostatic activity against *Meloidogyne* spp. [67-70], *Heterodera glycines* and *Rotylenchulus reniformis* [71, 72]. A significant relationship was revealed between positive mycotrophy and an increase in plant resistance to *Meloidogyne javanica* and *M. incognita* upon inoculation of the culture with an arbuscular mycorrhizal fungus (*Glomus interradices*) [73].

Due to the use of a continuous system of cultivation of rice and wheat with high doses of inorganic fertilizers, the soil agroecosystem is disrupted, requiring the integration of legumes as green manure into crop rotations. It is known

that adapted tropical legume plants accumulate greater dry biomass, nitrogen and potassium in the soil per 1 ha [74-77] compared to other types of winter legumes already in the virginal period of ontogenesis (35-60 days) [28, 59, 78]. It has been shown that crotalaria as a predecessor significantly increases the yield of rice, wheat, rye and corn in crop rotation [56, 76, 79-81. *C. juncea* has also been proposed as a green manure for organic strawberry production [82]. *C. juncea* as a green manure for different crops exibits a significant positive correlation between plant heights, green and dry biomass, and number of root nodules [83].

Due to land shortages, fodder crop cultivation is not attractive to many Indian farmers, leaving cattle owners in India dependent on expensive concentrates [84]. The nutritional value of crotalaria is no less than that of clover and alfalfa [85, 86]. Its hay contains a significant amount of protein, from 18 to 22%. However, due to alkaloids (trichodesmin, junsein, apigenin-7-4'-0-diglucoside, apigenin-7-glucuronide, lectin, senecionine, seneciphylline and monocrotaline) in the dry biomass, the crop is used to a limited extent for silage, no more than 10% of volume. Toxic alkaloids are contained in the form of free bases in seeds and shoots, so their inclusion in the diet of animals requires a special attention and separate study [87-89]. Plant seeds may also contain toxic amino acids (α-amino-β-oxylaminopropionic, α-aminoxylaminobutyric and/or α,γ-diaminobutyric) [87, 88]. In animals. toxicants lead to refusal to eat and general weight loss, an increased excitability, lameness and constant diarrhea. Horses may develop tenesmus and, less commonly, pulmonary adenomatosis and severe dyspnea. Nephrosis occurs in pigs, hair loss and difficulty breathing in sheep [90, 91].

The main non-toxic crotalaria cultivar currently used in the United States is Tropic Sun released in 1983. In other varieties, the accumulation of toxic concentrations of alkaloids in the biomass occurs at the flowering stage, 45-60 days after sowing [92, 93]. To meet the needs of most livestock and avoid the toxic effects of these compounds, plant biomass for silage should be harvested at a specified time [53].

In experiments with bacterial biofilms, the crotalaria alkaloid kaempferitrin, a flavonol glycoside exhibited antimicrobial properties against the Gram-positive pathogenic bacterium *Staphylococcus aureus* [94]. Aqueous extracts of the crotalaria plants have an allelopathic effect against weeds [95-98]. Moreover, extracts from 4-week shoots reduced the number of cereal weeds, broad-leaved weeds, and sedges, having a stronger inhibitory effect than extracts obtained later in the growing season [97].

C. juncea has some potential for resistance to heavy metals, particularly tolerance to the accumulation of cadmium, nickel, and chromium ions [99-102]. To a certain extent, it can be used in the technology of phytoreclamation (phytoextraction) of technogenically-disturbed lands [74, 103]. Inoculation with toxicant-resistant strains of rhizobacteria from the genus *Streptomyces* significantly increases phytoextraction of cadmium from a crop [104].

C. juncea seeds contain 45.2% carbohydrates, 36.4% protein, 4.2% fat, 10.8% moisture and 3.3% ash. A small proportion (up to 0.1%) of toxic dehydropyrrolizidine alkaloids is also present [105]. Polysaccharides (galactomannans) resulted from degumming of seed oil can be used as physiologically active growth-stimulants for exogenous treatment of other plants, in particular cereals [106]. Their further purification by precipitation produces a viscous colloidal solution, the gum. This gum is a natural food additive or a thickener (stabilizer) in addition to the high-molecular gum extracted from guar *Cyamopsis tetragonoloba* (L.) Taub, another leguminous crop non-traditional for Russia. The *C. juncea* gum is used in the mining industry to break down oil-bearing formations [107].

The *C. juncea* can be used in pharmacology and medicine [108]. The seeds

cleans the blood, treat impetigo, psoriasis, other skin diseases, it also stimulates menstrual cycle. The juice from the leaves is used to relieve swelling and treat leprosy. In Indian folk medicine (Ayurveda), the leaves are used as an emetic, laxative, abortifacient, analgesic meanse, and to treat diarrhea and bleeding disorders. Crotalaria flowers are useful in the treatment of gonorrhea and blood diseases [108].

Secondary metabolites isolated from crotalaria leaves, flowers, and seeds provide a rich source of carbohydrates, steroids, triterpenes, phenols, flavonoids, saponins, glycosides, tannins, anthraquinones, chebulic acid, ellagic acid, gallic acid, chebulic acid, and volatile oils [109]. The plant has hypolipidemic, antioxidant, antibacterial, antifungal, antidiarrheal, anti-inflammatory, hepatoprotective, hypolipidemic and pharmacological effects. We especialy highlight the results of assessing the antibacterial activity of alcoholic extracts from the seeds and flowers of *C. juncea* against *Citrobacter freundii*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella flexneri*, S*taphylococcus aureus*, *Shigella dysenteriae* and *Vibrio cholerae* when grown on an agar medium. It was shown that an ethanol extract from seeds had a higher antibacterial effect than an extract from flowers [110]. The zone of growth inhibition varied from 14.00 to 18.00 mm. Although the results were positive, still they turned out to be worse than for ciprofloxacin used in the experiment as a standard [111].

Compounds extracted from *C. juncea* seeds in sodium phosphate buffer are highly active against the bacterial pathogen *Xanthomonas oxanopodis* pv. *punicae* [112]. Analysis of antifungal activity in vitro revealed the peptides cj-AFP and cp-AMP in seeds, which are capable of inhibiting the growth of filamentous fungi Fusarium oxysporium (113, 114). These proteins were subjected to de novo amino acid sequence analysis, finding no homology with existing proteins in the data bank. The discovery of these peptides may contribute to the development of biotechnological products — transgenic plants resistant to fungal diseases.

Another practical application of *C. juncea* is the production of low-cost biofuel from seed oil [115]. It has been noted that the anaerobic fermentation of cow manure added with of 20% crotalaria serves as a potential source of additional biogas production [116]. Since plant fiber consists of lignocellulosic biomass and is difficult to microbially transform, alkaline pretreatment is necessary [117]. Alkali destroys lignin, neutralizes the acidity of the biomass and increases the methane (CH_4) production. Sodium hydroxide is the best in terms of alkaline decomposition of agricultural waste [116]. It has been shown that when plant raw material is processed before fermentation, the CH_4 production is 89% higher than without pre-treatment [115].

Further prospects for research. Currently, there is a growing need for fiber production and the development of organic farming technology. Because crotalaria can be grown on a large commercial scale as a cover crop and bast crop [118], many countries around the world are gradually introducing it into diversified crop rotations as a green manure to improve the soil health and reduce root-knot nematode abundance. An effective agrotechnical technique that increases the adaptation of a crop to the conditions of a certain soil-climatic zone can be the use of biological products based on a consortium of beneficial nodule and associative rhizobacteria, as well as arbuscular mycorrhizal fungi. Dur to creation of a legume symbiosystem, the mineral nutrition and tolerance of the macrosymbiont to various stresses improves [119, 120].

Efforts should be focused on introduction of the crop into various agroclimatic zones of our country. The limiting factor for now remains the high cost of seeds accounting for 4.5USD per 1 kg.

As a multi-purpose crop, crotalaria is of interest as a raw material for the

production of high-quality fiber, a source of lignite cellulose in the production of biofuel, and green manure. The potential is being assessed of using crotalaria for livestock feed and as a remediator plant, accumulating in green biomass toxicants from the soil ecosystem during its gradual phytorecultivation (sanitation).

The pharmaceutical use of the crop is a separate area. In this case, it is permissible to cultivate plants in both fields and greenhouses on soilless substrates with minimal use of mineral fertilizers, herbicides and other chemical protectanys, but with organic growth regulators and microbial biological products. The vast majority of alkaloids found in the genus *Crotalaria* must be studied to evaluate their pharmacodynamic properties and to develop new alkaloid-based drugs [121].

Information on the crop genetic variation is still limited. In recent years, genetic and breeding studies have been carried out on crotalaria grown in Brazil [121-124]. Since the pollination in the crop can usually be controlled, the results of these studies indicate the possibility of using heterosis.

A germplasm bank can serve to maintain the genetic purity of the crop and to select more resistant lines and varieties through micropropagation followed by cultivation in greenhouse microclimates [122]. However, before this, it is necessary to assess the variability of the chromosome set in the *C. juncea* varieties, especially their ploidy, in order to obtain hybrids with high fertility. In addition, methods for growing the resulting seedlings must be standardized for different hydroponic systems (chemo-, aggregate-, and ionoponics).

Thus, this review is the first in the domestic special literature that summarizes data on physiology and cultivation of *Crotalaria juncea*, a non-traditional leguminous crop in the Russian Federation. In the southern Russian regions, *C. juncea* may serve as an intermediate crop in new diversified crop rotations and as a raw material for gum production instead of or in addition to guar. The abiotic factors limiting cultivation of this tropical crop in Russia are the lack of heat and, in some sites, moisture supply, photoperiod, soil pathogens and the absence of soil nitrogen fixing bacteria. Field conditions and the use of biologicals based on a consortium of beneficial nodule and associative rhizobacteria complementary to the genus *Crotalaria* will partially avoid these problems. Organic additives, e.g., the compounds based on humic acids of various origins (peat, coal, sapropel, zoohumus, etc.), can be used as physiologically active plant growth stimulants. However, the decisive role in the creation of stable legume-rhizobium symbiosystems based on *C. juncea* plays its genotype. So far, the main suppliers of *C. juncea* seeds for Western countries are India, Africa and Pakistan. The Hawaiian variety Tropic Sun is among the promising varieties for research. Nevertheless, creation of Russian crotalaria varieties possessing all the necessary biological and economic characteristics is relevant. The plants can be adapted for cultivation for seeds in greenhouses and hotbeds equipped with neural vision and a system for automatic control of growth parameters. Soilless potted culture excudes the limiting influence of soil matrix and adsorption processes on plant development and absorption of nutrients from substrates. Solid substrates that replace soil are mineral wool, coconut fiber, zeolite, vermiculite, etc. Probably, in such phytotechnical complexes, *C. juncea* plants will be shorter due to shorter internodes. Neertheless, due to precise control of the microclimate, the plants will confidently produce beans.

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POTATO JUICE vs. TRADITIONAL POTATO USE — A NEW INSIGHT
(review)

E.P. SHANINA ✉, D.A. OBERIUKHTIN, A.E. CHERNITSKIY

Ural Federal Agrarian Scientific Research Centre UB RAS, 112a, ul. Belinskogo, Ekaterinburg, 620142 Russia, e-mail shanina08@yandex.ru, oberuhtindenis@gmail.com, cherae@mail.ru (✉ corresponding author)
ORCID:
Shanina E.P. orcid.org/0009-0000-5818-3813
Oberiukhtin D.A. orcid.org/0009-0006-1497-5740
Chernitskiy A.E. orcid.org/0000-0001-8953-687X
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Abstract

Traditionally, potatoes are consumed in a heat-treated form, e.g., boiled, fried, baked, with a significant part of its beneficial properties lost (A.D. Fabbri et al., 2015; J. Tian et al., 2016). Such processing greatly changes the mineral and vitamin composition of the product, the content of dietary fibre and the activity of secondary metabolites (J. Tian et al., 2016; A.T. Popova, 2019). Freshly squeezed potato juice can be a healthy alternative to heat-treated potatoes. Its use in folk medicine has been known since the early XIX century (J.E. Vlachojannis et al., 2010), while only a few scientific studies describe the physiological effects of potato juice consumption on experimental animals and on humans. One of the unique components of potato juice is resistant starch (L. Copeland et al., 2009). Resistant starch is not digested in the human body (P.J. Butterworth et al., 2011), positively affects the intestinal microbiota (I. Martínez et al., 2010), and normalizes insulin and glucagon-like peptide-1 in blood serum (A.A. Rashed et al., 2022). Of all plant proteins known to date, potato protein is the most balanced in essential amino acids and bioavailable to humans (M. Hussain et al., 2021). Its protease inhibitors are able to regulate digestion and have therapeutic effects in obesity (S. Komarnytsky et al., 2011; S. Nakajima et al, 2011), patatin has hypolipidemic (J. Wu et al., 2021), hypotensive (Y. Fu et al., 2019), antioxidant and antiproliferative properties (Y. Sun et al., 2013). Raw potatoes and their juice contain high concentrations of ascorbic acid (K.A. Beals et al., 2019), B vitamins, potassium, phosphorus, calcium, magnesium, iron and zinc (K. Zaheer et al., 2016; G.I. Piskun, 2023) which are essential for good health. Potato varieties with purple-, red- and yellow-coloured tubers are the richest source of polyphenols, primarily phenolic acids and anthocyanins (E.P. Shanina, 2013; H. Akyol et al., 2016; I.V. Kim et al., 2020). The potato glycoalkaloids solanine and chaconine remain the most controversial in terms of possible health benefits. On the one hand, their average content in potato tubers is low to cause symptoms of poisoning in humans (K. Nishie et al, 1971). On the other hand, experiments with pure extracts of glycoalkaloids proved their anticholinergic, anticholinesterase (V.A. Voronov et al., 2023) and cytotoxic effects o (M. Friedman, 2015; D.K. Zhao et al., 2021; M.L. Lanteri et al., 2023). In the review, we discuss the likely danger of the identified effects for human health vs. the prospects for the immunodeficiency correction, as well as prevention and treatment of cancer diseases (D.K. Zhao et al., 2021; M.L. Lanteri et al., 2023). We also focus on current methods of biodegradation of potato glycoalkaloids (R.C. Hennessy et al., 2020). Selected studies on the biological effects of potato peel extract (N. Singh et al., 2008) and potato juice (R. Muceniece et al., 2008; V. Bartova et al., 2018) are described. The above information shows that potato juice contains all the useful substances of intact raw potatoes. The prospects for using potato juice in functional nutrition are obvious, but it remains to determine the optimal technological methods for its mass production while preserving the biological activity of the components.

Keywords: potatoes, potato juice, starch, protease inhibitors, patatin, polyphenols, flavonoids, phenolic acids, vitamin C, solanine

In dietetics, there has long been controversy about the benefits and harms of potatoes (*Solanum tuberosum* L.) as a food product. In 2018, the World Health Organization (WHO) published healthy eating guidelines that adults should consume daily 400 g of fruits and vegetables, excluding potatoes [1]. As the main arguments that do not allow considering potatoes as a healthy food product, experts cite the following facts: low fiber content [2], high starch content which during long-term storage of potatoes is hydrolyzed to simple carbohydrates (mainly D-glucose, although as a result of cold saccharification, D-fructose also accumulates in tubers) [3, 4], a high glycemic index [5], and the possible presence of the glycoalkaloid solanine which is toxic to humans [6, 7]. Potatoes are not recommended for people suffering from cardiovascular diseases [8], diabetes [9], and obesity [10]. However, to date, the beneficial properties of potatoes have also been cited. These are its unique mineral composition (primarily high potassium content and low sodium content) [11] and protein composition (balanced combination of amino acids, including the essential amino acids arginine, phenylalanine, valine, lysine, unique protease inhibitor proteins and patatin) [12], as well as secondary metabolites (vitamin C, polyphenols, phenolic acids, glycoalkaloids, etc.) [13] which may have a potential therapeutic and preventive effect in a number of socially significant diseases [14].

The first medical records of the use of raw potatoes belong to the Swiss physician M. Bircher-Benner (1867-1939) who discovered the antacid and antispasmodic effects of potato juice in gastrointestinal diseases [15]. Later in the studies of J.E. Vlachojannis et al. [16[potato juice has been shown to relieve symptoms of dyspeptic disorders. Given the complex multicomponent composition of potatoes, the key factor determining its physiological effects may be the method of consumption of the product and the presence or absence of heat treatment [17]. Traditionally, potatoes are consumed boiled, stewed, fried, baked, steamed, or microwaved [18].

In a review by J. Tian et al. [19] it was noted that during heat treatment, mineral composition of potatoes changes (during the cooking process, up to 50% of potassium is lost as a result of leaching). Water-soluble vitamins (ascorbic and nicotinic acids, thiamine) are lost both as a result of leaching and atmospheric oxidation. Protein denaturation occurs; the content of dietary fiber increases slightly due to the formation of bonds between polysaccharides and proteins. To one degree or another depending on the method of preparing potatoes and the time of thermal exposure, the content and activity of secondary metabolites — polyphenols (including anthocyanins), carotenoids and glycoalkaloids are reduced.

Freshly squeezed potato juice can be a healthy alternative to cooked potatoes. Most of its beneficial properties are preserved with this form of use [18, 19]. The use of potato juice in folk medicine has been known since the beginning of the 19th century [16]. However, only a few scientific studies describe the physiological effects of consuming raw potatoes and its components on the body of experimental animals and humans.

The purpose of this review is to systematize knowledge about the biologically active components of raw potatoes and substantiate the use of potato juice in functional human nutrition.

The search for sources was carried out in PubMed, Google Scholar and eLibrary services for the period from 2013 to 2023. Out of 300 articles found for the key queries “potato juice” and “potato juice,” we selected 80 sources devoted to the study of the composition of potato juice and its biological activity in in vitro

and in vivo experiments. We did not include publications concerning technologies for obtaining, purifying and concentrating potato juice in the process of starch production, as well as the study of sweet potato juice (sweet potato, *Ipomoea batatas* L.).

Composition and calorie content of raw potatoes. The nutritional value of raw potatoes is determined by the balanced ratio of the most important nutrients. 100 g of tubers contain less than 1 g of fat, 18 g of carbohydrates and 3 g of protein. The calorie content of raw potatoes is about 75 kcal [20]. During thermal cooking of potatoes, ~ 6% of fats, 9% of carbohydrates and 5% of proteins are lost [21].

The main carbohydrate of potatoes, starch [2-4, 7], consists of two fractions, the amylopectin (branched-chain glucose polymer) and amylose (straight-chain glucose polymer) in a constant ratio of 3:1 [22]. Raw starch is practically not digestible by humans [23], but in freshly boiled potatoes more than 95% of all starch is converted into an easily digestible form [24]. The remaining part is so-called resistant starch, it is intensively fermented by the microbiota of the large intestine to produce short-chain fatty acids which lower the pH of the intestinal contents, reduce the toxic effect of ammonia, and act as a prebiotic [20, 25]. Using pyrosequencing technologies, I. Martнnez et al. [26] showed that resistant starch increases the population of *Actinobacteria* sp. and *Bacteroidetes* sp in the intestine whyle *Firmicutes* sp. decreases.

The review by A.A. Rashed et al. [27] describes the positive effect of resistant starch on the patients with type 2 diabetes mellitus, i.e., an increase in the blood levels of insulin and glucagon-like peptide-1 and a 2-fold decrease in postprandial glycemia (the amount of glucose in the venous blood after a meal). The observed effects suggest an antidiabetic effect of resistant starch, although the detailed molecular mechanisms of this action remain to be studied.

Proteins. Plant proteins serve as a source of essential amino acids [28]. Increasing your intake of high-quality plant protein instead of animal protein has been shown to reduce the risk of type 2 diabetes [29]. Because plant proteins are very cheap [30], their consumption by the population has increased over time [31].

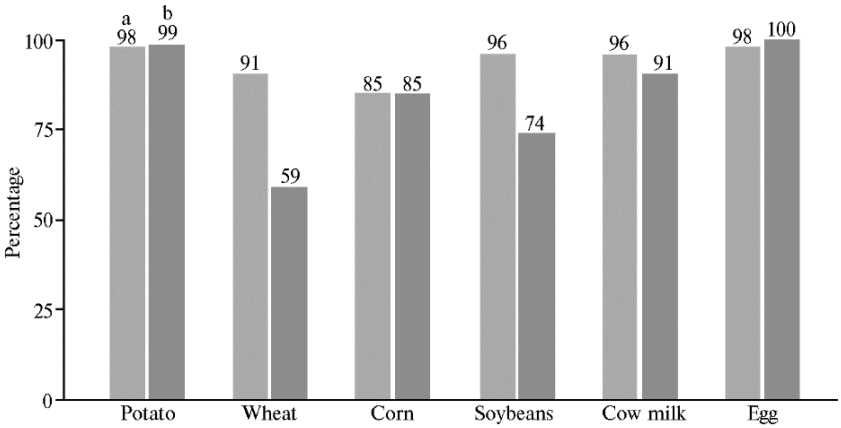

Fig. 1. Digestibility (a) **and biological value** (b) **of proteins from various sources.** To construct the diagram, we used the experimental data of M. Hussain et al. [34].

The protein content of potatoes is higher than that of most tubers of other plants [20]. When talking about protein quality, the concept of "biological value" (BV) is often used, taking into account its amino acid composition and bioavailability [32]. Egg albumen is considered the reference protein with biological value taken as 100% [33]. Potatoe BV is relatively high, above 90%. compared to other

key plant protein sources (Fig. 1) [34].

Potato protein consists of 19 amino acids, including lysine, methionine, threonine, and tryptophan (Fig. 2) [20, 34-36]. The amino acid composition can vary significantly between varieties. An analysis of 22 varieties and hybrids showed that the content of some amino acids (arginine, tyrosine and phenylalanine) depends on the genotype, and the total protein content in potatoes is directly related to the agroclimatic conditions of cultivation [35]. It was found that in the Leader potato variety grown in the Urals, the protein is $^1/_3$ essential amino acids arginine (0.644%), phenylalanine (0.430%), valine (0.369%), and lysine (0.340%). The remaining $^2/_3$ are nonessential amino acids of which aspartic (1.77%) and glutamic (1.44%) are mainly found [35-36].

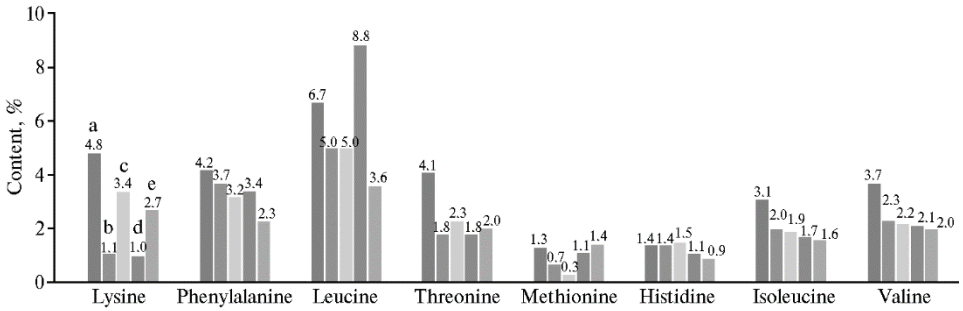

Fig. 2. Content of essential amino acids (% of total protein) **in potatoes** (a)**, wheat** (b)**, soybeans** (c)**, corn** (d) **and eggs** (e)**.** We compiled the diagram based on the experimental data of M. Hussain et al. [34].

Potatoes contain protease inhibitors (50% of total proteins), patatin (40%) and other proteins (10%), but their percentage varies greatly depending on the variety and growing conditions [34].

Protease inhibitors are water-soluble 4-25 kDa proteins [37]. There are 7 classes of potato protease inhibitors, the inhibitor I, inhibitor II, serine protease inhibitor, cysteine protease inhibitor, aspartic protease inhibitor, Kunitz type protease inhibitor, carboxypeptidase inhibitor and inhibitors of other serine proteases [38]. All of them actively bind to trypsin, despite the acidic environment of the stomach, presumably due to the large number of β-sheets in the secondary structure of the protein. Trypsin inhibition prevents the proteolytic inactivation of endogenous trypsin-sensitive cholecystokinin-releasing peptides, promoting the release of cholecystokinin [39, 40]. Studying the kinetics of interaction between protease inhibitors and trypsin, Q. Li et al. [41] found a nonspecific type of inhibition. In this type, the inhibitor binds to the ester group outside the active site and does not affect the enzyme-substrate interaction.

Cholecystokinin plays a central role in the regulation of nutritional homeostasis. It is secreted by neuroendocrine cells located in the mucosa of the small intestine [42]. The earliest physiological effect of this hormone is to stimulate contraction of the gallbladder and secretion of the exocrine pancreas. Bile is necessary for the formation of micelles during the digestion of fats, and pancreatic enzymes are involved in the digestion of fats and proteins. In addition, L.J. Miller et al. [43] found cholecystokinin receptors in afferent neurons of the intestinal vagus nerve (cholecystokinin receptor type 1) and on gastric parietal cells (cholecystokinin receptor type 2). Thus, cholecystokinin increases intestinal motility and mediates the secretion of gastric juice. The described mechanisms allow us to consider potato juice protease inhibitors as cholecystokinin agonists and an effective therapeutic agent against obesity (Fig. 3).

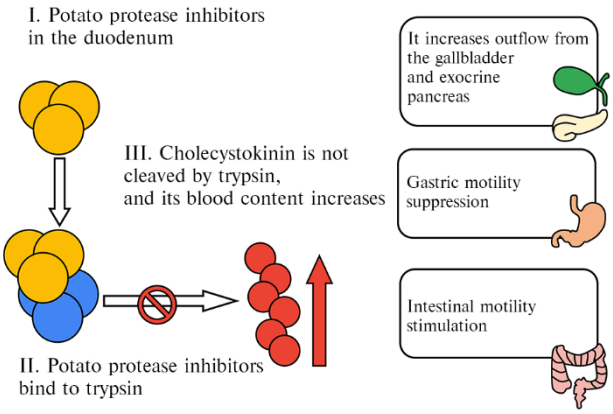

Рис. 3. The effect of a potato protease inhibitor on the gastrointestinal tract functioning. We compiled the diagram based on the experimental data from L.J. Miller et al. [43].

Another in vivo study [44] showed that peptides derived from potato protease inhibitors by enzymatic hydrolysis could reduce blood cholesterol and triglycerides through sterol-binding capacity. In the blood serum of rats that consumed this hydrolysate, the amount of total cholesterol, low-density lipoprotein cholesterol and triglycerides decreased compared to animals from the control group [44]. In earlier studies of the biological activity of potato protein hydrolysates, analysis of rat liver mRNA showed increased synthesis of proteins responsible for lipoprotein clearance [45].

Patatin is a glycoprotein with a molecular mass of 40-45 kDa [46]. Purified patatin contains 6 essential amino acids — lysine, phenylalanine, threonine, isoleucine, leucine and valine. The essential amino acid index (EAAI) is 76%. Patatin monosaccharides contains mannose, rhamnose, glucose, galactose, xylose, arabinose, and fucose [47]. The ratio of proteins and carbohydrates in patatin is 64 and 36%, respectively [48]. The biological effects of patatin are interesting. In studies on *Danio rerio* fish, patatin exhibited nonspecific acyl hydrolase activity on triglycerides, activating lipolysis. Moreover, patatin is able to inhibit pancreatic lipase and regulate lipid absorption in the small intestine [47]. The findings suggest that patatin has great potential for use as a functional product in weight loss programs.

In addition, patatin has been assessed in silico [49] as a precursor of angiotensin-converting enzyme (ACE) and renin inhibitory peptides. Such peptides have the ability to bind to ACE and renin, causing their conformational changes through a mixed mechanism [49, 50]. Effective inhibition of two key enzymes of the renin-angiotensin-aldosterone system is one of the promising approaches to the treatment of arterial hypertension [51].

Other biological effects of patatin have also been described. For example, antioxidant and antiproliferative activity against B_{16} mouse melanoma cells, in which pathanin initiated cell cycle arrest in the G_1 phase, and against Caco-2 and HT-29 intestinal cancer cells [48, 49, 52].

Potato juice can be used in the diet of people prone to allergies. Compared to gluten, a wheat protein to which children and adults are often allergic, the protein found in potato juice has lower IgE-binding capacity, even at high concentrations. Patatin is the only fraction of potato protein that can provoke an allergy, but its intensity will be significantly lower than for wheat, cow's milk or egg proteins [34]. In 2018, Nestle (Switzerland) patented a formula of milk substitute based on potato proteins for children with an allergy to cow's milk protein [53].

During the thermal processing of food products, a sugar-amine condensation reaction (Maillard reaction) occurs. In 1912, French chemist L.C. Maillard (1878-1936) accidentally discovered that a solution containing sugars and amino acids darkened and acquired a characteristic odor when intensely heated [54]. The brown pigments produced in the Maillard reaction are called melanoidins. They are formed as a result of the interaction of ketone groups of sugars and amino groups of amino acids [55]. Since potatoes are a high-carbohydrate product that contains proteins, prolonged heat treatment produces an extremely undesirable Maillard reaction product, toxic acrylamide [56]. It has been proven that acrylamide has a pronounced cyto- and genotoxic effects [57]. Exposure of cells to acrylamide initiates oxidative stress, leading to mitochondrial-type apoptosis [58]. In experiments on BALB/c mice [59], it was found that dietary fiber from potatoes can reduce the side effects of acrylamide. In a group of animals receiving a potato dietary fiber preparation, ther was a decrease in the negative effects of acrylamide on the histological structure and innervation of the small intestine [58].

Thus, plant proteins contained in potato juice in their native form have high biological activity and are able to regulate digestive processes. In addition, hydrolysates of these proteins have hypolipidemic, hypotensive, antioxidant and antiproliferative properties. However, these effects are characteristic only of native proteins and proteins obtained through enzymatic hydrolysis, while conventional cooking with heating to 100 °C and above destroys native proteins.

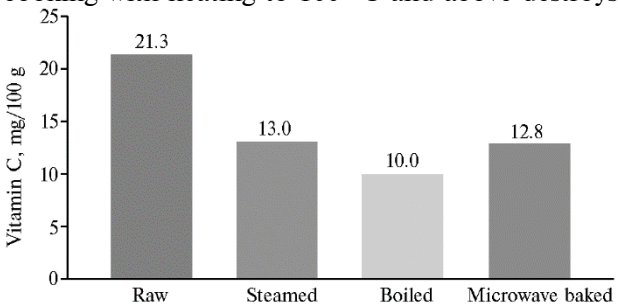

Fig. 4. Vitamin C content depending on the way to prepare potatoes. We compiled the diagram based on the experimental data from A.T. Popova [62].

Vitamins and minerals. During the heat treatment of potatoes, the activity of many vitamins contained in them is lost [60], in particular vitamin C in the form of ascorbic acid. A medium-sized raw potato (150 g) contains 28 mg of vitamin C [20], or approximately $^1/_3$ of an adult's daily requirement [61].

A.T. Popova [62] investigated how much the vitamin C content decreases depending on the ways of cooking potatoes (Fig. 4).

In addition to ascorbic acid, raw potatoes and potatoe juice are rich in B vitamins (B_1, B_2, B_3, B_6) and minerals, the potassium, phosphorus, magnesium, calcium, iron, sodium and zinc [25, 56].

1. Some vitamins and minerals found in raw potatoes [20, 61]

Nutrient	Content, mg/100 g of raw potatoes	Percentage of daily physiological needs for adults per 300 g of raw potatoes
Vitamin C	18.3	55
Vitamin B_1	0.08	16
Vitamin B_2	0.02	3
Vitamin B_3	1.09	16
Vitamin B_6	0.14	21
Folic acid	0.0163	12
Potassium	420.6	36
Calcium	13.6	4
Magnesium	22.4	16
Iron	0.75	16
Zinc	0.27	7

Table 1 shows the contents of some vitamins and minerals in raw potatoes

and the percentage of daily physiological needs according to the current Methodological Recommendations MP 2.3.1.0253-21 "Norms of physiological needs for energy and nutrients for various groups of the population of the Russian Federation") [61] when consuming juice from two medium-sized raw potatoes.

Thus, potato juice, convenient for consuming potatoes raw, retains all the vitamins and minerals contained in potatoes in their native form and in their original concentrations.

P o l y p h e n o l s. Potatoes contain significant amounts of polyphenols. In 150 g of fresh raw potatoes ther are 36 mEq gallic acid, total antioxidant activity is equial to 124.5 mg vitamin C [63]. In addition to ascorbic acid, pigmented potato varieties contain other substances with antioxidant activity, such as carotenoids, flavonoids, tocopherol, and α-linoleic acid [20, 63]. Distribution of polyphenol in potatos uneven, their maximum amount is determined in the peel and gradually decreases towards the center of the tuber [64]. Potato varieties with purple and red pulp possess the highest antioxidant activity, and it is less in varieties with yellow and white tubers [20, 63].

In plants, polyphenols provide the processes of photosynthesis, respiration and protection of the genetic apparatus from ultraviolet radiation, and therefore are continuously synthesized in cells [65].

In the works of S.V. Luca et al. [66] and H.-F. Chiu et al. [67] the effects of using polyphenols are quite fully described experimentally and clinically. In their pure form, polyphenols are widely used as biologically active food additives [68]. The effects of polyphenols in animals and humans are numerous. Thus, in mammals, flavonoids are oxidized into quinones that can interact with functional groups of enzymes, thereby affecting the kinetics of biochemical reactions [69]. In addition, flavonoids have chelating properties. In their active form, they bind transition metal ions, forming chelate complexes [70]. Due to the formation of such complexes in the cell, free radical processes are inhibited [71]. Due to their unique structure, polyphenols have multiple physiological effects, e.g., restorative, anti-inflammatory, hepatoprotective, choleretic, antitumor [71, 72). Moreover, polyphenols can enhance the effect of certain medications. For example, K. Zhai et al. [73] demonstrated the synergistic effects of traditional chemotherapy drugs and some polyphenols (chrysin, catechin, formononetin, hispidulin, icariin, quercetin, rutin, and silibinin) against an aggressive brain tumor glioblastoma.

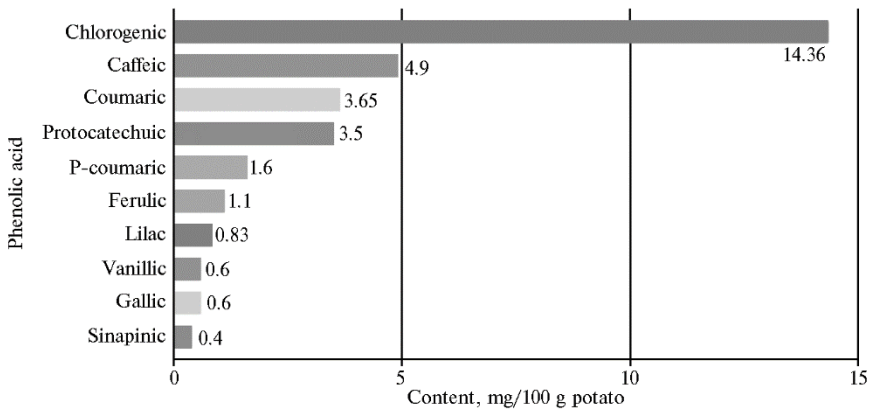

Fig. 5. Contents of phenolic acids in raw potatoes. We compiled the diagram based on the experimental data from H. Akyol et al. [74].

All potato polyphenols can be divided into phenolic acids and flavonoids, including flavonols, flavanones and anthocyanins). Potatoes contain the most phenolic acids of which up to 90% are chlorogenic acid (Fig. 5) [74].

Among potato flavonoids, the most common are anthocyanins, as well as catechin, quercetin, kaempferol, and rutin (Table 2) [74]. Thanks to anthocyanins, the peel and pulp are colored purple, red and yellow. Potato anthocyanins include pelargonidin, peonidin, petunidin, and malvidin [74, 75].

2. Average content of flavonoids in potato dry matter [74]

Flavonoid	Concentration, mg/100 g DM
Anthocyanins	283,4
Katekhin	41,7
Rutin	2,9
Quercetin	2,5
Kaempferol	1,1

Thus, the juice obtained from potato tubers with pigmented pulp will have additional biological effects due to high content of flavonoids.

Glycoalkaloids. Glycoalkaloids are secondary plant metabolites that can accumulate in flowers, leaves, fruits, and tubers [76]. Potatoe plants synthesize predominantly two alkaloids, the α-solanine and α-chaconine (chaconine) (77). In chemical structure, both are a compound of the aglycone solanidine with a carbohydrate side chain responsible for interaction with cell membranes [78]. The structure of glycoalkaloids is similar to mammalian steroid hormones [79]. Consumption of large doses of glycoalkaloids may cause an intoxication syndrome [80].

Although the glycoalkaloid content of potatoes varies considerably depending on variety and growing conditions, in general, comparison can be made of the solanine and hakonine contents in fresh raw tubers with the human semi-lethal doses LD_{50} (Table 3) [81, 82].

3. Content of glycoalkaloids in potato peel and pulp and average semi-lethal dose for humans (LD_{50}) [81, 82]

Glycoalkaloid	In potato peel, mg/kg	In potato pulp, mg/kg	LD_{50} per or, mg/kg
α-Solanin	89	12	2,8
α-Hakonin	173	18	

Glycoalkaloids are actively synthesized in tubers in the presence of pests, dueing long-term storage, especially when exposed to light (even artificial) and high temperature [83, 84]. Therefore, to avoid high levels of glycoalkaloids in tubers, potatoes must be properly grown, transported, and stored before consumption [85].

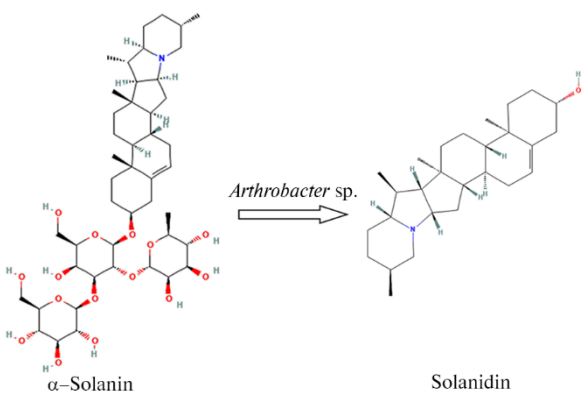

Fig. 6. Biodegradation of glycoalkaloids on the example of α-solanine. Formulas are taken from PubChem [91, 92].

However, in addition to toxic effects, therapeutic effects have been described for pure glycoalkaloids from potatoes [86-89]. In vitro and ex vivo, an anticholinergic effect was shown due to antagonistic activity towards M_3-cholinergic receptors and an anticholinesterase effect [86], a cytotoxic effect against cells of neuroblastoma SH-SY5Y [87], colon cancer HT-29, liver Hep G2, cervix uterine HeLa and lymphoma U937, cancer of stomach AGS and KATI II [88], prostate LNCaP and PC3, and other cell lines [89]. The data obtained indicate that glycoalkaloids can be considered as promising

agents for antitumor therapy.

Biotechnological methods for reducing the toxicity of potato glycoalkoloids are described in the literature. Thus, in the bacteria *Arthrobacter* sp. enzymes capable of biodegrading α-solanine and α-chaconine were discovered. These enzymes can remove the trisaccharide responsible for the interaction of glycoalkaloids with animal cell membranes from α-solanine and α-chaconine molecules. Such biodegradation (Fig. 6) provides formation of low-toxic solanidine [90].

Potato processing products and their biological effects. Potato skin contains the maximum amount of polyphenols [64], so N. Singh et al. [93] proposed studying extracts from it. In an experiment on laboratory rats, researchers showed that potato peel extract was able to significantly reduce acute liver damage due to antioxidant activity.

Potato juice contains compounds that can influence GABAergic activity in the brain, displacing γ-aminobutyric acid (GABA) from its receptors [94]. In addition, V. Bartova et al. [95] found that potato juice, due to its unique proteins, exhibits pronounced antimycotic activity, and the strength of the effect could be modulated by temperature.

Moreover, in a pilot study conducted in 2006, S. Chrubasik et al. [96] used potato juice manufactured by Biotta company (Switzerland) in the treatment of patients with dyspepsia syndrome. The following dosage regimen was recommended: 100 ml twice a day, half an hour before meals in the morning and in the evening before bed. The results of the clinical study showed that at least $^2/_3$ of the patients had improvement after 1 week, which confirms the promise of using potato juice in clinical gastroenterology.

Prospects for the development of potato juice as a functional food product. Potato juice is the only product that allows preservation of all natural components — proteins, starch, vitamins, minerals, polyphenols, glycoalkaloids (Table 4).

4. The main components of potato juice and their biological effects

Component	Biological effects	References
Starch	Source of glucose and fructose, the body's most important energy substrate	[2, 3, 20, 22, 23, 25, 26]
"Resistant starch"	Intestinal microflora substrate; suppression of the growth of pathogenic flora; antidiabetic effect	[20, 22, 24, 26, 27]
Prosthetic inhibitors	Source of essential amino acids; strengthening of digestion processes; obesity prevention; hypolipidemic effect	[37-41, 45]
Patatin	Source of essential amino acids; obesity prevention; hypolipidemic effect; antihypertensive effect; antioxidant effect and antiproliferative activity	[34, 47-53]
Vitamin C	Antioxidant, immunomodulatory, adaptogenic effects; increased iron absorption; participation in the formation of collagen fibers	[61]
Vitamin B_1	Regulation of carbohydrate and energy metabolism	[61]
Vitamin B_2	Redox reactions; promotes increased color sensitivity by the visual sensory system and dark adaptation	[61]
Vitamin B3	Regulation of redox reactions; cofactor for several enzymes	[61]
Vitamin B6	Regulation of protein, lipid and nucleic acid metabolism; immunomodulatory effect; regulation of processes of inhibition and excitation of the nervous system; participation in the processes of erythropoiesis	[61]
Folic acid	Participation in the exchange of nucleic acids and amino acids	[61]
Potassium	The main intracellular ion that maintains membrane potential; participation in electrolyte metabolism	[61]
Calcium	Maintaining the structure of bone tissue, participating in the transmission of nerve impulses, muscle contraction, blood clotting processes	[61]
Magnesium	Cofactor for a number of enzymes, stabilizer of biomembranes, regulates muscle contractions, maintains homeostasis of calcium, potassium and sodium	[61]

Continued Table 4

Iron	Part of hemo- and myoglobin, cytochromes, catalase and peroxidase; regulates the occurrence of redox reactions; depending on the concentration, it has a pro- or antioxidant effect	[61]
Zinc	Part of the enzymes involved in the metabolism of carbohydrates, proteins, lipids and nucleic acids; regulates gene expression;	[61]
Polyphenols	Antioxidant effect and protection of biomembranes	[20, 63-75]
Glycoalkaloids	Intoxication syndrome; antiproliferative effect	[76-89]

However, for the industrial production of potato juice, it is necessary to resolve a number of issues regarding the requirements for raw potatoes, their processing and packaging the juice. In addition, it is worth considering adding preservatives and antioxidants to the juice. An organoleptic assessment of the resulting product is also necessary to understand whether additional components are necessary to give the juice a more attractive taste. Despite the technological difficulties, potato juice can become a complete functional product to be introduced into the diet of all age groups to maintain and improve public health [97].

The described effects of potato juice in vivo can be achieved due to the synergy of its components, which opens up broad prospects for the use of this product in nutrition and medicine [98-100]. Systematic consumption of potato juice can become an important element in the prevention of such socially significant diseases as malignant neoplasms, diabetes mellitus and arterial hypertension [101, 102]. Potato juice can also be recommended as an adjuvant therapy for people who already have these diseases.

Therefore, potato juice contains all the beneficial substances that make up raw potatoes in their native form, i.e., unique proteins, ascorbic acid, B vitamins, potassium, phosphorus, calcium, magnesium, iron, zinc, polyphenols (primarily phenolic acids and anthocyanins). The accumulated information opens up broad prospects for using potato juice for functional nutrition. Experiments revealed the positive effect of potato juice components on digestive processes, intestinal microbiota, the blood content of insulin and glucagon-like peptide-1. Hypolipidemic, hypotensive, antioxidant and antiproliferative effects have also been described. The most controversial in terms of benefits for human health and requiring further study are the potato glycoalkaloids solanine and hakonine. Potato juice is becoming an attractive product for the food industry and dietetics. The bioavailability and high activity of its components together with the described effects, suggest that this product can be used in the prevention of malignant neoplasms, diabetes, arterial hypertension and other diseases. Further research should determine optimal technological methods for mass production of potato juice while maintaining the biological activity of its components.

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PLANT ANTIOXIDANTS AND THEIR NON-TRADITIONAL SOURCES
(review)

Yu.K. GONCHAROVA[1, 3] ✉, S.V. GONCHAROV[2], E.M. KHARITONOV[1], Yu.V. FOTEV[4], V.V. SIMONOVA[1], N.A. OCHKAS[1, 3]

[1]*Federal Rice Research Center*, 3, Belozernii, Krasnodar, Russia 350921, e-mail yuliya_goncharova_20@mail.ru (✉ corresponding author), evgeniyharitonov46@mail.ru, viktoriasimonovaa@mail.ru, ochkasnikolay@mail.ru;
[2]*Trubilin Kuban State Agrarian University*, 13, ul. Kalinina, Krasnodar, 350044 Russia, e-mail serggontchar@mail.ru;
[3]*Aratay LLC,* 15/1, ul. Kotlarova, Krasnodar, 350016 Russia, e-mail yuliya_goncharova_20@mail.ru;
[4]*Central Siberian Botanical Garden SB RAS,* 101, ul. Zolotodolinskaya, Novosibirsk, 630090 Russia, e-mail fotev_2009@mail.ru
ORCID:
Goncharova Yu.K. orcid.org/0000-0003-2643-7342
Goncharov S.V. orcid.org/0000-0002-6317-7173
Kharitonov E.M. orcid.org/0000-0002-4049-6173
Fotev Yu.V. orcid.org/0000-0002-0299-3689
Simonova V.V. orcid.org/0000-0001-6734-616X
Ochkas N.A. orcid.org/0000-0003-4852-3356
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Abstract

The viability of eukaryotes largely depends on a biochemical defense system that protects the body from damage. Antioxidants that neutralize free radicals are significant components of biochemical protective system (M.G. Uzbekov, 2014). Oxidative stress underlies many diseases, e.g., oncological, rheumatoid, bronchopulmonary, cardiovascular, and premature aging (S. Miwa et al., 2016; J.G. Geisler, 2019). There are more than 5,000 antioxidants which differ in chemical composition, antiradical and antiproliferative activity. Many studies show the synergism or additive effect of antioxidants (V. Polonsky et al., 2018). That is, to effectively protect the body, the range of antioxidants consumed must be quite broad. In this regard, it becomes urgent to search for new sources of biologically active substances and increase their content in already cultivated species. This work provides a classification of antioxidants. Among exogenous ones, carotenoids, polyphenols (flavonoids), and trace elements are considered in more detail. The various antioxidant activities of these substances are considered. Flavonoids are the most significant antioxidants. The antiradical activity of flavonoids can be 50 times higher than that of many plant substances, vitamins E and C are notably inferior to them (Y. Yao et al., 2010). Black grain rice varieties are rich sources of flavonoids (U.K.S. Kushwaha, 2016). Carotenoids are another effective antioxidants, the distinctive feature of which is interaction with other substances of this nature which increases the biological activity of the compounds (W. Stahl et al., 2004; C. Hu et al., 2020). Sources with high antioxidant potential and significant accumulation of carotenoids can be red grain varieties of rice, momordica, amaranth (Yu. Fotev et al., 2018; D. Shafigullin et al., 2018). The intraspecific diversity observed at the phenotypic level in terms of color characteristics is associated with both regulatory and structural genes (E.K. Khlestkina et al., 2014). The increased content of proanthocyanidins in the seed coat determines resistance to germination on the root, and the presence of anthocyanins contributes to better preservation of seeds after long-term storage and increased plant resistance to stress (T.L. Korotenko, 2018). Antioxidants increase plant resistance to biotic and abiotic stresses. However, this aspect has not been sufficiently studied in rice varieties with colored pericarp. The study of genetic mechanisms that control plant color traits is relevant in connection with the antioxidant and antimicrobial properties of pigments and their colorless precursors (Y. Qin et al., 2018). These compounds provide the prevention of cancer, reduce the risk of cardiovascular diseases, atherosclerosis, type 2 diabetes, increase immunity, improve the synthesis of visual pigments, activate metabolic processes, and slow down aging (C. Xu et al., 2017). Color variations and grain quality traits in rice samples is controled by 41 loci. The *Ra* (*Prp-b* for varieties with purple pericarp) and *Rc* (brown pericarp and aleurone layer) genes mainly contribute to the phenotypic effect on rice grain color and nutritional quality (Y. Shao et al., 2011). These genes are located on chromosomes 9, 10 and 8 in the regions of the markers RM228 (amplification product size 90-154 bp), RM339 (166-148 bp), and RM316 (160-210 bp) location (T. Furukawa et al., 2007). Molecular characterization of key genes involved in the biosynthesis of the above compounds will allow breeders to control and accelerate selection for color traits, important for improving the nutritional value of functional products.

Keywords: rice, momordica, stained pericarp, flavonoids, carotenoids, antioxidants, anthocyans, regulatory genes, structural genes, marker-assised selection, SSR markers

An increase in O_2 concentration on the Earth's surface 2 million years ago stimulated plant and animal evolution to form a defense system capable of protecting from destruction by free radicals in the presence of oxygen in the atmosphere. The system of biochemical protection against free radicals includes substances that neutralize their effects. These substances constitute a system of antioxidants which includes low molecular weight compounds and complex groups of enzymes [1].

Free radicals are formed during redox reactions. In the body of a healthy person, the content of free radicals is quite constant. Disturbances in the functioning of the body or its defense systems provoke excessive formation of free radicals and lead to cell aging [2-4]. When there is an imbalance in the functioning of the antioxidant defense mechanisms, there is an excessive accumulation of free radicals, fat oxidation products, and other peroxidation products, which leads to oxidative stress [5-7].

Oxidative stress can be provoked by external factors. The formation of free radicals occurs when taking certain drugs or oxygen therapy, irradiation (ultraviolet, laser, radiation therapy), under the influence of environmental factors. In addition, susceptibility to oxidative stress may be genetic [8-10]. Many diseases and pathological processes, including rheumatism, diabetes, heart and vascular diseases, inflammatory diseases, and early aging, begin with the oxidative stress [11-13].

This review examines non-traditional plant sources of antioxidants.

Classification of antioxidants. All antioxidants (AO) are divided into substances of indirect and direct action. Based on their origin, AO are divided into two groups, the enzymatic antioxidants (EAO), e.g., glutathione peroxidase (GP), catalase, glutathione reductase, superoxide dismutase (SOD), and non-enzymatic antioxidants (NEAO) [14-16]. NEAO include substances of endogenous origin, for example, α-lipoic acid, glutathione, coenzyme Q_{10}, and exogenous origin. The latter include carotenoids, vitamins A, C, E, trace elements (selenium), polyphenols (flavonoids) and their synthetic analogues — low molecular weight compounds (ubiquinone, glutathione) [17-19].

EAO are highly specific, their concentration is relatively constant (except for pathological conditions), and they act strictly against activated oxygen metabolites which serve as substrates. Ions of zinc, silver, selenium, manganese, and iron increase the efficiency of reactions [20-22].

Some of the most powerful radical scavengers are phenolic antioxidants. Many of the several thousand known antioxidants, such as phenocarboxylic acids, are of plant origin and enter the body only with food [23-25]. Plants colored in red and brown tones, even black and purple, usually contain carotenoids and flavonoids. Carotenoids are effective antioxidants that scavenge singlet molecular oxygen and peroxyl radicals. More than 850 natural carotenoids are known [26-28].

Antiradical activity. Antiradical activity characterizes the effect-iveness of a particular antioxidant in neutralizing free radicals. Among natural antioxidants, flavonoids have the highest antiradical activity with a high rate of free radical neutralization [29-31]. AO flavonoids are also capable of inhibiting a number of enzymes that increase oxidative stress [32-34]. It has been shown that the maximum antiradical activity is characteristic of theoflavin, quarcetin and cyanidin. The activity of rutin is weaker, and it is minimal in flavones and flavone glycosides [35-37]. When assessing the beneficial properties of plants, special attention is paid to the so-called P-activity, which is largely determined by the phenolic component of the substances they contain. This group includes rutins,

quercetins, isoquercetins, anthocyanins, leukoanthocyanins and catechins. Each plant species and even variety has its own unique composition of antioxidant pools [38-40].

The maximum protective effect is due to both the high antiradical activity of plant metabolites with antioxidant properties and the variety of natural antioxidant substances, even with less antiradical potential, since their targets are often different [41-43].

Sources and properties of flavonoids. Rich sources of flavonoids are plants with dark-colored organs. Their pharmacological value varies depending on the chemical composition of the accumulated substances. Color can be a criterion for the accumulation of flavonoids, for example, anthocyanins or carotenoids in a plant, but it provides little information about the chemical composition of beneficial substances [44-46]. Antioxidants not only protect the human body, but also contribute to the preservation of food, for example, they stabilize food fats, can replace food preservatives, and improve nutritional value [47, 48].

In addition to AO properties, phenolic substances have anti-inflammatory, antimicrobial, and antispasmodic effects [49-51]. It has been noted that mixtures of carotenoids are more effective than each compound alone [52, 53].

Anthocyanins, coloring plant generative organs and fruits, are involved in attracting pollinators and seed distributors. In vegetative organs, anthocyanins are involved in adaptation reactions to environmental conditions. Anthocyanins are able to interact with regulatory proteins and components of signaling pathways, thus modulating physiological processes [54, 55[. The main sources of anthocyanins are dark-colored fruits, including elderberries, chokeberries, pomegranates and blueberries, currants, and black-colored tomatoes [53]. Recently, dark-colored cereals, amaranth, soybeans, grains, and tubers have been considered as sources of anthocyanins [54]. They are even more attractive as sources of these compounds, since they are characterized by longer storage, availability and the possibility of everyday consumption, unlike seasonal berries and fruits. Studies of the consumer characteristics of products made from rice and wheat grains that contained anthocyanins have shown that they are not inferior, and in some parameters are superior to the reference products that do not contain anthocyanins [56-58].

Soy is another source of antioxidants, namely isoflavonoids, which are a subgroup of flavonoids. In soybean seeds, the content of isoflavonoids varies from 0.1 to 5 mg/g, depending on the type of isoflavonoids and plant growing conditions [59, 60]. Soybean products have a preventive effect against cancer, inhibiting the growth of cancerous tumors due to the high content of genistein, a natural inhibitor of tyrosine-specific protein kinase, as a result, soybean products are considered functional [61, 62]. The accumulation of isoflavones in soybean seeds in the technical ripeness phase is 0.69 mEq/g (as per quercetin). By the end of the biological ripeness phase, it increases in vegetable soybean varieties to 0.90 mEq/g, which is 9.7% more than in grain varieties.

Sources of antioxidants in the diet are important for improving health and increasing human lifespan. As already noted [11-13], various pathologies are associated with oxidative stress, including the risk of carcinogenesis. Aging may be largely due to the accumulation of oxidants, the by-products of normal metabolism produced by mitochondria [63]. Oxidative damage to proteins and lipid membranes disrupts the structure of key enzymes, and due to lipid peroxidation, the level of mutagenic aldehydes increases [63].

Red grain rice varieties and *Momordica* charantia L. are plants with high antioxidant potential and significant accumulation of carotenoids. According to

the Central Botanical Garden of the Siberian Branch RAS, momordica is capable of accumulating carotenoids in the leaves up to 545.1 mg% per fresh weight, in the aryllus of the fruit 68.9-177.6 mg%, in the mesocarp 5.1-9.0 mg%. In the fruits of the orange-fruited tomato variety Top Model (control), the content of carotenoids (1.8 mg%) was more than 300 times lower than that in the leaves of momordica. For comparison, in carrots (a kind of standard for high carotene content among vegetable crops), the amount of carotene on average across 32 varieties was 16.6 mg%, and in the leaves of green vegetable plants, according to a study conducted in India, it was 3.85-130 mg% [44, 64].

The effect of antioxidants on health and life expectancy. Many natural antioxidants are common components of human food, which has made it possible to recommend large doses of them to slow down aging and increase life expectancy. However, half of the planet's population is deficient in one or another AO, which leads to conditions close to radiation aging. It is hypothesized that widespread insufficient AO intake leads to DNA damage through a mechanism similar to the effects of radiation and chemicals [63-65].

It is known that the complex of micronutrients entering the human body with food determines the development of microbial associations in the gastrointestinal tract (GIT), the microbiota of which plays a key role in the homeostasis of the host organism [66]. Dietary polyphenols have prebiotic properties and act against pathogenic gut microbiota, providing benefits in a variety of disorders. In particular, polyphenolic compounds can modulate the composition and function of microorganisms in the gastrointestinal tract, affecting membrane permeability and increasing the susceptability of bacteria to xenobiotics. When the composition of food ingredients changes, the composition of the microbiota also changes within 24 h, which affects the functional state of the body and its resistance to environmental factors [67, 68].

In mice, antioxidants could change average lifespan by suppressing tumors. The effect of antioxidants on tumors, in turn, has been linked to the involvement of free radicals in the regulation of proliferation and differentiation of both cancer cells and immune cells, as well as their other regulatory functions [69]. Optimizing the content of antioxidants in the diet is a reserve for increasing human life expectancy. There is a connection between demographic indicators and the content of antioxidants in foods that are commonly consumed in different countries For example, a Mediterranean diet rich in vegetables and fruits has been shown to increase life expectancy [70]. The so-called French paradox, that is, relatively low age-related mortality from cardiovascular diseases combined with high consumption of atherogenic foods in France, is associated with the high content of polyphenolic antioxidants in dark-colored wine. Timely administration of AO, which allows delaying cardiovascular diseases, can in many cases prolong life [71].

It should be noted, however, that the experimental results both confirm the benefits of antioxidants and do not prove their positive effects. Experiments are often carried out without taking into account all the factors influencing the result. More than 5000 antioxidants are known, which differ in chemical composition, antiradical and antiproliferative activity [72]. For many, an effect on proliferative activity was detected in some tumor cell cultures and much weaker in others. Synergism or additive effect of antioxidants is shown. That is, to protect against various forms of cancer, the composition of consumed antioxidants must be quite broad [73, 74]. Diversification of nutrition is necessary to effectively protect the body from the increasingly destructive influence of the environment. Each plant contains its own complex of useful substances. Therefore, limiting the range of fruits and vegetables in the diet reduces the effectiveness of the biologically active substances they contain.

Importantly, there are experimental data that indicate the limitations of

the free radical theory of aging. Thus, in the absence of oxidative stress, antioxidants did not have a positive effect on aging. Approximately the same result was obtained in mice, in which an increase in life expectancy under the influence of antioxidants was observed only in a conventional vivarium, whereas this did not occur in a pathogen-free vivarium in the absence of stress [65, 75].

Nevertheless, the search for new sources of biologically active substances and increasing their content in cultivated species remain important tasks. When studying the antioxidant properties of substrates, one must take into account the fact that cell cultures are similar to an isolated system not subject to stress, which significantly differs from the conditions inside an organism subject to all kinds of stress. In other words, in some cases, the undetermined effect of antioxidants may be a consequence of an incorrect methodological approach to assessing their properties.

Black and red grain rice as sources of antioxidants. Rice with a black pericarp color was known in China more than 3 thousand years BC. Even then it was served only at the imperial table, since it was believed to ensure health. In recent years, studies have been conducted that have confirmed the healing properties of this rice. Thus, black rice bran contains no less AO than blueberries and currants, and their antiradical activity is higher. Anthocyanins give rice grains their black color. Their benefits in the prevention of cancer and heart disease have been proven [74-77]. Chemical composition of black rice includes phytic acid, B vitamins, microelements, oryzanol, anthocyanins and vitamin E. Dozens of black rice varieties have been created in the world, differing both in the chemical composition of antioxidants and in antiradical activity. The most reactive antioxidants in black rice are cyanidin-3-glucoside and peonidin-3-glucoside. Their content varies over varietiesfrom 19 to 141 mg/100 g and from 11 to 13 mg/100 g, respectively [78, 79].

The color of a sample is directly related to its chemical composition, with darker pericarp indicating higher polyphenol content [80, 81]. The yellow or orange tint is due to carotenoids, aurones, flavones and flavonols, and flavonol glycosides. The red or dark brown color is due to flavonoid compounds — proanthocyanidins and phlobaphenes. The same pigments can determine different colors in different plant tissues due to a tissue-specific system for regulating the synthesis of these compounds [82].

Red grain rice is characterized by the presence of proanthocyanidins, whereas black rice by the accumulation of mainly cyanidin 3-glucoside and peonidin 3-glucoside [83]. Rice with red pericarp contains 166 to 732 mg/100 g of phenolic compounds [84]. Glutinous black-colored varieties accumulate from 260 to 2540 mg/100 g of anthocyanins. Varieties with colored pericarp contain more microelements (zinc, calcium, manganese, iron, and copper) [85]. In terms of total phenol content, red-grain varieties are 8 times inferior to black-grain varieties, in the accumulation of anthocyanins, approximately 60 times inferior, and in antiradical activity 45 times inferior [86].

Functional food products. Flour of a certain composition (from black rice, red rice, amaranth, black wheat, soybeans) is a functional product that helps improve the health. Rice flour can be white (from milled rice) or whole grain (from hulled rice). Polished rice flour is snow-white in color and almost devoid of smell and taste. Coarser whole grain flour is characterized by the presence of a seed germ, darker color and nutty aroma, it contains more vitamins, microelements, and antioxidants. Minimally processed hulled rice is especially beneficial because only the flower scales are removed. Subsequent processing (grinding and polishing) removes the germ and aleurone layer, which increases the shelf life of rice grains, but significantly reduces its nutritional value [87-89].

Rice flour's lack of gluten allows its use as an alternative to wheat flour for those with celiac disease or on a gluten-free diet [90, 91]. Gluten enteropathy, or celiac disease, is a chronic disease in which eating foods containing gluten (wheat, rye, barley) causes poor digestion and absorption due to damage to the mucous membrane of the small intestine.

Rice flour is lower in calories, easier to digest, and acts as a soft sorbent in the intestines, cleansing the body of toxins [92, 93]. The content of essential amino acids in rice flour is higher than in wheat and corn flour, and is slightly inferior to amaranth flour. The leader in their content is soy flour. In appearance, consistency, color, and smell, rice and amaranth flour are similar to wheat flour. Corn flour has a yellow color and a special aroma. Soy flour imparts a bean-like aroma and a brownish tint to bakery [94, 95].

Colored and white grain rice varieties do not differ significantly in protein content. Amino acids are a material for the synthesis of proteins, the deficiency of which disrupts the synthesis of vitamins, pigments, and hormones. An unbalanced composition of amino acids in food weakens a person's cognitive abilities and reduces immunity. The relationship with the likelihood of diabetes has been established for several amino acids, namely, serine, alanine, arginine. The content of lysine, an amino acid that limits the digestibility of cereal protein, is higher in rice than in wheat, corn and sorghum [92, 96].

The most valuable property of rice flour is its low asparagine content, which reduces the risk of the formation of carcinogenic substances in baked foods. There is evidence that the content of asparagine and soluble sugars is associated with the formation of acrylamide, a substance that causes cancer [96]. The main groups of products in which acrylamide is formed are French fries and chips, coffee, cookies, confectionery and bakery products. Acrylamide accumulates as a result of the interaction of asparagine with sugars (glucose and fructose) at temperatures above 120 °C and low humidity. The amount of asparagine in wheat grain varies from 75.5 to 2150 mg/kg, in oats from 51 to 1390 mg/kg, in corn from 71 to 2900 mg/kg, in rye from 310 to 900 mg/kg, in rice from 14.9 to 24.9 mg/kg. That is, on average, the amount of asparagine in rice is 3 times less than in wheat and corn, and 2 times less than in oats [97]. This property is used by adding rice flour to baked goods and confectionery products to reduce the carcinogenicity of hazardous products. The identified varietal variability by trait makes it possible to increase the usefulness of products with rice flour [94, 95].

Amaranth seed flour has a high protein content, 18.82%, which is 8.5% more than wheat flour. Amaranth seed flour contains almost 7 times more fat than wheat flour, and there is less starch and digestible carbohydrates. It is also characterized by a high potassium content up to 1500 mg%, which is 1378 mg% higher than wheat, and a significantly higher content of iron, calcium and magnesium.

Amaranth flour and flour from colored rice varieties added into the recipe enriche the products with vitamins and microelements [97, 98].

The use of whole grain flour is one of the trends in the production of healthy and functional products. Consuming whole grains reduces the risk of cardiovascular disease, obesity, diabetes, and some types of cancer [98].

Genetic mechanisms regulating the antioxidant properties of black pericarp rice. The study of genetic mechanisms that control color in plants is relevant given the antioxidant and antimicrobial properties of pigments and their colorless precursors. These compounds provide prevention of cancer, reduce the risk of cardiovascular diseases, atherosclerosis, type 2 diabetes, increase immunity, improve the synthesis of visual pigments, and activate metabolic processes [76, 84]. It has been established that intraspecific diversity in pericarp color is due to a complex of regulatory and structural genes [99, 100]. An increased content of

proanthocyanidins in the seed coat is associated with resistance to germination on the root, and the presence of anthocyanins in the pericarp contributes to better preservation of seeds after long-term storage and increased plant resistance to stress [101, 102]. That is, plants with a high content of antioxidants posess significant competitive advantages. Antioxidants increase plant resistance to biotic and abiotic stresses, but this aspect has not been sufficiently studied in rice varieties with colored pericarp [103, 104].

In rice, antioxidant compounds such as oryzanols, tocopherols, and phenolic acids reduce the risk of developing chronic diseases [105]. Among the various phenolic compounds in the grain of colored varieties, there are ferulic acid (56-77%) found in endosperm, bran and whole grains, p-coumaric acid (8-24%), sinapic acid (2-12%), gallic acid (1-6%), protocatechinic acid (1-4%), p-hydroxybenzoic acid (1-2%), vanillic acid (1%) and syringic acid (1%) [105]. Understanding the genetic nature of the traits that determine the antioxidant properties of rice is important for breeding. Variations in grain color and nutritional quality were studied in 416 accessions, including red and black rice. A total of 41 loci were identified for quality-determining traits.

Ra, *Prp-b* genes for varieties with purple pericarp and *Rc* (brown pericarp and aleurone layer) were confirmed to be major contributors to the phenotypic effect on rice grain color and nutritional quality [106, 107]. These genes are localized on chromosomes 9, 10 and 8 in the regions where markers RM228 (90-154 bp amplification product), RM339 (166-148 bp), RM316 (160-210 bp) are located. A total of 11 markers were identified for four pericarp color traits and one marker (RM346) is associated with phenolic content. The *Wx* gene locus was identified as a chromosomal region that determines color intensity. The identified markers can be used to improve the beneficial properties of rice via marker-assisted selection (MAS) [108].

Another study identified QTL (quantitative trait loci) for 5 traits of color, phenolic content, flavonoid content and antioxidant capacity [109]. Correlation analysis showed that the color traits of rice, namely, color intensity (L), red tint (a), yellowness (b), color tint (C) were interrelated. Phenol content positively correlated with flavonoid content and antioxidant capacity ($p < 0.001$), while flavonoid content was not associated with antioxidant capacity, but positively correlated with L color intensity. Three QTL located between markers GA285 and CT580 on chromosome 2, were associated with parameters L, b and C; the last two traits were also influenced by QTL on chromosome 8. Two other QTL on chromosome 2 (qPH-2 and qFL-2-1) flanked by markers CT87 and G1234 were identified as loci for the content of phenols and flavonoids with additive effects determining 16.91 and 12.71% of the phenotypic effect. Three QTL located in the same region of chromosome 7 between markers G379A and CT360 affected the color parameter a and antioxidant capacity. They may be allelic for the *Rd* gene which is responsible for pigmentation in brown rice [110].

To increase the nutritional value of products from functional rice varieties with colored pericarp, it is important to combine selection for traits that determine adaptability, quality and productivity [102, 111].

Genetic mechanisms regulating carotenoid biosynthesis in *Momordica charantia* L. Carotenoids were quantified in various organs of *M. charantia*, and genes responsible for the accumulation of carotenoids were identified. Using the momordica transcriptome database, identification was performed of a cDNA fragment clone encoding geranyl-geranyl-pyrophosphate synthase (McGGPPS2) and several clones of full-length cDNA encoding geranyl-geranyl-pyrophosphate synthase (McGGPPS1), zeta-carotene desaturase (McZDS), lycopene beta cyclase (McLCYB), lycopene epsilon cyclase (McLCYE1 and

McLCYE2), beta-carotene hydroxylase (McCHXB) and zeaxanthin epoxidase (McZEP). In various organs of *M. charantia* (leaves, flowers, roots, fruits) and at four stages of fruit ripening, the expression of mRNA encoding these eight putative enzymes of carotenoid biosynthesis, as well as the accumulation of lycopene, α-carotene, lutein, 13Z-β-carotene, E-β-carotene, 9Z-β-carotene, β-cryptoxanthin, zeaxanthin, anthraxanthin and violaxanthin. The transcripts constitutively express at high levels in leaves. Taken together, these results indicate that the enzymes McGPPS2, McZDS, McLCYB, McLCYE1, McLCYE2, and McCHXB may be key factors to control carotenoid content in momordica. In the future, overexpression of carotenoid biosynthesis genes in *M. charantia* can be used to increase the yield of these nutritionally and medically important antioxidants [46, 111].

Thus, our analysis of scientific publications showed the promise of using black and red rice, momordica and amaranth as non-traditional sources of antioxidants and microelements. The high content of antioxidants in these crops and their high antiradical activity have been confirmed in many studies. Cyanidin-3-glycoside and pionidin-3-glycoside are the main antioxidants of black rice. For red rice, momordica, and amaranth, high antiradical activity is associated with carotenoids. In black rice, flavonoids are among of the main antioxidants. The antiradical activity of flavonoids can be 50 times higher than that of many plant antioxidants; they are significantly inferior to vitamins E and C. Carotenoids are also effective antioxidants. The distinctive feature of carotenoids is their interaction with other substances of this nature, which increases the biological activity of the compounds. The intraspecific diversity in color traits observed at the phenotypic level is associated with both regulatory and structural genes. In addition to nutritional value, the increased content of anthocyanins contributes to better preservation of seeds after long-term storage, prevents germination on the root, and increases the adaptability of plants to biotic and abiotic stresses. Studying the genetic control of the biosynthesis of flavonoids, carotenoids and other-er antioxidant compounds will facilitate breeding for target traits to produce functional foods.

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POISONOUS PLANTS AND PHYTOTOXICOSES IN HORSES
(review)

A.A. KSENOFONTOVA ✉, N.P. BURYAKOV, D.A. KSENOFONTOV, V.G. KOSOLAPOVA

Russian State Agrarian University — Timiryazev Moscow Agricultural Academy, 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail tmetre@rgau-msha.ru (✉ corresponding author), n.buryakov@rgau-msha.ru, smu@rgau-msha.ru, v.kosolapova@rgau-msha.ru
ORCID:
Ksenofontova A.A. orcid.org/0000-0003-1920-2326
Ksenofontov D.A. orcid.org/0000-0003-2926-3085
Buryakov N.P. orcid.org/0000-0002-6776-0835
Kosolapova V.G. orcid.org/0000-0002-4409-0306
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Abstract

A large number of plants contain chemical compounds that have a toxic effect on animals (E.M. Kurenkova et al., 2018). Feed-born phytotoxins cause severe pathology in horses. Phytotoxins are diverse in species composition, distribution, mode of action and lethal effect. Poisoning of horses by poisonous plants is a relatively common veterinary problem that can occur when a fresh plant is ingested by an animal on pasture or when the plant contaminates hay, silage and other feed (F. Caloni et al., 2015). Plant toxicity is also a serious problem, as animal poisoning results in significant economic losses (L. Curtis et al., 2019). Depending on the degree of toxicity and the amount of plant eaten, the effect varies from mild illness to disruption of the activity of organs and body systems, which can lead to animal death (M. Wickstrom, 2002). Poisoning caused by poisonous plants is difficult to diagnose and differentiate from other pathologies, since clinical signs are usually not specific and can be observed in other diseases (K.E. Panter et al., 2012). Data on the true incidence of equine poisoning by plant toxins are sparse or absent due to lack of centralized poisoning reporting and monitoring system in place (K. Welch, 2019). Despite the fact that most toxic plants have an unpleasant taste for horses, there are many factors that increase the risk of poisoning, e.g., the influence of the growing season on the taste of some poisonous plants, lack of feed on pasture, toxic plants getting into the hay, monotonous habitat, curiosity, etc. (F. Caloni et al., 2015). It is important to constantly update the knowledge of veterinarians and animal owners about the poisonous plant diversity, phytotoxins, and phytotoxicoses. The review summarizes the most common plant species in Russia which causes poisoning in horses. The places are indicated where plants that are poisonous to horses grow, the mechanisms of action of the toxic substances they contain and their clinical effects in animals are described in detail, including disturbances in the digestive, cardiovascular, excretory, respiratory and nervous systems and many other sings. Poisonous plants are classified according to the mechanism of action of toxic substances into groups containing alkaloids, neurotoxins, photosensitizing substances, cyanogenic glycosides, and cardiac glycosides (M.I. San Andrés Larrea et al., 2024). The need for careful monitoring of the botanical composition of pastures and hay, and avoiding accidental consumption of poisonous plants by horses, is emphasized.

Keywords: poisonous plants, horses, phytotoxins, mechanism of toxicity, clinical signs, habitat

A potential threat to the health of horses arises from toxic compounds in plants eaten by animals due to violations in the management of stables and pastures [1], which can lead to decreased productivity, exhaustion, chronic intoxication and even death of the animal [2]. The increasing rate of weed spread in hayfields and pastures around the world is leading to the colonization of new regions by these species, posing additional threats to animals [3, 4]. There are hundreds of plant species that can be toxic to horses, with some only growing in

certain areas. To prevent poisoning, horse owners and managers of horse breeding enterprises need to be informed about species that have a toxic effect on animals, so that when studying the botanical composition of pasture grass, hayfields and areas adjacent to the stable, they can identify potentially dangerous plants [5].

In Russia, according to 2010 data, 4,730 species of meadow and pasture plants have been studied, more than 750 are classified as poisonous and harmful [6], and the phytotoxins contained in many of them cause poisoning of horses [7]. Poisonous plants are classified according to the target organ and the corresponding effect caused by the toxins they contain (cardiotoxic, hepatotoxic, neurotoxic, teratogenic and other effects), according to the active substances (containing cyanides, nitrates, oxalates, alkaloids, glycosides, etc.), by family and genus [8]. The formation and accumulation of toxic substances in plants is influenced by a number of factors, such as the growing area, soil, climatic and weather conditions, and phases of the growing season [9].

When horses are exposed to phytotoxins, pathological processes develop, characterized by varying severity and a variety of clinical signs. Providing information to horse owners and veterinarians about the potential hazards of feed contamination from poisonous plants can be a measure that will prevent animal poisonings and assist to performe anamnezing and diagnosis. Poisoning by poisonous plants, as a rule, does not have a specific treatment, and therefore the survival of the animal largely depends on supportive and symptomatic care. In some cases, the causes of death of the animal are not diagnosed until autopsy and identification of plant fragments in the stomach [10, 11]. Educating equine professionals about toxic plants and understanding the factors that influence the risk of poisoning will help ensure animal safety [12].

This review provides information on phytotoxins in plants that are poisonous to horses and various ways of poisoning. Here, we also summarize data on the nature of the effects of different classes of phytotoxins and describe the clinical signs and physiological and biochemical changes in acute and chronic toxicosis.

In most cases, horses avoid eating poisonous plants in toxic quantities. However, some plants are readily eaten by horses, and some species can cause poisoning if accidentally consumed in small amounts. The toxicity of some species is influenced by seasonal conditions, and therefore their negative effects on animals vary depending on the time of year. Since most poisonous plants have an unpleasant taste, horses try not to eat them, but with a lack of alternative feed, animal's feed selectivity decreases. Keeping horses in a monotonous environment that does not provide sufficient physical activity and/or sensory stimulation and the lack of satisfied zoosocial needs increase the likelihood of consuming poisonous plants out of boredom. Moving horses to a new location where the botanical composition of the pasture is unfamiliar to the animal may be another reason for eating plants that contain toxic substances or their precursors [13].

Poisonous plants should be considered a potential cause of disease if pasture feed supplies are poor due to overgrazing, drought, or poor early-season growth, if the animals have recently been moved to a new pasture, when hungry animals were released to new pasture, if a new feed or batch of feed (e.g., hay) are used [14]. The presence of such plants in hay can pose a serious threat to most stabled horses, as they generally do not have the choice unlike the animals that have access to pasture. Some horses will separate hay, refusing to consume suspicious plants, but most animals tend to eat them, despite the specific taste or smell, because due to the lack of constant access to hay they demonstrate a high rate of eating. A horse that is not very hungry can also eat hay contaminated with poisonous plants, since the intensity of the offending taste or smell can be reduced during drying without reducing toxicity [15].

Plant poisons, or phytotoxins, are products of plant metabolism and most often perform protective functions, scaring away possible consumers, since they have the properties of biological contaminants, the compounds that have high biological activity with a negative effect on animals [16]. Phytotoxins include a group of diverse low- and high-molecular bioactive compounds which include non-proteinogenic amino acids, isoprenoids, glycosides, alkaloids, phenolic compounds, steroids, lectins and other substances [17].

Plants containing alkaloids. Alkaloids are secondary metabolites found in approximately 20% of plant species. Alkaloids represent a diverse group of compounds linked by the presence of a nitrogen atom in a heterocyclic ring, including pyrrolidine, pyrrolisidine [18], tropane [19], pipyridine [20, 21], quinolizidine [22], indolizidine, quinoline [23] and other groups [24].

Pyrrolizidine alkaloids are one of the most important classes of naturally occurring toxins due to their widespread occurrence and high risk of accidental animal consumption of contaminated feed [25]. More than 350 pyrrolizidine-based alkaloids have been found in plants, with some plants containing multiple types of toxins. Pyrrolizidine alkaloids are a large group of cytotoxic [26], neurotoxic [27], tumorigenic [28], hepatotoxic, pneumotoxic and genotoxic compounds [29-31].

Pyrrolizidine alkaloids themselves are not toxic, but when absorbed from the gastrointestinal tract (GIT), they reachthe liver, their first target organ. The of hepatotoxicity caused by pyrrolizidine alkaloids is due to several mechanisms, such as activation of pyrrole ester, formation of reactive oxygen species, and activation of glutathione-degrading enzymes. The latter are found in most tissues, especially in high concentrations in the liver, and play an extremely important role in protecting hepatocytes, red blood cells, and other cells from toxic damage [32-35]. Pyrrolizidine alkaloids are also metabolized by liver microsomal enzymes to pyrrole derivatives which inhibit replication and protein synthesis. As a result, the cells are unable to divide and instead continue to grow, forming megalocytes which are eventually replaced by fibrous tissue [36].

The extent of damage to the liver or other organs depends on the specific type of pyrrolizidine alkaloid and its amount. Pyrrolizidine alkaloid poisoning can be acute or chronic, with the acute form being extremely rare because it occurs when large amounts of toxic plants are accidentally ingested with contaminated hay or when toxic plants become the dominant species in the pasture [37]. Chronic poisoning occurs when a horse consumes small amounts of the plant over a long period. The onset of clinical signs of chronic pyrrolizidine alkaloid poisoning is delayed and does not appear until several weeks or months after initial exposure. Thus, there may be cumulative and progressive effects of repeated administration of the toxin in small doses. This makes it difficult to determine when and under what conditions a horse was exposed to toxins [38].

Liver diseases with accompanying clinical signs in the form of progressive liver failure are becoming the most common signs of pyrrolizidine alkaloidosis. Acute intoxication leads to sudden death from hemorrhagic liver necrosis and visceral hemorrhages [39]. The animal may die within a few days or weeks. Horses with significant signs of acute pyrrolizidine alkaloid poisoning rarely recover. Clinical signs of chronic intoxication in horses include deterioration of condition, anorexia, lethargy, constipation or diarrhea, hepatic encephalopathy, jaundice (yellow discoloration of the mucous membranes), bloating (colic), and behavioral changes, e.g., head shaking, yawning, aimless wandering, head resting into the wall, hyperexcitability and aggressiveness [40].

Secondary, or hepatogenic, photosensitization may occur, caused particularly by disruption of detoxification in the live of phylloerythrin, the product of

chlorophyll metabolism. Phylloerythrin is produced by intestinal microflora and, during intoxication, accumulates in the skin where due to UV activation free radicals are formed that damage cell membranes. At the initial stage, erythema of non-pigmented hairless areas (nasal tract, eyelids and visible mucous membranes) appears, and later swelling develops, turning into dermatotonecrosis [41]. As the liver gradually loses function, the symptoms intensify, and when the liver is critically damaged, liver failure progresses rapidly. As a result death can occur suddenly or after the animal remains in a prone position for a long time, which is accompanied by hepatic coma and a high blood concentration of ammonia. Less common clinical signs described in pyrrolizidine toxicoses are inspiratory dyspnea in ponies due to laryngeal and pharynx paralysis, and dyspnea due to interstitial pneumonia in horses [42, 43].

Tropane alkaloids are a class of bicyclic alkaloids and secondary metabolites that occur naturally in many plants of the *Solanaceae* family and have anticholinergic effects. They reduce the metabolic effects of acetylcholine, a key neurotransmitter, by acting in mammals as antagonists of central and peripheral muscarinic acetylcholine receptors and, therefore, can cause a distinct toxic syndrome [44]. More than 200 tropane alkaloids are known, but the most studied are atropine and scopolamine [41]. Symptoms associated with tropane alkaloids are dryness of the upper gastrointestinal tract and respiratory tract, constipation and colic, dilated pupils (mydriasis), changes in heart rate, and central nervous system effects such as restlessness, irritability, ataxia, seizures and respiratory depression. In severe cases, death may occur from respiratory paralysis, heart failure, or gastric rupture within minutes, hours, or days after the animal consumes the toxic plant [45].

Piperidine alkaloids are extremely toxic to adult animals and also have a teratogenic effect. The mechanism of the teratogenic potential of these compounds is the stimulation of muscle-type acetylcholine receptors with subsequent desensitization and inhibition of fetal movements, which leads to the development of musculoskeletal pathologies [46, 47]. Acute toxicoses caused by plant piperidine alkaloids are due to their ability to reduce the sensitivity of acetylcholine receptors. As a consequence, paralysis of the endings of sensory and motor neurons occurs, as well as damage to the central nervous system (first its excitation occurs, and then paralysis) [48].

In Russia, horses are especially at risk of poisoning by such species of alkaloid-containing plants as *Convolvulus arvensis* L., plants of the genus *Aconitum* L., *Colchicum autumnale* L., *Hyoscyamus niger* L., *Datura stramonium* L., *Conium maculatum* L., *Senecio jacobaea* L., *Jacobaea vulgaris* L. [24, 49].

Field bindweed is a perennial herbaceous weed in cultivated areas. Tropane and pyrrolidine alkaloids were found in its shoots and roots [50]. Horses are very sensitive to toxic substances of field bindweed that cause impaired intestinal motor function, leading to colic [51], barycardia (slow heart rate) and dilated pupils [52].

Aconites are a genus of perennial poisonous herbaceous plants of the *Ranunculaceae* family, numbering over 50 species. The plants are distributed throughout Russia, growing in damp places along river banks and along roadsides, in humus-rich soils and mountain meadows [53]. Animals generally do not eat these plants, so poisoning in the field is rare, but they may contaminate the hay. All vegetative organs of plants contain alkaloids, primarily the highly toxic aconitine, which has cardio- and neurotoxic effects due to short-term stimulation of the central nervous system (especially the respiratory center) and peripheral nerves (the endings of the motor, sensory, secretory and recurrent nerves), followed by

their oppression. The mechanism for the development of pathophysiological reactions is due to the effect of the toxin on voltage-gated sodium channels in neurons, the conduction system of the heart and muscles, which causes a constant influx of sodium (that is, persistent depolarization) and prevents adequate repolarization, leading to seizures and arrhythmias. In the heart, excess sodium influx activates calcium metabolism, and intracellular hypercalcemia increases both the inotropic effect (changes in the force of cardiac contraction) and the likelihood of arrhythmias [54]. All parts of the plants are extremely poisonous to horses. If even small amounts are ingested, severe symptoms appear within minutes, and if lethal doses are ingested, death usually occurs within 6 h. Signs of intoxication in horses include gastrointestinal symptoms, including diarrhea and colic, as well as cardiac manifestations, e.g., slow heart rate, hypotension, and arrhythmias leading to suffocation which often causes death in animals [55]. Although there is not enough information about the toxic dose of aconite, a fatal case of poisoning of horses has been reported after eating 0.075% green plants by weight, that is, only 10-12 g. The most poisonous part of aconite is the rhizome, but cases of poisoning of horses with it have not been recorded. Poisoning by abovegraound parts of the plant is possible on mountain pastures and when grazing in gardens where aconites are grown as ornamental plants. There are known cases of poisoning with silage containing aconite plants [56].

H. niger and *D. stramonium* are poisonous plants of the *Solanaceae* family and growing mainly in the European Russia. Both species are classified as weeds; they can be found near roads, near housing, in fallow lands and fields. *H. niger* grows scatteredly or in small groups, and since the plant does not form thickets, it is difficult to detect at the early stage of the growing season. Datura is rarely found as a single plant; it often forms small groups (curtains) [57]. All parts of these plants contain the tropane alkaloids hyoscyamine and scopolamine the toxicity of which persists during drying [58], ensiling and haylage [59]. Animal consumption of henbane and datura may be accompanied by bloating, difficulty breathing, convulsions, cyanosis, tachycardia, incoordination, dilated pupils, and restlessness [60].

Poisoning of horses by *D. stramonium* has been widely studied. An outbreak of intractable, long-lasting, recurrent colic due to colonic and/or cecal obstruction without any other antimuscarinic signs occcur in horses consuming hay contaminated with this plant [61]. Toxic effects of this plant have also been documented in horses fed a sunflower-based supplement heavily contaminated with Datura seeds, one horse suddenly died from acute dilatation and rupture of the stomach, and in other horse an intestinal obstruction occurred which resulted in the animal being euthanized [62]. Spontaneous intoxication was described in 34 horses after eating freshly cut corn intended for silage and heavily contaminated with young Datura plants. Intoxication was accompanied by erythrocytosis, leukocytosis, regenerative neutrophilia with a shift to the left, lymphopenia, eosinopenia, increased hematocrit, low erythrocyte sedimentation rate, hyperglycemia, bilirubinemia, hypoproteinemia and increased activity of the enzymes alanine aminotransferase and aspartate aminotransferase. Autopsies and pathological studies of two dead horses revealed toxic liver degeneration, heart damage and significant degenerative and necrotic processes in the kidneys [63].

C. maculatum contains 8 alkaloids of which the most toxic are coniine and γ-coniceine, which have nerve paralytic and teratogenic effects. This is a widespread plant growing in the temperate climate zone; its natural range in Russia covers almost the entire European part, the Caucasus and Western Siberia. The plant is found everywhere, i.e., on forest edges, water meadows, limestone slopes,

as a weed in crops and vegetable gardens, in fallow lands and wastelands, near housing, along roads and fences, in landfills [64]. All parts of the plant are poisonous, and their consumption causes respiratory failure in less than 3 h. Horses can eat young plants when other feed is not available. The toxicity of spotted hemlock persists in hay, but gradually decreases during storage. Horses can be poisoned by eating this plant in amounts as low as 0.25% of their body weight. The alkaloids contained in the plant initially have a stimulating effect, followed by long-term depression of the function of the central nervous system. Common clinical signs of poisoning in horses include nervousness, frequent urination and defecation, trembling, staggering, ataxia, hyperpnea (disturbed respiratory rhythm) and tachycardia, followed by depression and inability to rise from a prone position. Coma also develops, and the animal may collapse due to respiratory failure [65, 66]. Teratogenic effects are possible when the plant is consumed by pregnant mares [67].

J. vulgaris is found in all regions of central Russia in meadows and clearings, along forest edges, in pine forests and in populated areas [68]; the plant is toxic to most species of productive animals. The active spread of this species in recent years has been facilitated by an increase in the area of fallow land, increased nitrogen levels in the air, targeted planting in roadside green spaces, and climate changes such as drought [69]. This has led to increased plant populations in pastures and grasslands, resulting in hay contamination [70]. Horses are particularly sensitive to the plant's toxic effects and generally avoid this weed, but poisoning can occur when animals graze poorly maintained pastures or when feed such as hay or silage is contaminated [71]. The alkaloids it contains cause liver damage followed by damage to the central nervous system, accompanied by ataxia and secondary photodermatitis [72-74]. The drying reduces the bitter taste of this plant, reducing feed selectivity in horses [75, 76]. An important factor influencing selectivity in horses when consuming feed is the formation in foals during early ontogenesis of feed habits which are based on imitation of the feeding behavior of their mothers [77]. Early diagnosis of poisoning is extremely difficult because many common biochemical and histopathological features of the disease are nonspecific and cannot distinguish poisoning from other immune, infectious or toxic diseases [78]. If liver disease is advanced, the prognosis is generally poor and treatment options are only palliative [79]. Poisoning of animals is accompanied by a number of clinical manifestations. Particularly, horses lose weight, signs of jaundice appear, hyposensitivity to external stimuli is observed (animals often stand with their heads down and may stop chewing feed in the mouth), tremors (especially in the head and neck area), frequent yawning and difficulty swallowing occur which can cause feed to be aspirated (inhaled) or regurgitated through the nasal cavity. Affected horses walk in a straight line or in circles, bumping into objects. Pressing on the head can often cause an animal to have a panic attack and run away uncontrollably. The disease is usually fatal, lasting from a week to several months [80].

Meadow saffron is found in the western and southern regions of Russia, usually growing in damp meadows. All parts of the plant are poisonous, especially the bulbs, since they contain the highly toxic tropolone alkaloid colchicine, which causes intractable multiple organ failure [81]. Colchicine is mutagenic and has the greatest effect on cell division and inhibition of tubulin polymerization, preventing spindle formation during mitosis. The main targets are organs with high mitotic potential, including the gastrointestinal tract, liver and kidneys [82]. Clinical signs develop approximately 48 h after ingestion and typically include drooling, dysphagia, colic, diarrhea, and malodorous stool with tenesmus, and may also include a history of bloody urine and cough [83]. In Germany, a case of horse poisoning

was recorded after feeding a batch of hay heavily contaminated with meadow saffron (apprx. 1.48% of the total mass). Three out of 17 horses developed colic; after a few days, one animal died; at autopsy, intense accumulation of serous or serous-hemorrhagic fluid was noted in the chest and abdominal cavity [84]. One study found that horses can consume contaminated hay when provided with free access to good quality hay [85].

Plants containing neurotoxins. Plant metabolites that have a neurotoxic effect have different chemical natures and specific mechanisms of action on the body, which underlie pathological changes caused by shifts in biochemical processes. One such compound is the unsaturated aliphatic alcohol cicutoxin, extracted from Cicuta species, which targets biomembranes that control the import and export of metabolites and ions in cells [86, 87]. Cicutoxin blocks Na^+ and K^+ channels and has a cholinergic effect on the central nervous system by acting as a noncompetitive antagonist of gamma-aminobutyric acid that binds to the beta domain of its receptor. This deactivates the receptor and disrupts the flow of chloride ions across the membrane, leading to unrelenting neuronal depolarization. Excessive stimulation of motor neurons leads to generalized seizures, resulting in death from respiratory failure [88]. At autopsy, lesions of the cardiac and skeletal muscles are observed, which are limited to pale areas, while multifocal degeneration of the myocardium is noted due to necrosis of myofibrils and replacement fibrosis in the tissues of the heart. Depending on the severity of convulsions, serum concentrations of lactate dehydrogenase, aspartate aminotransferase, and creatine kinase increase [89].

Neurotoxic effects have also been observed when horses consume plants containing the enzyme thiaminase I, which breaks down vitamin B_1 (thiamine), which plays an important role in the metabolism of carbohydrates, fats and proteins, and acts as a cofactor in enzymatic pathways responsible for energy production. Thiamine is an important cofactor in the decarboxylation of pyruvate to acetyl-CoA, which subsequently enters the tricarboxylic acid cycle. Deficiency of this vitamin impairs cellular energy processes and limits certain metabolic pathways, leading to systemic accumulation of pyruvate and lactate and, as a result, cerebrocortical necrosis (polioencephalomalacia) due to neuronal degeneration and death [90].

The poisonous plant *Cicuta virosa* L. of the *Apiaceae* family is one of the most toxic plants in the world. The range of this species includes the northern border of the forest zone. *C. virosa* grows in all regions of the European Russia, in Siberia and the Far East, rarely in Central Asia and the Caucasus, in swamps, damp meadows, marshy banks of rivers and lakes [91]. All parts of the plant contain cicutoxin, its toxicity decreases throughout the growing season, but the roots remain highly toxic all year round. Cicutoxin is not destroyed by high temperature and long-term storage. Poisoning most often results from grazing animals on pastures with depleted grass, in places where this species grows abundantly, as well as uncontrolled movement of animals. Additional factors contributing to the consumption of this plant are hunger and fatigue of animals, as well as their need for green food after the winter diet. In most cases, poisoning occurs at the early stage of the plant's growing season, when horses, tearing off young leaves and stems, remove the plant along with the rhizome from the soft, damp soil and eat it. In horses, severe poisoning occurs if they eat 1-2 rhizomes or 200-250 g of plant mass. Clinical gastrointestinal symptoms of poisoning include increased salivation and colic, cardiovascular and respiratory symptoms are tachycardia (rapid heartbeat) and tachypnea (rapid breathing). The animal becomes fearful, excited, coordination is impaired, and muscle tremors occur. Within 15 min of the first symptoms appearing, the horse may collapse [92].

Equisetum arvense L. is distributed throughout Russia everywhere except deserts, semi-deserts and the Far North. It prefers poorly drained sandy, fairly rich, moderately moist soils, and therefore is found in forests, upland and floodplain meadows, the edges of swamps, pebbles, sandbanks, fields, pastures, along the banks of rivers and streams [93]. It can seriously suppress field crops and other plants, and therefore often alone or together with cereals dominates the grass cover of water meadows and fallow lands. *Pteridium aquilinum* (L.) Kuhn. grows in coniferous and deciduous forests of the European Russia, Siberia, the Far East and the Urals. It is found on forest edges, open hills and in thickets of bushes, preferring light and poor soils, often dominating the grass cover. These plants contain the enzyme thiaminase, which catalyzes the hydrolysis of vitamin B_1 (thiamine) produced by microbial synthesis in the intestinal tract of horses, thereby causing deficiency [94]. The clinical signs of poisoning reflects the state of vitamin (thiamine) deficiency. Symptoms begin slowly and the first signs may include a general unkempt appearance, weight loss (without significant loss of appetite), diarrhea and slightly uncoordinated movements. If the animal is left untreated, the disease progresses to the point where the horse loses muscle control, has an unsteady gait, and has severe balance problems. The horse becomes restless, may lie down and be unable to get up, and convulsions occur. Ultimately, the animal may die from exhaustion within approximately 1-2 weeks [95-97]. *P. aquilинum* also contains the toxin ptaquiloside, found in the highest concentrations in young growing parts of the plant, which is thought to be responsible for bone marrow suppression and carcinogenic activity [98, 99].

Plants containing photosensitizing substances. Some plant species, such as buckwheat (*Fagopyrum esculentum* Moench) and St. John's wort (*Hypericum perforatum* L.), are capable of accumulating various biologically active substances, e.g., hypericin, fagopyrin, phyloerythrin, furocoumarin, etc., which, when ingested by animals, cause primary photosensitization, or light-induced photodermatitis of exogenous origin [100]. Possessing fluorescent and resonant properties, these substances become phototoxic when exposed to ultraviolet radiation. Clinical signs of photosensitivity dermatitis usually develop within a few hours of exposure to sunlight. Photodynamic agents, or chromophores, are absorbed into the gastrointestinal tract and transported through the circulatory system to organs and tissues. Hair and skin pigments have protective properties because they absorb light energy before it activates chromophores and damages skin tissue. The photosensitizing effect of these substances causes an increase in the sensitivity of non-pigmented areas of the skin, visible mucous membranes and eyes of horses to ultraviolet radiation, since its exposure activates the accumulation of toxic compounds. This initiates a destructive cascade of chemical reactions against susceptible substrates within cells (e.g., lysosomes, mitochondria, cell membranes, lipids, proteins, nucleic acids) and resulted in localized foci of inflammation [101].

Photodynamic substances are activated by photons and converted into high-energy compounds that cause tissue damage by releasing inflammatory mediators and producing free radicals, also known as reactive oxygen species. These are highly reactive molecules that damage membranes, disrupting cell permeability and homeostasis, leading to cell death [102]. As a result, inflammatory reactions develop in non-pigmented areas of the skin, which are accompanied by redness (erythema), swelling, itching and peeling [103, 104]. Large lesions may progress to blistering, ulceration, exudation, skin necrosis, and sloughing of necrotic tissue, which may lead to secondary bacterial infection. In horses, the most severely affected areas are poorly pigmented areas that are not well protected by the coat and exposed to the sun, especially the face, ears, eyelids, vulva and coronary bands; the tongue and oral cavity may also be affected. In severe cases of the

disease, photosensitivity can develop even in animals with dark and thick hair. Photophobia is also a clinical feature of the disease [105].

St. John's wort is widespread in Eurasia, often growing as a weed in forests, abandoned fields and pastures, along roads and reservoirs, and is poisonous at all stages of growth. In the spring, horses are attracted to young, tender shoots, and hay contaminated with them can cause poisoning in the winter [106]. Cases of photodynamic dermatitis have been reported in the Czech Republic when horses grazing on pastures contaminated with St. John's wort [107, 108].

Buckwheat is a plant widely cultivated in southern and central Russia, which is often used as a precursor that has a beneficial effect on biological processes in the soil, improving its physical, mechanical, agrochemical properties and phytosanitary condition. Occasionally it is found as a weedy alien plant in crops and along roads [109].

Sporadic cases of outbreaks of primary photosensitivity have been reported in horses fed predominantly alfalfa hay [110].

Plants containing cyanogenic glycosides. Apprpximtely 2,600 plant species contain cyanogenic glycosides which are a potential cause of acute and chronic poisoning in animals. Cyanogenic glycosides most often belong to monoglycosides (durrin, prunasin, linamarin, lotaustralin), their main carbohydrate component is D-glucose, and the cyanohydrin part is stabilized by a β-glycosidic bond. The most dangerous plants for horses are Sudan grass *Sorghum sudanense* (Pers.) Stapf and black elderberry *Sambucus nigra* L. [111]. By the enzymatic hydrolysis of cyanogenic glycosides in the gastrointestinal tract, a highly toxic compound, the hydrogen cyanide is formed which binds to the ferric iron of the cytochrome oxidase of the mitochondrial respiratory complex and forms the cyanide-cytochrome oxidase complex. As a result, the transport of electrons to molecular oxygen stops, the entire respiratory chain is blocked, which leads to cellular hypoxia, or cytotoxic anoxia. Because of the high content of hemoglobin saturated with oxygen and due to disruption of the process of oxygen absorption by tissues, the color of venous blood becomes bright red. In addition to cytochrome oxidase, in mammals, the activity of at least 40 enzyme (lactate dehydrogenase, catalase, peroxidases, etc.) is inhibited. Simultaneously with inhibition of the electron transport chain, the ATP synthesis, which is necessary to ensure biochemical processes in cells, is disrupted [112].

Clinical signs appear very quickly after animals consume large amounts of cyanogenic plants. In acute cyanide poisoning, death occurs within a period of several minutes to several hours. Animals exhibit signs of agitation and generalized muscle spasms, an unsteady gait, followed by severe clonic convulsions, as well as respiratory distress (shortness of breath), which develops as a compensatory response to tissue hypoxia, that is, the frequency and depth of respiratory movements increase. There is also lacrimation and drooling, mydriasis (dilated pupils) and bright color of the mucous membranes [113].

When small amounts of toxins are consumed within a few weeks, clinical symptoms associated with spinal cord and nerve damage begin to appear due to the destruction of the myelin sheath of peripheral nerves. Horses, due to impaired proprioceptive function, gradually develop ataxia (disorder of motor coordination), which is most noticeable when the animal is asked to turn. As the disease progresses, paralysis of the tail and hind limbs may occur. Due to damage to the nerves innervating the bladder, animals develop equine cystitis-ataxia syndrome, accompanied by incontinence (urinary incontinence) which in females leads to burns of the hind legs and, in some cases, to inflammation of the bladder and kidneys. In pregnant mares, toxins can cause abortion or cause skeletal malformations in the developing fetus. Once neurological symptoms appear, nerve

damage is irreversible and the prognosis for the animal is poor [114].

Sudan grass is a valuable forage pasture and hay crop. On the territory of Russia, it is cultivated in the southern and southeastern regions of the European part, in the Altai Territory and in the Far East. Sudangrass toxicity is associated with the formation of free hydrocyanic acid in the plant after drought or frost [115]. Cyanides are present in the form of glycosides mainly in the leaves and seeds and only in trace amounts in the stems. Hay is generally safe because the glycosides are hydrolyzed during storage [116, 117].

Black elderberry grows in the south of the European Russia, as an undergrowth in coniferous and deciduous forests, easily runs wild and spreads along roads, in populated areas and in wastelands. All parts of the plant are poisonous, with the exception of flowers, shell and pulp of ripe berries [118].

Plants containing cardiac glycosides. Many plants of different families synthesize toxins that directly or indirectly affect the functioning of the cardiovascular system. So-called cardiotoxic plants contain cardiac glycosides (gitoxin, digoxin, convallamarin, etc.), which cause acute pathological processes and sudden death of horses [119]. Species containing cardiac glycosides grow on the territory of Russia, among which the most dangerous for horses are *Convallaria majais* L., oleander *Nerium oleander* L. and representatives of the genus *Digitalis* L. [120].

Cardiac glycosides inhibit adenosine triphosphatase in cell membranes, causing electrical conduction disturbances in the heart muscle [121]. They inhibit the active transport of Ca^{2+} and K^{+} ions across plasma membranes, which increases the amount of Ca^{2+} inside cardiomyocytes, leading to cell death and secondary arrhythmias [122, 123] due to disruption of Hiss bundle conduction. Cardiac lesions are the main diagnostic marker of oleander poisoning in horses [124]. Animals experience short-term bradycardia, then the heart rate increases and arrhythmias appear. Glycosides have a local irritant effect on the gastrointestinal mucosa, and therefore clinical symptoms include hypersalivation and colic. Glycosides also take part in the development of pathological processes in the respiratory system, slowing down its functioning, which is clinically expressed by difficulty breathing. There is an increased release of exudate, an inflammatory process develops, accompanied by an increase in the number of opportunistic microflora, which contributes to secondary intoxication and leads to the appearance of muscle tremors, ataxia and the inability of the animal to stand. In addition, an irregular and weak pulse due to decreased cardiac output leads to hypothermia of the limbs, and the animal often experiences clonic convulsions before death [125]. Cardiac glycosides can pass into mother's milk and pose a risk to foals [126].

C. majais is a herbaceous plant growing in the European Russia, in Transbaikalia, the Amur region, Primorye, Sakhalin and the Kuril Islands [127]. All parts of the plant are toxic, with the highest concentration of cardiac glycosides in the roots. The plant also contains various saponins. *C. majais* is considered one of the most potent cardiotoxic plants as more than 38 cardiac glycosides have been identified in it, including convallatoxin, convallarin, and convallamarine [128].

Oleander is an evergreen ornamental shrub, common on the southern coast of Crimea, the Black Sea coast of the Caucasus and in Transcaucasia [129]. All parts of the plant, both fresh and dried, are poisonous and contain varying amounts of cardiac glycosides, especially oleandrin. The toxicity of oleander varies depending on the color of the flower and the time of year. Fatal intoxication for most mammals is possible with consumption of the plant in amounts as low as 0.005% of the animal's body weight [130, 131]. The plant also contains saponins and terpenoids. Although horses rarely eat green oleander leaves because they are unpalatable, there is a possibility that dried leaves accumulate in long-grass

pastures or end up in a horse's daily diet and may be ingested by the animal. Horses ingesting a lethal dose of oleander leaves are often found dead within 8-10 h, and symptoms of poisoning rarely last more than 24 h before death [132].

Digitalis is a genus of herbaceous plants containing the cardiac glycoside digoxin. On the territory of Russia, foxglove species grow in the Caucasus, in the European part and Western Siberia, in meadows, deciduous and mixed forests [133]. All parts of the plant are poisonous; the toxic dose for horses can be several hundred grams of the fresh plant, and when dried, this amount is significantly reduced [134].

In summary, poisoning of horses by poisonous plants is a relatively common phenomenon worldwide, causing serious economic harm to animal owners [135-138]. The main reason for horse consumption of these plants is the lack of control over the botanical composition of pastures and hayfields, which is especially important in the context of the spread of invasive species [138]. Clinical signs of plant poisoning in horses range from mild distress to sudden death, and diagnosis can rarely be made based on the clinical syndrome alone [139, 140]. Accurate diagnosis usually requires a history of exposure to the plant, making accurate identification necessary.

So, phytotoxins from poisonous plants are substances diverse in structure and mechanism of action on the body. The most common plants in Russia that are dangerous for horses contain alkaloids, neurotoxins, photosensitizing substances, cyanogenic and cardiac glycosides. Plant species containing different groups of alkaloids include bindweed, aconite, meadow saffron, henbane, datura, spotted hemlock and meadow groundsel. Pyrrolizidine alkaloids are hepatotoxic, and horses may experience secondary photosensitization when exposed to sunlight. Tropane alkaloids have an anticholinergic effect, acting as an inhibitor of the neurotransmitter acetylcholine. Piperidine alkaloids are toxic to adult horses and cause teratogenic effects. A neurotoxic effect is exerted by cicutoxin contained in the poisonous stem, as well as thiaminase produced by horsetail and bracken. The development of primary photosensitivity in horses is observed when consuming buckwheat and St. John's wort. Sudan grass and black elderberry contain cyanogenic glycosides which cause cellular hypoxia and disrupt the activity of the central and peripheral nervous system. Convallaria, oleander and foxgloves synthesize cardiotoxic substances that disrupt the functioning of the cardiovascular system of horses. Since there is no antidote for most toxins found in poisonous plants, treatment of poisoning is primarily symptomatic and supportive. In this regard, the best measure to combat phytotoxicoses should be considered prevention, which should first include a thorough check of hay and silage, and the removal of toxic plants from pastures.

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BOLTING IN SUGAR BEET (*Beta vulgaris* subsp. *vulgaris* var. *altissima* Döll): TRIGGERING, GENETIC MECHANISMS AND PREVENTION
(review)

A.Yu. KROUPINA ✉, P.Yu. KROUPIN, G.I. KARLOV, M.G. DIVASHUK

All-Russian Research Institute of Agricultural Biotechnology, 42, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail annshirley@yandex.ru (✉ corresponding author), pavelkroupin1985@gmail.com, karlov@iab.ac.ru, divashuk@gmail.com
ORCID:
Kroupina A.Yu. orcid.org/0000-0003-3654-8310
Kroupin P.Yu. orcid.org/0000-0001-6858-3941
Karlov G.I. orcid.org/0000-0002-9016-103X
Divashuk M.G. orcid.org/0000-0001-6221-3659
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Abstract

Sugar beet is a significant crop that is used in the production of sugar, alcohol, livestock feed, confectionery, etc. Sugar beet is a biennial plant that form a root-crop in the first year. In the second year, after winter storage, it produces a bolt with inflorescences. Bolting refers to the ability to form both peduncle and flowers within the first year of life. The formation of bolts in sugar beets is triggered by vernalization (exposure to low positive temperatures) and long daylight hours. Flowering is significant in beet-growing regions with cold springs and long daylight hours as it can result in reduced yield and sugar content. From a genetic perspective, flowering is controlled by a complex system of genes that regulate the transition from the vegetative phase to the generative phase of development. The interaction between the *BvBTC1* and *BvBBX19* genes plays a central role in this process. The functional products of these genes stimulate the expression of the flowering inducer gene *BvFT2* and inhibit the expression of the flowering repressor gene *BvFT1* (N. Dally et al., 2018). In the beet genome, several Arabidopsis orthologue flowering genes have been identified. These genes are characterized by differential expression and methylation, which are influenced by vernalization and vary between flowering-resistant and non-flowering genotypes (M.-V. Trap-Gentil et al., 2011; Z. Pi et al., 2021). The main physiological regulator of flowering in sugar beets is gibberellic acid, which is also involved in vernalization through the regulation of synthesis regulator genes (E. Mutasa-Gottgens et al., 2009). The primary methods for controlling flowering involve implementing suitable agrotechnical practices and developing resistant varieties and hybrids through breeding and genetic techniques. Agrotechnical practices include selecting the appropriate sowing date to avoid exposing plants to low temperatures, choosing recommended varieties for the cultivation zone, removing early flowering plants, and using chemical treatments on seeds and vegetative plants (I.A. Oksenenko et al., 1987; K.S. Devlikamov et al., 2016; M. Sadeghi-Shoae et al., 2017). Breeding methods involve creating an analytical framework for the negative selection of flowering material. This includes practices such as ultra-early and sub-winter sowing, selection under long-day conditions, sowing with vernalized seeds, and sowing in soil treated with herbicides (A.V. Kornienko et al., 1983; A.V. Logvinov et al., 2021, 2022). It is crucial to assess genetic collections from global repositories of cultivated and wild accessions in order to identify new sources of resistance to flowering (E.S. Kutnyakhova et al., 2016; V.I. Burenin et al., 2018). An important method for generating new non-flowering alleles is mutagenesis using ethyl methanesulfonate. Markers for allelic variants (haplotypes) of functional flowering genes, as well as quantitative trait loci and single-nucleotide polymorphisms associated with resistance to bolting can be used in marker-assissted selection (B. Büttner et al., 2010; Y. Kuroda et al., 2019; S. Ravi et al., 2021). Great prospects for accelerated sugar beet selection and seed production can be achieved through the "seed to seed" scheme. This involves stimulation of bolting under artificial climate conditions by carefully controlled growing parameters, including the vernalization stage. Important parameters for successful vernalization are temperature, the phenophase of vernalization initiation, and the duration of the photoperiod.

Keywords: sugar beet, vernalization, flowering, bolting, selection, marker-mediated selection, gene networks, agricultural technology, accelerated selection

Sugar beet (*Beta vulgaris* subsp. *vulgaris* var. *altissima* Дцll) is a universal industrial agricultural crop. Despite the fact that the only purpose of its cultivation is to obtain sugar from root crops, the plant is processed with virtually no waste. Thus, the tops remaining during harvesting are placed in the soil as organic fertilizer and sent to feed cattle and pigs; molasses is used in confectionery, in the production of yeast, alcohol, citric acid, etc.; pulp and molasses are also used for feed; defecate can be used as lime fertilizer [1].

Sugar beets are of great importance in crop rotation as a precursor for corn, legumes, annual grasses, millet, and early spring grains, which produce higher yields due to the soil-improving and phytosanitary positive effects of sugar beets [2]. However, the value of sugar beet as a precursor depends on climatic and soil conditions.

Sugar beets are divided into three groups, the high-yield (large root crops with a low sucrose content), high-yield-sugar (medium-sized root vegetables with an average sucrose content) and sugary (relatively small root vegetables with increased accumulation of sucrose). Thanks to the work of breeders, the sugar content in beet roots has risen from 1.3% from the time it was discovered in the roots to 17-20% [3, 4].

In 2017-2022, the world produced an average of 275 million tons of sugar beet annually, with a total planted area of about 4.6 million hectares [5, 6]. For this period statistics show that the European Union as a whole can be called a leader in the production of sugar beets with average gross harvest of approximately 113 million tons from an average sown area of 1.5 million hectares. However, when analyzing the each state separately, the Russian Federation demonstrates primacy in the industry with average gross harvest of approximately 44 million tons from an average sown area of 1.1 million hectares [5-7]. In France over the same period, the annual harvest of this root crop was approximately 36 million tons, in Germany and the USA approximately 30 million tons [6]. Sugar beets account for approximately 20% of world sugar production [5], and in Russia, this crop remains the only source of domestic raw materials for sugar production [8].

In 2022-2023 in Russia, sugar beets were grown in 24 regions and processed at 65 sugar factories in 18 regions. The gross harvest of sugar beets in the Russian Federation increased by 15% and returned to the values of the first post-Soviet peak in 2011 [9]. The most favorable regions for the crop are chernozems in the south of Russia, e.g., Krasnodar Territory, Volga region, Chernozem region, the North Caucasus and the Volga region.

One of the pressing problems of field commercial two-year cultivation of the crop is the so-called bolting, that is, flowering in the first year of the plant's life. Flowering is necessary to produce beet seeds, but when growing root crops, the varieties should not enter this stage of development.

Our goal was a systematic review of publications on bolting, the causes of its occurrence and methods to avoid the problem in sugar beets.

Tasks and challengs of sugar beet breeding and seed production in Russia. Sugar is an irreplaceable resource of quickly accessible energy for humans. As the world's population grows, the global demand for sugar also increases. Despite the fact that the sugar beet sown area in the Russian Federation is the largest in the world, the harvest here is several times less than in countries with smaller sown areas. On average for 2017-2022, the yield of sugar beet in Russia was 425 c/ha vs. 812 c/ha in France, 745 c/ha in Germany, 735 c/ha on average in the European Union, 682 c/ha in the USA, that is, approximately 1.5-2 times higher than in Russia [5-7].

In addition, with Russia's leading position in the area of sugar beet crops in recent years, more than 90% are sown with imported seeds [10]. Among other reons,

this is due to progressive agricultural technologies that exclude manual labor, and the resulting seed quality, i.e., grinding, calibration, pelleting, germination approaching one hundred percent, etc. [11].

However, despite the fact that the yield of imported hybrids and varieties is higher than that of domestic ones, it turned out that foreign hybrids are susceptible to diseases, such as root rot under conditions of excessive moisture, and do not accumulate the percentage of sugar content declared by their manufacturers. Domestic varieties and hybrids, compared to foreign ones, are more resistant to abiotic and biotic stress factors of the environment and have better shelf life, that is, they have less sugar loss during storage [12, 13]. This breeding potential must be use, so the lag in the methodology of scientific research and the use of breeding and seed advances are unacceptable, since this will limit the dependence of our farms on foreign seed suppliers, negatively affect-ing the economic and technological sustainability of the country's beet-sugar complex [10, 14]. In 2016, out of 33 new hybrids in the State Register of Breeding Achievements, only three were domestic. These varieties are Azimuth bred at the Pervomayskaya selection and experimental station of sugar beet (Krasnodar Territory) for the Kuban region with average yield of 496 c/ha, Konkurs bred at the Lgov experimental breeding station (Kursk Province) for the Central Black Earth region wirh a yield of 421 c/ha, and RMS 127 bred at the Mazlumov All-Russian Research Institute of Sugar Beet (Voronezh Province) with a yield of 324 to 720 c/ha depending on the cultivation area [15].

In Russia, within the framework of the Federal Scientific and Technical Program for the Development of Agriculture for 2017-2025, the program "Development of selection and seed production of sugar beets" is being implemented.. This state program focuses on breeding high-yielding and high-quality hybrids resistant to local abiotic stresses and diseases, on effective sugar beet cultivation, storage and processing, and on providing with high-quality seeds to reduce dependence on foreign hybrids. When creating single-seeded hybrids based on cytoplasmic male sterility (CMS), it is important to reach multi-germinate varieties and hybrids in terms of seed shape, germination energy and field germination [16-18]. Agrochemically active sugar beet varieties will produce more organic matter per unit of fertilizer applied [19]. Tolerance of hybrids to a specific herbicide will limit the herbicide use [20], and tolerance to stressful acidic soils, drought and heavy metals will expand the crop cultivation area [21, 22]. Wild beet relatives are involved in crosses as donors of valuable traits to create varieties resistant to diseases and unfavorable conditions [23, 24]. Important tools that help improve thesuar beet breeding are the molecular markers for genotyping lines and hybrids [25]. We need hybrids with a wide geographical area of cultivation, including in the northern regions, that is, cold resistant, with high productivity, product quality and not prone to bolting [26].

Bolting of sugar beets. Sugar beet has a two-year life cycle. In the first year it forms vegetative organs (shortened stem, root crop, leaves), in the second year a peduncle with seeds appears. In sugar beet traditional commercial breeding, non-planting, planting and transplanting (steckling planting) methods have become widespread to produce seeds. In the first year of life, mother roots (mother plants) are grown from the sown seeds, and in the second year of life, the seed yield is obtained [11]. Typically, leaves and flowering shoots grow in root crops planted in the soil in the second year of the growing season from dormant axillary buds formed in the first year at high temperatures. A further decrease in temperature to 0...+8 °C provokes the development of the latter. Under natural conditions, this decrease occurs in winter, and in the spring of the second year, the buds produce flowering shoots. However, in some cases, this can occur in the first year of plant life, which leads to bolting.

For every 1% of bolting plants, there is a reduction in yield by 0.5-0.7% [27]. In the root crops of bolting beets, the cell walls are compacted with an increased amount of lignin. Such root vegetables are difficult to cut into shavings due to excess fibrousness. The root crops of bolting beets differ significantly from ordinary ones not only in their chemical composition, but also in the increased mass of the head (20-22% of the root crop mass vs. 11-13%), increased woodiness and the content of molasses-forming substances, reduced sugar content and lower purity of beet juice. Bolting beets, especially early ones, are more susceptible to diseases and are unstable during storage due to greater damage by black rot [28-30].

In the fields, even a seemingly insignificant number of bolting plants can create big problems. Particularly dangerous is early bolting which becomes the precursor of a malicious weed, the weedy (wild) beets, since the fallen seeds can remain in the soil for decades without loss of germination ability. Seeds that have undergone vernalization germinate randomly in rows and between rows, bolt in 100% of cases, form numerous seed plants, and again repeatedly weed the fields, winning the fight for nutrients and inhibiting cultivated beet plants. This leads to yield losses and can paralyze the sugar mills. In weed-infested fields, specific beet diseases and pests spread [31].

Studies in model plants have shown that the regulation of flowering involves multiple pathways that depend on both environmental and endogenous signals [32]. The causes of bolting can be external (e.g., due to the influence of temperature and light conditions, mineral nutrition, herbicides, etc.) and internal, the genetically determined.

External factors for bolting. The bolting of sugar beets can be caused by very early sowing, prolonged low-temperature influence, the so-called vernalization (0...+10 °C for 1-6 weeks, often in lowlands, especially when cotyledon leaves and the true leaves appear) [33], illumination for more than 12 h, and depending on quality and intensity of illumination [34].

One of the main reasons for beet bolting is sowing too early, prolonged cold springs with a sharp cold snap without precipitation. In this case, the sown seeds lie in the ground for up to 40 days and manage to go through the stage of vernalization, i.e., acquiring or accelerating the ability to shoot and flower as a result of a long stay at low temperatures. For example, in 1974 in the Vinnitsa region of Ukraine, spring was dry and cold. The field was sown with beets on March 28 and partially reseeded on May 13 due to poor seedlings. During harvesting, 27% of beet planrs in the early sowing area were bolting, while there was none at all in the reseeded areas [28].

According to the All-Union Research Institute of Sugar Beet [2], with the spread of crops to the north, bolting increases due to lower air temperatures, lengthening daylight hours and far red spectrum of light with long waves which provokes shadow avoidance syndrome, when the stem sharply elongates towards the light source. For example, when sowing the same beet variety in the Vologda Province wirhdaylight period of 20 h 05 min in June, and in Kyrgyzstan with 15 h 10 min daylight period, the bolting rate was 10.2 and 0.01%, respectively [28].

Several researchers point to a connection between plant growth and premature bolting. For example, there is a positive correlation between the proportion of bolting plants and soil fertility or between the proportion of bolting plants and watering. In addition, a connection has been noted between the use of fertilizers, herbicides, mineral nutrition [35, 36], especially after vernalization, and the development of flower stalks, with nitrogen fertilizers having the strongest effect [28]. Reduced plant density has also been reported to result in more bolting plants [37]. It seems that favorable growing conditions, especially after vernalization, promote premature

bolting of sugar beets, with bolting being more pronounced at higher yields [36, 38]. Although there are several indications of a relationship between rapid growth and bolting, growers are unlikely to limit flowering by radically changing growing practices, since anti-flowering measures may reduce yields.

It is important to note that in addition to vernalization, the phenomenon of devernalization has been established. In years with the return of spring frosts after emergence, but with the subsequent rapid onset of a warm period, the percentage of bolting plants turned out to be lower than in years when there were no frosts, but the plants were exposed to low temperatures for a long time [36].

Single-seeded beet varieties and hybrids have lower cold resistance and,there of, higher bolting rate than multi-seeded varieties [28]. The most bolting are single-seeded tetraploid plants, followed by single-seeded diploid plants, multi-seeded tetraploid plants. In the literature, along with the concept of single- and multi-seeded plant, there are the terms dioecious and monoecious, single- and multi-sprouted forms. Multi-seeded diploid varieties are the least prone to bolting due to more focused elaborated breeding [18]. In addition, the size of fruitlets influences the bolting rate. Large ones appear earlier, and, therefore, produce the seedlings faster providing their longer vernalization [38].

Mathematical models for bolting prediction. Predicting the percentage of bolting plants in crops depending on external factors, e.g., daylight hours and temperature that largely depend on the sowing date, is important for planning agrotechnical measures. Planting time can influence sugar content, yield, and harvest time, which in turn is related to sugar mill operations [39-41].

In the UK, a “cool day” model was used to determine the expected proportion of bolting sugar beet plants in a field, in which the percentage of bolting plants is explained via the number of days with a maximum air temperature of less than 12 °C [27].

Later G.F.J. Milford et al. [34] proposed the equation to calculate the correction factor for the duration of vernalization (vernalization weighting) (1):

$$y = -1.256 + (1.260 + 0.131x) \cdot 0.9357^{x}, \quad (1)$$

where y is the correction factor for the duration of exposure by which the time (hours) of vernalization is multiplied; y depends on x, the observed temperature at a particular hour [34].

Typically, a massive transition of plants to bolting in a plot or in a growing season occurs after a certain threshold value of the sum of weighted vernalization hours, when the proportion of bolting plants increases sharply. This parameter is called “vernalization requirement” (VR).

The expected proportion of bolting plants (y) is determined according to equations (2) and (3):

$$y = 0 \text{ when } VI \leq VR, \quad (2)$$

$$y = BS\ (VI \cdot VR) \text{ when } VI > VR, \quad (3)$$

where BS (bolting sensitivity) is the proportional increase in bolting plants with each 10-hour increase in above-threshold vernalization, VI (vernalization intensity) is the accumulated number of weighted hours of vernalization between sowing and the end of June.

T. Chiurugwi et al. [42] used this model to determine the earliest time for sowing sugar beet in the UK that would have a 95% chance of avoiding bolting. Note that to use the model in Russia, recalculation of indicators is necessary. In addition, the model requires the inclusion of new coefficients, such as daylight hours, so that it can be used for both field forecasts and controlled climate chamber experiments.

E. Mutasa-Gottgens et al. [43] determined the bolting time as a function of

the height of the peduncle and the thermal time accumulation at a temperature threshold of 3 °C as the number of days after vernalization with a temperature above 3 °C multiplied by the average temperature per day. They used the equation (4), developed by J. Goudriaan et al. [44]:

$$H = (c/r)\ln(1 + \exp[r(\theta - \theta b)], \quad (4)$$

where H is the height of the peduncle, θ is the thermal time accumulation since the end of vernalization, r is the initial relative growth rate, c is the maximum absolute growth rate, θb is the accumulated thermal time at which the peduncle transition from exponential to linear growth occurs.

The model can be useful in experiments with different genotypes to determine the thermal time required for bolting after vernalization.

Internal factors for the occurrence of boltig. The genetic control of bolting in sugar beets is complex and has not yet been fully studied, despite a significant amount of data from various groups of researchers. To date, several models of gene networks regulating bolting have been proposed, in which, in addition to genes and protein regulators that mutually terminate or activate each other through cis- and trans-interactions, epigenetic and hormonal mechanisms are involved, triggered by external signals, such as photoperiod and vernalization.

The central gene in the genetic system of transition to flowering in sugar beet is *BvBTC1* (*BOLTING TIME CONTROL 1*), located in the *B* locus. *BvBTC1* belongs to the pseudoresponse regulator (*PRR*) genes and is homologous to the *Arabidopsis* gene *PSEUDO RESPONSE REGULATOR 7* (*PRR7* is the closest homologue of the sensitivity gene to photoperiodism in cereals *PPD1*). *BvBTC1* encodes a protein that carries a receiver response regulator (REC) domain [45], and photoperiodism sensitivity domains CONSTANS (CO), CONSTANS-Like, and TOC1 (CCT) [46].

Another bolting gene was cloned from the *B2* locus and named *BvBBX19* (*DOUBLE B-BOX TYPE ZINC FINGER*). In the gene network, *BvBBX19* is upstream of *BvBTC1* and influences it epistatically [47]. Plants that simultaneously carry functional alleles *BvBTC1* and *BvBBX19* are characterized by a one-year life cycle. Both BvBBX19 and BvBTC are homologous to the *Arabidopsis* CO protein which induces *FT* gene expression. However, unlike CO, BvBBX19 carries two zinc finger domains (B-box) but lacks a CCT domain; the BvBTC protein, on the contrary, carries a CCT domain.

Two *FLOWERING LOCUS T* (*FT*) genes, *BvFT1* and *BvFT2*, belong to the phosphatidylethanolamine-binding protein (PEBP) gene family located in the gene network downstream of *BvBTC1* and *BvBBX19*, therefore, the expression of *BvFT1* and *BvFT2* is controlled by the expression products of *BvBTC1* and *BvBBX19* [47, 48]. *BvFT1* and *BvFT2* are antagonist genes, while *BvFT2* promotes flowering and is required for flower development like its *Arabidopsis* ortholog gene *FT*, *BvFT1* acts as a repressor of flowering unlike *Arabidopsis FT*). N. Dally et al. [49] suggested that the functional proteins BvBTC and BvBBX19 form a heterodimer containing both CCT and B-box domains. It acquires the ability to increase the expression of the flowering inducer gene *BvFT2* and inhibit the expression of the flowering repressor gene *BvFT1*, which determines the annual type of development. With dysfunctional mutations, BvBTC and BvBBX19 lose this ability, resulting in either a two-year phenotype or a complete loss of the ability to form a peduncle.

Based on the analysis of coexpression of multiple sugar beet genes in leaves, a two-module model was proposed to describe the plant transition to flowering [50]). The first module includes four genes of the photoperiodic pathway (*BvELF3*, *BvGI*, *BvTOC1* and *BvBOA*), three genes of the autonomous pathway (*BvFVE1*, *BvFLD* and *BvFCA*) and *BvBTC1*. All genes serve as positive regulators of each other, with

the exception of *BvFVE1*. Its expression is negatively correlated with *BvELF3* [51]. In the second module, *BvFT1* and *BvFT2* were associated with *BvLHY*, *BvGATA22* and *BvFVE2*. *BvGATA22* showed negative feedback with the flowering activator *BvFT2* and positive feedback with the flowering inhibitor *BvFT1*. The expression of the latter was also positively correlated with the expression of *BvLHY* and *BvFVE2*.

It has also been established that sugar beet genes, the orthologs of which in *Arabidopsis* are associated with hormonal status, change theier expression during vernalization and/or in genotypes resistant to bolting. Among them, there are the gibberellin pathway genes *BvGA20ox1*, *BvGA20ox2*, *BvRAV1*-like, *BvRAV1*, *BvDELLA* and *BvRGA*, as well as the cytokinin-dependent gene *BvGATA22* [42, 51-53].

Iin response to vernalization, differential expression of vernalization pathway genes, such as *BvVRN1*, *BvVRN1*-like, *BvVAL1*, *BvVAL2*, *BvVIN3*, occurs [54, 55]. In addition, mall interfering RNA miR156 and long non-coding RNA MSTRG.26204.1 participate in vernalization [56, 5]. It has been revealed that the methyltransferases BvDNMT and BvRNMT which are factors of epigenetic modifications of DNA and RNA, respectively, are involved in vernalization [54, 58, 59]. Based on an integrated approach regarding both differential methylation and expression, a model was developed centered on the *BvBTC1-BvFT1-BvFT2* "core." The upstream flowering blocker *BvFL1* is activated by *BvRNMT* and inhibited by *BvFVE* and long-term vernalization (for 9 weeks). The activator of flowering *BvFT2* is positively regulated by *BvCOL1* and, in turn, positively regulates the expression of *BvAGL24* and *BvFUL*. *BvCOL1* and *BvBTC1* are also positively regulated by photoperiod length. Moreover, in boltin-unresistant genotypes, the *BvRNMT*, *BvFVE*, *BvFL1*, *BvFT1* and *BvFT2* genes are hypermethylated [59].

The study of lines derived from ethyl methane sulfonate (EMS)-induced mutagenesis and the natural allelic diversity of sugar beet and its wild relative, sea beet (*Beta maritima* L.), made it possible to find new loci that determine the requirements for vernalization, one- or two-year life cycle or flowering time. On chromosome II, two unlinked loci, *LB* and *LB2*, were identified, which in the recessive state form a late-flowering phenotype [60, 61]. B. Büttner et al. [62] identified two loci, *B3* and *B5* that affect flowering timing and are not linked to the *BvBTC1* locus. S.F. Abou-Elwafa et al. [63] discovered the *B4* locus, determining the requirements for vernalization, at a 11 cM distance from the *B* locus on chromosome II. Y. Kuroda et al. [64] identified the dominant gene *BLOND*, its carriers form seeds in 4 months under 24-hour daylight without vernalization. N. Pfeiffer et al. [65] identified QTL (quantitative trait loci) *BR1* on chromosome IX, associated with resistance to bolting after winter, for which C. Tränkner et al. [66] identified *BvCPSF73-Ia* as the most likely candidate gene and also identified an additional compensatory gene *BvCPSF73-Ib*.

N. Pfeiffer et al. [67] identified three QTL, on chromosomes III (*DTBnat1-DTBart1*), V (*DTBnat2-DTBart2*), and IX (*DTBnat3*) that influenced the timing of bolting transition. In beets, a tandemly duplicated locus *Bv_22330_orky* was discovered on chromosome VI, in the intron of which SNP183 (single nucleotide polymorphism) was associated with the predisposition of sugar beets to flowering [68]. Y. Kuroda [69]) identified QTL *qB6* in close proximity to this SNP, associated with resistance to bolting. The author believes that it may correspond to the previously described genes *BvFL1* or *Bv_22330_orky*. S. Ravi et al. [70] found two SNPs associated with a low propensity of sugar beet to bolting, The first is SNP_36780842 on chromosome I in the 3′ UTR of a gene homologous to the genes of the chaperone-J-domain superfamily which involved in the control of flowering, The second is SNP_48607347 on chromosome II in the exon 3 xylose isomerase genes, probably

involved in the modulation of the endogenous amount of sugars, important for signaling during the transition to flowering. Y. Kuroda [69] showed minor QTL associated with bolting, including *qB1* on chromosome I near QTL SNP_36780842. Minor QTLs *qB8* on chromosome VIII and *qB9* on chromosome IX were also found.

In addition to nuclear genes, the mitochondrial genes *ORF152*, *ORF102b*, *ORF192*, *ORF104*, and *COX2* have also been shown to be differentially expressed and/or methylated [59].

The transition to flowering is accompanied by complex changes in the hormonal status of the plant. Among the hormones associated with vernalization, gibberellic acid (GA) plays a significant role. This is shown in works on the influence of hormones on the transition of sugar beet plants to bolting and flowering, depending on the genotype and growing conditions, especially temperature and day length.

E.S. Mutasa-Gottgens et al. [71] demonstrated that genotypes *BB* and *Bb* require long daylight hours for bolting, regardless of gibberellin status, while biennial genotypes *bb* require vernalization for the GA-mediated transition to bolting. For transition from bolting to flowering, both genotypes require long daylight hours, and the GA content is not a limiting factor. Y. Koda et al. [72] found that exogenous jasmonic acid (JA) leads to thickening of the main and, to a greater extent, lateral roots, inhibition of bolting caused by GA treatment and vernalization. The JA content in the apical leaves of plants in the field increased during the summer, reaching a peak in August and decreasing in September. N. Liang et al. [73] demonstrated in plants grown from vernalized roots that after vernalization there is an increase in the amount of GA and indolylacetic acid, associated with the accumulation of auxin signaling protein GH3.1 and gibberellin signaling protein GA3OX1. Bolting probably occurs when a certain concentration of these hormones is reached.

Expression of the *BvRAV1-like* gene increased 2.5 times after vernalization and an additional 3 times after treatment of sugar beet plants with gibberellins. Without vernalization, treatment with gibberellins reduced the expression of this gene [53]. E.S. Mutasa-Gottgens et al. [53] identified 19 genes differentially expressed by GA treatment. According to L. Zhao et al. [52], vernalization suppresses the expression of *BvABFs* and *BvMYC2s*, implying inhibition of asbcisic and jasmonic acid signaling.

Agrotechnical methods for combating bolting. The main methods remains the optimal sowing time, compliance with the requirements of agricultural technology, the use of resistant hybrids and varieties in the regions according to recommendations [28]. Even the most genotypically productive hybrid will show unsatisfactory characteristics if seeds are used that are poorly prepared at the seed plant and obtained in violation of agrotechnical requirements [74].

The Russian Federation is a country with different soil and climatic conditions in each beet growing zone, that is, varieties and hybrids mustmeet certain specific requirements. Drought-resistant and early-ripening varieties are needed for the Central Black Earth zone, the varieties responsive to irrigation, late-ripening and resistant to cercospora are needed for the southern territories, and non-bolting varieties for the northern regions [18]. It has been established that beet varieties that were created for northern latitudes do not form bolting plants, while those produced for middle latitudes can produce up to 10% bolting plants, those originating from southern countries — 10-50%, and from the most southern and hottest countries up to 100% [36]. As a rule, timely destruction of early bolting plants in the fields during the period from bolting to budding prevents crop rotation from being clogged with wild beets. This is an effective and cheap method, similar to manual weeding on grain crops. It is also advisable to identify and consider bolting plants in trials along

with productivity assessments [31].

Several methods of chemical treatment of sugar beet seeds have been patented to combat bolting and to breed forms resistant to premature flowering. This includes treating seeds with a treflan solution with storage at +8...+10 °C and subsequent winter sowing. In the spring, bolting plants are eliminated, and non-bolting plants are preserved until the end of the growing season and are used as source material with resistance to bolting [75]. Another way is to treat the seeds with a solution of chlorocholine chloride (TUR retardant) which delays the initial formation of the seedling, as a result of which the spring development of beets occurs at a higher temperature, the number of bolting plants decreases by 1.6-4.2 times, and the yield increases. The method is recommended for northern regions [76]. The use of paclobutrazol, a plant growth regulator and gibberellic acid inhibitor, is also proposed to reduce the percentage of bolting, to increases the sugar content and to improve the quality of root crops, depending on the genotype of sugar beet [77, 78].

Breeding methods for creating varieties resistant to bolting. To select non-bolting forms, the pre-winter sowing is used when the air temperature is approximately 0 °C and the soil temperature drops to +2...+4 °C. This method is used at the Mazlumov All-Russian Research Institute of Plants and Plants in some experimental breeding stations. Another methods are ultra-early sowing; selection of vernalized seedlings under long-day (the method has been developed by N.A. Negovsky); sowing seeds vernalized for 45 to 60 days (it is especially effective in the western and northwestern regions); negative selection of early ripening plantings; selection under polar day conditions at the VIR polar station. Selection within a population is most effective [18].

A.V. Logvinov et al. [36] developed and put into practice in the conditions of the Krasnodar region reliable methods for assessing and selecting bolting-resistant initial breeding material and commercial hybrids. After pre-winter and early spring sowing dates, bolting plants were detected in June and September before harvesting. Dioecious forms exhibited bolting (mostly early) to a greater extent than monoecious forms. Vector and Atamansha hybrids showed the best resistance to bolting (0%). Further studies have demonstrated that an effective method for assessing and obtaining breeding material resistant to bolting is provocative early spring sowing with seeds germinated at +9 °C or treated with an aqueous solution of the herbicide Burefen FD-11 (emulsion concentrate, active ingredient desmedipham 80 g/l and phenmedipham 80 g/l, FSUE VNIIHSZR, Russia) at a working solution concentration of 5 ml/l [36].

A comparative study and assessment of sugar beet breeding material for resistance to bolting was carried out by A.V. Logvinov et al. [30] (Pervomaisk Selection and Experimental Station of Sugar Beet, Krasnodar Territory, and Experimental Scientific Station for Sugar Beet, Republic of Belarus) using a specially developed provocative technique. Pre-winter and early spring sowing was carried out with seeds pre-soaked in water and kept for 20 days at +3 °C. the hybrids Pervomaisky and Korvet expressed the greatest resistance to bolting [30]. Sowing of vernalized germinated seeds in a greenhouse under additional lighting may be used to isolate non-bolting forms from the beet population [38]. A.V. Kornienko et al. [79] propose provocative conditions by introducing into the soil a mixture of herbicides Eptam and Lenatsil, which enhances bolting by 20%, followed by selecton of non-bolting forms. To maintain the so-called ‘stubborn ones’ in genetic collections, a cultivation method with a multi-level rejection system has been developed [80].

Studying the genetic diversity of sugar beet allows identification of new donors of resistance to flowering. E.S. Kutnyakhova et al. [81] in 2012-2014 in evaluation of sugar beet hybrids bred by Lion Seeds Co., Ltd. (Thailand) and Mazlumov

VNIISS found out that half of the samples showed boling from 0.4 to 1%. V.I. Burenin et al. [82], when assessing the VIR collection, found that samples from Sweden were characterized by the greatest resistance to bolting, the breeders from Germany have also successfully created dioecious specimens resistant to bolting.

Marker-assissted selection of bolting-resistant plants. MAS for any trait is based either on functional markers of allelic polymorphisms of genes with known sequences, or on markers linked (associated) with traits. If in the first case the gene sequence and phenotypic manifestation of the alleles are known, then in the second case information about the structure of the gene and the functional role of the found nucleotide polymorphism is most often absent. A feature of the studied functional genes that regulate flowering in sugar beets is the presence of many polymorphisms between allelic variants, including SNPs and indels, which allows them to be called haplotypes.

B. Bьttner et al. [83] developed a codominant marker, GJ1001c16, that distinguishes the dominant *BvBTC1* allele (one-year life cycle) from the recessive one. The marker has been tested in many studies with segregating populations to search for alternative vernalization genes [53, 63, 67, 84].

For the allele resulting from EMC-induced mutagenesis and leading to a two-year phenotype, the CAPS marker CAU4206 (primers NH619 + NH620 and restriction enzyme HinfI) was developed [85]. Y. Kuroda et al. [86] developed primers F2/R2 to amplify the sequence between exons 7 and 9. The use of the HhaI restriction enzyme made it possible to distinguish between alleles *a* (biennial developmental type), *g* and *o* (one-year developmental type). In sugar beet, the allelic diversity of *BvBTC1* is well described, its nucleotide sequences are publicly available [85-88], so the development of new molecular markers of polymorphisms characteristic of certain alleles are expected. For anonymous genome regions associated with resistance to bolting, we can note the TaqMan marker of single nucleotide polymorphism SNP18. Its allelic variant *T* is associated with resistance to bolting, *C* with susceptibility [68]. Another markers are two HRM (high resolution melting) markers, the SNP /SNP_36780842 (*G* allele is associated with boting resistance, *C* allele with susceptibility) and SNP21/SNP_48607347 (*C* allele is associated with bolting resistance, A allele with bolting susceptability) [70].

Mutagenesis and genetic engineering in the creation of plants resistant to bolting. To create new highly adaptive breeding forms of sugar beet, it is necessary to expand its allelic diversity, including genes that determine the requirements for vernalization, sensitivity to long daylight hours and resistance to bolting. It is possible to create fundamentally new alleles or use new genes in the beet genome using mutagenesis, genetic engineering and genome editing.

Mutagenesis is a fundamental method for studying the structural and functional characteristics of a gene, as well as one of the available methods for increasing genetic diversity and obtaining new promising breeding forms, in particular sugar beets [89].

The TILLING method (the targeting-induced local lesions in genomes) is based on point mutations using EMS-induced mutagenesis with subsequent identification of the target gene in the resulting lines by the reverse genetics method [90].

U. Hohmann et al. [91] used EMS-induced mutagenesis to create a collection of sugar beet lines based on the early flowering line 930190. Experiments with mutant lines identified loci *B2*, *B3*, *B4*, and *B5* [62, 63, 85]. A model of the interaction between the BvBTC1 and BvBBX19 proteins was constructed [47, 49], and a new allele *BvBBX19h* was obtained [49]. S.L. Frerichmann et al. [92] using the EcoTILLING method with restriction enzyme CELI to search for mutations, detected 20 silent SNPs and one nonsynonymous SNP in the *BTC1*, *BvFL1*, and

BvFT1 genes, resulting in 55 haplotypes. The authors also found associations of nucleotide polymorphism in *BvFL1* with winter bolting and winter hardiness.

Another approach to improving sugar beets is the creation of transgenic plants. In order to study the influence of hormonal status on bolting and flowering, E. Mutasa-Gottgens et al. [43] obtained transgenic lines of sugar beet with genes for hormonal metabolism of beans and *Arabidopsis*. A transgenic sugar beet line with the bean gene *PcGA2ox1*, which is involved in the degradation of biologically active forms of GA, required an additional 20 days for the transition to bolting, had a dwarf phenotype and was sterile, but male fertility was restored by spraying with GA. The *Arabidopsis* transgene *gai*, which is an allelic variant of the DELLA protein lacking the DELLA domain and weakly sensitive to GA, caused a delay in bolting in a sugar beet plant for 11-14 days while maintaining fertility [43].

Genome editing allows new alleles to be created based on existing genes, and the resulting plants do not carry transgenes. To date, there is only one report of CRISPR/Cas9 editing of sugar beet in relation to resistance to beet curly top virus [93]. Since allelic variants that lead to the formation of a two-year phenotype requiring vernalization arise as a result of disruption of the functionality of proteins involved in the transition to bolting and flowering, genome editing as a tool for obtaining non-functional alleles is promising for the creation of sugar beet forms resistant to bolting.

To summarize, it should be noted that beet growing remains one of the most popular, knowledge-intensive, technologically and organizationally complex industries. The problem of import substitution of seed and varietal material of sugar beet requires effective interaction between representatives of various scientific fields. The combination of modern agrotechnical, biotechnological, molecular genetic methods (including genomics and epigenomics, transcriptomics, metabolomics and proteomics), speed breeding technologies and classical selection methods in the creation and cultivation of sugar beet hybrids will increase their productivity and the quality of domestic seed material. Vernalization of seeds in the mother plant and the biotechnological method of rescuing embryos seem to be promising methods.

Thus, bolting is a problem faced by many sugar beet growers. The most effective solution is to obtain genotypes in which resistance to bolting is combined with a complex of other useful traits, e.g., productivity, sugar content, resistance to abiotic and biotic stresses during the growing season, di- and monoecious forms, long-term storage, technological qualities, etc.). The use of varieties recommended for a specific zone and compliance with the regulations for their cultivation, including seed treatment, allows avoiding plant bolting. Bolting is a complex natural phenomenon, the physiological and molecular genetic mechanisms of which continue to be studied. Their understanding and assessment of genetic collections of cultivated varieties and wild species will allow us to identify and obtain new alleles for resistance to bolting. When creating haplotypes suitable for selection, genomic editing can be used along with classical mutagenesis. It is necessary to continue studying the allelic diversity of genes regulating the transition to flowering and the search for valuable nucleotide polymorphisms using genomic selection. Particular attention should be paid to the speed breeding to obtain seeds under controlled conditions due to the ability of sugar beets to form a flowering shoot from a rosette under the influence of vernalization and long daylight hours.

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ANALYSIS OF THE *Triticum aestivum* L. GENETIC DIVERSITY INDUCED BY THE CHEMICAL MUTAGEN PHOSPHEMIDE

N.A. BOME[1] ✉, S. SALEKH[1], M.U. UTEBAYEV[2], K.P. KOROLEV[1], L.I. WEISFELD[3]

[1]*Tyumen State University*, 6, ul. Volodarskogo, Tyumen, 625003 Russia, e-mail bomena@mail.ru (✉ corresponding author), corolev.konstantin2016@yandex.ru, samuel.biotech@yandex.ru;
[2]*Limited Liability Partnership A.I. Barayev Research and Production Centre of Grain Farming, Republic of Kazakhstan,* 021600, Akmola region, Shortandinsky District, Nauchny village, e-mail phytochem@yandex.ru;
[3]*Emanuel Institute of Biochemical Physics RAS,* 4, ul. Kosygina, Moscow, 119334 Russia, e-mail liv11@yandex.ru
ORCID:
Bome N.A. orcid.org/0000-0002-5467-6538
Salekh S. orcid.org/0009-0007-6907-7371
Utebayev M.U. orcid.org/0000-0003-0729-0592
Korolev K.P. orcid.org/0000-0001-9595-3493
Weisfeld L.I. orcid.org/0000-0002-8449-3979

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Abstract

Currently, climate change and growing demand for food necessitate effective methods for crop improvement. Induced mutagenesis is a promising tool to create breeding material. This work, for the first time, established the potential of the chemical mutagen phosphemide on spring soft wheat. Particularly, it was revealed that seed treatment with an aqueous solution of the mutagen in optimal concentrations effectively increases genetic variability to select economically valuable forms. Our goal was to increase the genetic diversity of spring bread wheat (*Triticum aestivum* L.) using the chemical mutagen phosphemide and to determine the biological potential of mutants (M_5, M_6) based on the variability of morphological and productive traits under the conditions of the Northern Trans-Urals. A total of 29 spring soft wheat samples selected from mutant populations of two cultivars, Cara and Skant 3, from a hybrid (Cara × Skant 3), as well as three control cultivars, the Tyumenskaya 25, Tyumenskaya 29, Novosibirskaya 31 were involved in the study. Mutant samples were generated using the chemical mutagen phosphemide. The seeds were treated with a 0.002 and 0.01 % phosphemide aqueous solution for 3 hours. Identification of mutations and testing their stability were carried out in the second (M_2) and third (M_3) generations. Here, we submit data for M_5 and M_6 mutants grown under contrasting meteorological conditions in 2021-2022. Sowing, observations, records, description of morphological characteristics and biological properties of plants were carried out at the experimental site of the biological station of the Tyumen State University Lake Kuchak (Nizhnetavdinsky District of Tyumen Province). Electrophoretic analysis of gliadins was carried out in caryopsis of the 2021 harvest (M_5). Based on grain electrophoretic analysis of the original and mutant plants, genetic formulas of gliadin were compiled, and the frequency of gliadin coding loci alleles (*Gli*) was determined. In field tests, significant differences occurred between genotypes in quantitative traits, e.g., plant height, linear dimensions and area of the flag leaf, number of productive stem per 1 m^2, ear length, grain number and weight per ear. Correlation analysis revealed that the strength of the relationship between yield and other traits depends on the meteorological conditions of the growing season. The dependence of grain yield on the number of plants ($r = 0.71$, $p < 0.05$) and productive stems ($r = 0.71$, $p < 0.05$) per 1 m^2, on plant height ($r = 0.82$, $p < 0.05$), chlorophyll content in flag leaf cells ($r = 0.28$, $p > 0.05$), and the number of grains per ear ($r = 0.73$, $p < 0.05$) was stronger under water and heat stress. Five mutants of spring soft wheat with a relatively high biological potential compared to other samples and standard cultivars were selected for a set of valuable traits. These mutants had the same allelic composition for gliadins. The yield was higher in 2022 and amounted to 396.1-518.2 g/m^2 for the best mutants, and 355.0-424.5 g/m^2 for the standard cultivars. Thus, the adaptation potential of spring soft wheat in the Northern Trans-Urals extreme conditions can be increased due to genetic variability of mutant populations. The biological effect of the chemical mutagen phosphemide has been proven to induce beneficial mutations in *T. aestivum*. Therefore, combination of mutational and recombination variability is effective in increasing genetic diversity.

Keywords: spring soft wheat, genotype, mutant forms, gliadin-coding loci, stress, resistance, quantitative characters, correlation

Climate change, leading to droughts, soil salinity, high temperatures, and the emergence of new diseases and pests, is a serious threat to global crop yields [1]. The growing demand to increase crop production to meet food demand necessitates effective methods for plant improvement.

Currently, plant improvement involves the integration of traditional and molecular methods. Among the available selection and genetic tools that allow the creation of donor plants with economically valuable traits, the induced (artificial) mutagenesis remains promising. Among varieties registered in the International Atomic Energy Agency database (FAO/IAEA, http://mvgs.iaea.org), there are 3402 mutant varieties of various crops, including 265 wheat varieties [2].

The effectiveness of molecular genetic analysis depends on the properties of the mutant population which determine the frequency of mutations, their diversity and quality. Considering that recombination variability can increase under environmental changes which is typical for the sharply continental climate of Western Siberia, we should expect an increase in the emergence of plant forms with transgressive expression of traits over generations.

To involve these genetic resources in breeding, it is necessary to assess their genetic and phenotypic variability [3], which will improve the breeding efficiency [4]. Morphological assessment of plants remains an important tool, despite the fact that morphological traits are controlled by different genes [5] and are influenced by environmental factors [6].

The search for chemicals that have mutagenic properties and can effectively change the hereditary nature of cultivated plants continues [7, 8].

This paper is the first to report the biological potential of the chemical mutagen phosphemide on spring soft wheat. We revealed that seed treatment with an aqueous solution of mutagen in optimal concentrations is effective for increasing genetic variability and selecting valuable forms.

Our goal was to expand the genetic diversity of spring bread wheat (*Triticum aestivum* L.) using the chemical mutagen phosphemide and to assess the biological potential of mutant samples (M_5, M_6) based on the variability of various traits in the conditions of the Northern Trans-Urals.

Materials and methods. Spring soft wheat *Triticum aestivum* L. accessions from the world collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR) were selected based on a preliminary study in the Tyumen Province in 2006-2010.

Variety Skant 3 (var. *lutescens*; originated by Research Institute of Agriculture of the Northern Trans-Urals and Kazakh Research Institute of Agriculture and Breeding) was created by individual selection from the F_3 population [F_1 (Shtorm × Saratovskaya 29) × Saratovskaya 29], registered in the Tyumen Province since 2003. Variety Cara from the world VIR collection (k-64381, var. *eritrospermum*, Mexico) is a carrier of the *Lr13* resistance gene according to GRIS (Genetic Resources Information System for Wheat and Triticale, http://wheatpedigree.net). Hybrid F_4 (Cara × Skant 3) was selected by a diallelic analysis of 5 parental and 10 hybrid forms produced at the Institute of Biology of Tyumen State University.

Spring wheat seeds were treated with a 0.002% and 0.01% aqueous solution of phosphemide for 3 h; control seeds were kept in distilled water. Phosphemidum, or di-(ethylenimide)-pyrimidyl-2-amidophosphoric acid, a white or yellowish crystalline powder soluble in water and alcohol, was synthesized at Lomonosov Moscow State University.

Phenotypic changes were assessed by the morphological characteristics of the ear, stem, leaves (color, pubescence, shape, size) and biological properties (late-ripening, early-ripening, winter-type plants, dwarfs). Selection in the second mutant generation (M_2) and testing for stability in the third (M_3) and subsequent generations were carried out for large, pyramidal, speltoid ear; bright yellow and anthocyanin colored straw; strong straw; wide flag leaf; tall plants; late ripening, early ripening.

Native electrophoresis of the storage protein gliadin was performed using grains of the 2021 harvest (M_5) according to common method [9] at the Analytical Center for Determining the Quality of Soil and Crop Products (LLP Baraev SRC of Grain Production). Vertical chambers for electrophoresis VE-20 (Helikon, Russia) and chemical reagents of the extra pure category (Sigma-Aldrich, USA) were used. Gliadins were identified according to a catalog of gliadin-coding loci alleles [10], the loci were *Gli-A1*, *Gli-B1*, *Gli-D1*, *Gli-A2*, *Gli-B2*, and *Gli-D2* [11].

In 2021-2022, 29 mutant samples M_5 and M_6 stored at the Institute of Biology of Tyumen State University were compared in field trials with the original varieties and the hybrid, and with varieties Tyumenskaya 25, Tyumenskaya 29 and Novosibirskaya 31 grown in the Tyumen Province.

Sowing, observations, records, description of morphological parametes and biological properties were performed at the experimental site of the Tyumen State University biological station Lake Kuchak (Tyumen Province, Nizhnetavdinsky District, 57°20′57.3″N 66°03′21.8″E) according to methodological guidelines [12, 13]. The soil of the site is soddy-podzolic sandy loam with 3.67% humus, pH 6.6. The experiment was designed in 4 repetitions with randomized 1 m^2 plots. Sowing density was 650 seeds per 1 m^2, or 6.5 million viable seeds/ha, with 20 cm row spacing. Sowing was carried out in the second decade of May, the crop was manually harvested at the stage of complete grain ripeness.

Plant height was measured from the soil surface, including the top leaf or ear depending on the phenological phase. The chlorophyll content in flag leaves of 10 plants was measured in sunny weather, between 11.00 and 14.00 with an optical counter SPAD 502 (Minolta Camera Co., Ltd., Japan). The area of the leaf blade was calculated by the formula [14]:

$$A = LWb_i,$$

where L is the length of the leaf blade, cm, W is the maximum width of the leaf blade, cm; $b_i = 0.835$.

After harvesting, plants and productive ears per 1 m^2 were counted, the grain yield, grain number and grain weight per ear for 10 plants in each replication were determined.

Environmental conditions were monitored at the experimental site (a professional local weather station IMetos IMT300, Pessl Instruments, Austria), information was also used on average daily air temperature and precipitation from the Weather and Climate reference and information portal (http://www.pogodaiklimat.ru/).

Statistical processing of experimental data was performed according to proven methods [12, 15] using the Microsoft Excel spreadsheet processor and STATISTICA 6.0 software (StatSoft, Inc., USA). The mean values (M), standard errors of the means (±SEM), coefficients of variation (Cv, %) were calculated, correlation analysis was performed. The significance of the differences between the mean values was assessed by Student's t-test.

Results. Preliminary cytogenetic studies on the phosphemide effects on

plants were carried out on the model plant *Crepis capillaris* L. which has three pairs of clearly distinguishable chromosomes. Dry seeds were treated with a phosphemide solution, and the types and number of chromosome rearrangements were analyzed on seedlings [16, 17]. It was important to determine how long the mutagenic effect could last. We found that with a single application of the phosphemide to the seeds of *C. capillaris*, chromosome rearrangements and the frequency of seedlings with mitoses remained detectable within 3 months. Therefore, it can be assumed that when storing treated seeds, the mutagen phosphemide does not decompose and its effect is practically not reduced [16, 17].

Based on these results, a single seed treatment was used in a study on varieties and hybrids of *T. aestivum*, followed by lab and field trials. When selecting a research object, we proceeded from the fact that the specified mutagen had not been used previously, and one of the tasks was to determine its effectiveness based on the frequency and spectrum of mutations. In this regard, we selected samples that differed in botanical and geographical origin and could presumably differ in their response to the action of phosphemide. In the M_2 generation, a wide range of mutations with modified plants (12 types) were identified with a frequency of 30.3% in the hybrid and 15.3-28.5% in the original varieties. In terms of the number of mutations that stably manifested the trait in the offspring, the phosphemide solution concentration of 0.01% had an advantage.

Table 1 describes spring soft wheat mutants we studied.

1. Phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants (experimental site of the Tyumen State University biological station Lake Kuchak, Tyumen Province, Nizhnetavdinsky District, 2021-2022)

Nos.	Designation	Nos.	Designation
1	$P_{1к}$ (Cara), contro;	17	P_2 (0.002 %) Skant 3
2	$F_{4к}$ (Cara × Skant 3), contro	18	P_2 (0.002 %) Skant 3
3	$P_{2к}$ (Skant 3), contro	19	P_2 (0.002 %) Skant 3
4	F_4 (0,01 %) Cara × Skant 3	20	P_2 (0.002 %) Skant 3
5	F_4 (0,01 %) Cara × Skant 3	21	F_4 (0.002 %) Cara × Скэнт 3
6	P_1 (0,002 %) Cara	22	P_2 (0.002 %) Skant 3
7	P_1 (0,002 %) Cara	23	P_2 (0.002 %) Skant 3
8	P_1 (0,002 %) Cara	24	P_2 (0.002 %) Skant 3
9	P_1 (0,002 %) Cara	25	P_2 (0.002 %) Skant 3
10	F_4 (0,01 %) Cara × Skant 3	26	P_1 (0.01 %) Cara
11	F_4 (0,01 %) Cara × Skant 3	27	P_1 (0.01 %) Cara
12	F_4 (0,01 %) Cara × Skant 3	28	P_1 (0.01 %) Cara
13	F_4 (0,01 %) Cara × Skant 3	29	P_1 (0.01 %) Cara
14	P_2 (0,002 %) Skant 3	30	P_2 (0.01 %) Skant 3
15	P_2 (0,002 %) Skant 3	31	P_2 (0.01 %) Skant 3
16	P_2 (0,002 %) Skant 3	32	F_4 (0.002 %) Cara × Skant 3

N o t e. F_4 is a fourth generation hybrid, P_1 is the original variety Cara, P_2 is the original variety Skant 3. The phosphemide concentration is indicated in parentheses.

2. Gliadin allele formulas of phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants

Nos	Gliadin coding loci (*Gli*)					
	A1	*B1*	*D1*	*A2*	*B2*	*D2*
1, 2 (control)	*c*	*l*	*d*	*n*	*p*	*b*
3 (control)	*a*	*e*	*b*	*f*	*t*	*a*
8, 10, 11, 12, 13	*c*	*l*	*d*	*n*	*p*	*b*
6, 7	*h*	*l*	*b*	*m*	*f*	*q*
17, 18, 19, 20	*o*	*f*	*a*	*l*	*p*	*n*
23, 24, 25, 26, 27, 28, 29, 16	*f*	*b*	*a*	*l*	*b*	*i*
5, 21, 22, 32	*c*	*e*	*b*	*n*	*p*	*q*
14, 30	*g*	*e*	*f*	*c*	*n*	*b*
4	*o*	*e*	*b*	*n*	*i*	*e*
9	*f*	*e*	*a*	*b*	*b*	*b*
15	*c*	*e*	*a*	*l*	*n*	*i*
31	*k*	*e*	*a*	*k*	*u*	*q*

N o t и e. The numbers correspond to the names of the samples given in Table 1.

Based on electrophoretic analysis of grains samples form the original and mutant, genetic formulas of soft wheat gliadin were compiled. It was found that the mutants often had identical spectra and, therefore, the gliadin formula (Table 2).

For all loci, there were alleles with the maximum frequency of occurrence. In the 1st homeological group (loci *Gli-A1*, *Gli-B1*, *Gli-D1*), the alleles *Gli-A1c* and *Gli-B1e* (34.5%), *Gli-D1a* (51.7%) were more common. In the 6th homeological group (loci *Gli-A2*, *Gli-B2*, *Gli-D2*), the *Gli-A2l* and *Gli-B2p* alleles (44.8%) and the *Gli-D2i* allele (31.0%) predominated. In total, gliadin electrophoresis revealed 4 alleles at the *Gli-B1* and *Gli-D1* loci, 5 alleles at the *Gli-D2* locus and 6 alleles at the *Gli-A1*, *Gli-A2* and *Gli-B2* loci (Fig. 1).

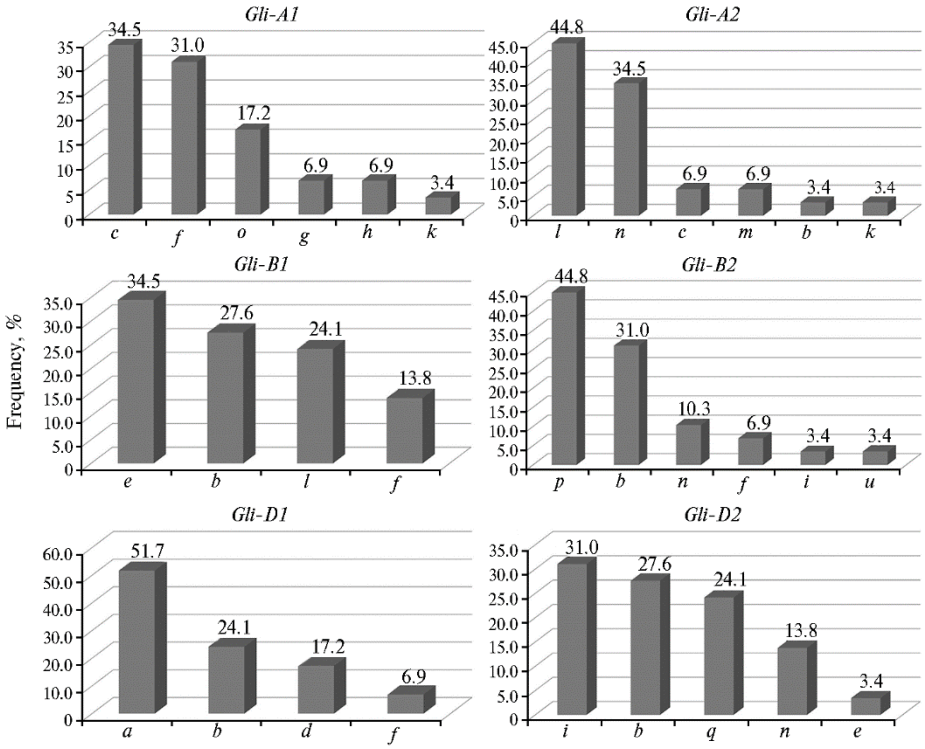

Fig. 1. Frequency of alleles of gliadin-coding loci (%) in phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants.

Analysis of gliadin formulas showed a high frequency of occurrence of the *Gli-A1c* allele (10 out of 29 samples). It is worth noting that this allele encodes the synthesis of a gliadin block, which is very close in number and mobility of components to the gliadins controlled by the *Gli-A1a* allele (Skant 3) [10], the difference lies only in the mobility of one protein band in the γ-zone of the electrophoretic spectrum. Rearrangements of the genetic apparatus under the influence of mutagen can occur that are expressed in the disappearance or appearance of one or two protein components in the gliadin spectrum [18]. It is likely that changes occurred in the genome due to the action of the mutagen modify the gliadin spectrum.

At the *Gli-B1* locus, the *Gli-B1e* allele had the maximum frequency of occurrence (see Fig. 1). It is worth noting that this allele is widespread in many Russian [19, 20] and Kazakh [21] wheat varieties and is probably associated with economically valuable traits.

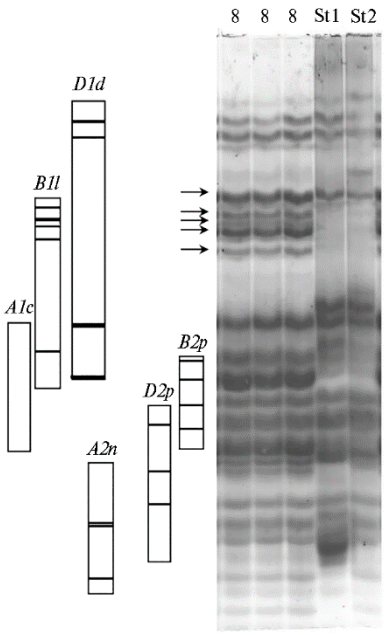

Fig. 2. Electropherogram and identified gliadin blocks in a phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutant: 8 — sample No. 8 P1 (0.002%) Cara, St1 — standard variety Bezostaya 1, St2 — standard variety Mironovskaya 808. Arrows indicate the main components marking the wheat-rye translocation.

The *Gli-B1l* allele is of interest, which controls a block of components, serves as a marker for the wheat-rye translocation 1RS.1BL and occurs with a frequency of 24.1% (Fig. 2). This allele is associated with plant resistance to a number of diseases, namely powdery mildew (*Pm8*), yellow rust (*Yr9*), stem rust (*Sr31*), and leaf rust (*Lr26*) [22, 23].

The predominant allele at the *Gli-D1* locus was *Gli-D1a* with a frequency of 51.7%. At the *Gli-A2* locus, alleles *l* and *n* were identified in mutants with a frequency of 44.8 and 34.5%, respectively. The distribution of the *n* allele among mutants is probably associated with the Cara variety which carries it in the genotype.

Therefore, treatment of parent varieties and hybrids with a chemical mutagen increased the genetic diversity in gliadin loci. This can later be used to create varieties with high grain productivity and quality, resistant to biotic and abiotic environmental factors.

Field testing of spring soft wheat mutants of the fifth and sixth generations (M_5, M_6), isolated by screening and further selection, was carried out in contrasting conditions of the growing seasons of 2021-2022 (Table 3).

3. Average daily air temperature and precipitations during the growing seasons 2021-2022 (experimental site of the Tyumen State University biological station "Lake Kuchak", Tyumen Province, Nizhnetavdinsky District)

Month	Average daily air temperature, °C			Total precipitation, mm		
	n	2021 год	2022 год	*n*	2021 год	2022 год
May	11.3	17.6	12.1	45.3	4.6	93.9
June	17.1	18.0	15.8	58.5	22.9	59.4
July	18.8	18.6	19.7	86.0	49.6	65.5
August	15.8	19.5	18.1	60.0	20.0	56.0
M, °C	15.8	18.4	16.4			
∑, mm				249.8	97.1	274.8

N o t e e. *n* — long-term averages (1968-2021), conditional norm.

The weather conditions of the growing season in 2021 provided selection of mutants that can withstand water and heat stress and form fully fledged grain. A record anomalous excess of the average daily air temperature vs. the norm (+6.3 °C) occurred in May under atmospheric and soil drought, when the amount of precipitation did not exceed 10.2% compared to the norm. With relatively favorable temperature conditions in June and July, the precipitation amounted to 39.1% and 57.6% of the norm, respectively. In August, grain ripened at elevated average daily air temperatures and deficit of precipitation (33.3% of the norm).

In 2022, the limiting factor for plant growth was the lack of moisture, but the harmfulness of dry periods was reduced due to average daily air temperatures slightly different from normal. Analysis of the average daily air temperature during the growing season revealed deviations from the norm in June (1.3 °C lower), July (0.9 °C higher), and August (2.3 °C higher). The amount of precipitation only in May significantly exceeded the long-term average. In other months, the indicator

varied from 101.5 (June) to 76.2% (August) compared to the norm.

Environmental factors significantly influenced quantitative traits (Table 4).

4. Morphophysiological traits of phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants under contrasting growing conditions (experimental site of the Tyumen State University biological station "Lake Kuchak", Tyumen Province, Nizhnetavdinsky District)

Trait	2021		2022		Comparison index, %
	M±SEM	*Cv*, %	*M*±SEM	*Cv*, %	
Plant height, cm	55.3±2.13	22.87	76.4±2.36*	18.38	38.2
Flag-leaf length, cm	10.8±0.21	11.20	17.4±0.28*	9.38	61.1
Flag-leaf width, mm	8.5±0.30	20.69	12.1±0.23*	11.18	42.4
Flag-leaf area, cm^2	6.4±0.30	28.06	14.3±0.38*	15.92	23.4
Chlorophyll content, Spad units	49.2±2.02	6.71	45.1±0.64	8.38	9.1
Productive stem number per 1 m^2	249.0±8.27	23.79	358.0±10.93*	20.89	43.8
Ear length, cm	6.1±0.46	16.64	8.1±0.17*	12.50	32.8
Ear grain number	15.0±3.00	41.83	30.2±1.18*	22.92	101.3
Ear grain weigtt, g	0.42±0.08	49.23	1.0±0.04*	28.88	147.6
Yield, g/m^2	138.1±4.88	20.96	279.4±7.75*	25.04	102.3

N o t e. For sample sizes, see Materials and methods section.

* Differences compared by year are statistically significant at $p < 0.01$.

The growing season of 2022 was more favorable compared to 2021 for the a number of traits. During the heading stage, the plants had an advantage in height and development of the flag leaf length, width, and area. More plants and productive stems were obtained for harvesting, which indicated increased plant survival and provided an increase in yield.

The drought influenced the physiological development of spring wheat plants. Plants responded to water and heat stress in 2021 by increasing the amount of chlorophyll in flag leaf cells. M. Yildirim et al. [24] reported that measuring chlorophyll content in leaves at the milky ripeness of the grain could be used in the selection of wheat plants with high yield potential both under relatively optimal conditions and under heat stress.

The variability of traits in most cases increased under the influence of stress factors, which was confirmed by the coefficient of variation. Differences across the years of the study were most significant in grain weight and the number of grains per ear, as well as in yield. A minimal decrease under stress occurred in the chlorophyll content in flag leaf and its area.

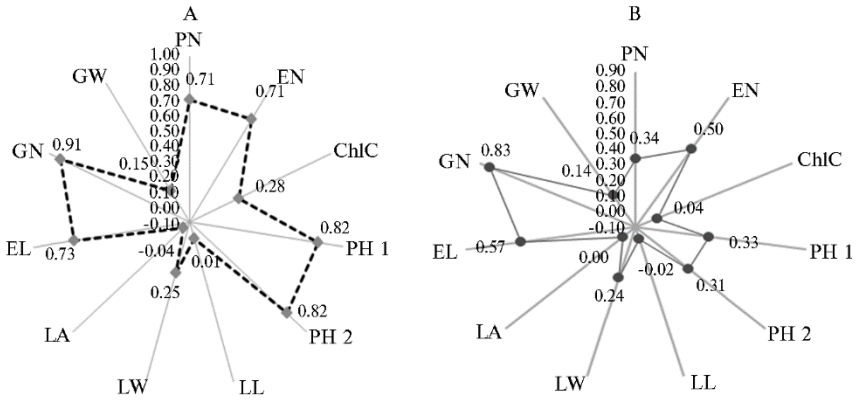

Fig. 3. Correlation of yield values with quantitative traits in 2021 (A) **and 2022** (B) **in phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants:** PN — plant number per 1 m^2, EN — ear number per 1 m^2, ChlC — chlorophyll content, Spad units, PH-1 — plant height, cm (records of 06/20/2021, 06/29/2022), PH-2 — plant height, cm (records of 07/10/2021, 07/17/2022), LL — leaf length, cm, LW — leaf width, cm, LA — leaf area, cm^2, EL — ear length, cm, GN — number of grains per ear, M3 — grain weight per ear, g (experimental site of the Tyumen State University biological station Lake Kuchak, Tyumen Province, Nizhnetavdinsky District).

The correlation between the yield of the mutant accessions studied and important quantitative traits depended on the genotype and environmental factors (Fig. 3). In the dry year of 2021, a significant correlation occurred between the yield values and plant height at booting and heading ($r = 0.82$, $p < 0.05$), plant number per 1 m^2 ($r = 0.71$, $p < 0, 05$), productive ear number per 1 m^2 ($r = 0.71$, $p < 0.05$), grain number per ear ($r = 0.73$, $p < 0.05$), and grain weight per ear ($r = 0.91$, $p < 0.05$). There was a direct relationship between yield and chlorophyll content in the flag leaf ($r = 0.28$, $p > 0.05$) and its width ($r = 0.25$, $p > 0.05$).

In 2022, the correlation between yield and grain weight per ear (r = 0.83, $p < 0.05$) remained high; a medium-strong relationship was found with the number of productive ears per 1 m2 (r = 0.50, $p < 0.05$) and the number of grains in the ear (r = 0.57, $p < 0.05$). The influence of other traits (except for the width and area of the flag leaf) on grain productivity decreased.

Based on a complex of valuable traits, five samples were selected from mutant populations that were significantly superior to the original forms and corresponded to the level of varieties grown in the Tyumen Province (Tyumenskaya 25, Tyumenskaya 29, Novosibirskaya 31) (Fig. 4, Table 5).

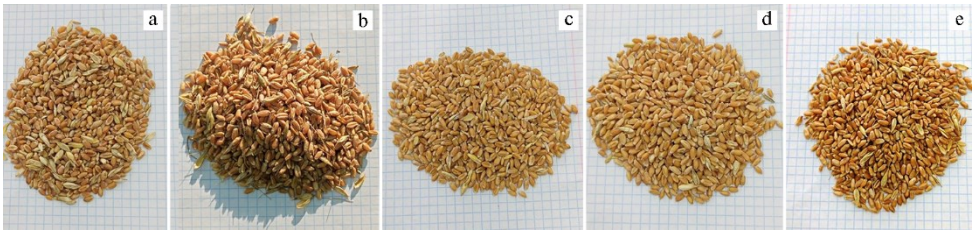

Fig. 4. Grain of phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants: a — sample No. 4 F_4 (0.01%) Cara × Skant 3, b — sample No. 5 F_4 (0.01%) Cara × Skant 3, c — sample No. 17 P_2 (0.002%) Skant 3, d — sample No. 20 P_2 (0.002%) Skant 3, e — sample No. 32 F_4 (0.002%) Cara × Skant 3 (experimental site of the Tyumen State Biological Station University "Lake Kuchak", Tyumen region, Nizhnetavdinsky district, 2022).

Three samples outstanded from the population of the hybrid Cara × Skant 3 after treating the seeds with phosphemide in two concentrations (0.01% and 0.002%), two samples were created on the basis of the Scant 3 variety (concentration 0.002%). The potential of grain productivity was more pronounced in more favorable growing conditions in 2022 (396.1-518.2 g/m^2); for standard varieties, the yield was 355.0-424.5 g/m^2.

Morphophysiological comparison of mutant samples with standard varieties revealed some features in the response to environmental stress factors. In height, the mutant plants obtained from the hybrid were significantly inferior in both years to the mutants selected from the Scant 3 variety and to the standards. The length of the flag leaf in 2021 was maximum in sample No. 5 F_4 (0.01%) Cara × Skant 3, in 2022 in No. 32 F_4 (0.002%) Cara × Skant 3. Among the standards, Tyumenskaya 25 (2021) and Novosibirskaya 31 (2022) stood out in turms of flag leaf length and width. The pattern revealed throughout the entire studied breeding material and manifested itself in an increase in the chlorophyll content in the flag leaf was confirmed by the data for the best mutant samples.

Lack of moisture and elevated air temperatures decreased plant viability, the number of productive stems per 1 m^2, the grains number and grains weight per ear. The maximum and minimum number of productive ears of mutants and standard varieties in 2021 had relatively small differences, 229-278 and 229-259 per 1 m^2, respectively. In 2022, this trait changed in mutants within 322-436 per 1 m^2, in standards within 272-317 per 1 m^2. With a smaller number of plants and productive stems at harvesting, the zoned varieties had an advantage in the number of grains per ear and their weight compared to the new samples. .

5. The best of phosphemide-induced spring bread wheat (*Triticum aestivum* L.) mutants compared to standard varieties under contrasting conditions of two-year growing seasons (*M*±SEM, experimental site of the Tyumen State Biological Station University "Lake Kuchak", Tyumen region, Nizhnetavdinsky district)

T	No. 4 F_4 (0.01 %) Cara × Skant 3		No. 5 F_4 (0.01 %) Cara × Skant 3		No. 17 P_2 (0.002 %) Skant 3		No. 20 P_2 (0.002 %) Skant 3		No. 32 F_4 (0.002 %) Cara × Skant 3		Tyumenskaya 25		Tyumenskaya 29		Novosibirskaya 31	
	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022	2021	2022
1	46.4±1.36a	62.2±2.24b	51.6±1.57a	66.5±1.80a	60.8±1.03a	91.7±1.86a	62.1±1.33b	81.9±1.60	53.3±0.64a	68.4±1.17	72.3+0.86	92.5±1.53	70.7+0.73	90.5±1.16	67.3+1.50	91.0±1.88
2	13.6±0.43	16.3±1.16a	12.2±0.54	17.8±0.51b	10.7±0.69	17.7±0.66b	10.9±0.62a	18.5±0.86	10.4±0.57a	18.9±1.08	13.2±0.53	17.4±0.87	12.3±0.56	19.5±1.02	13.0±0.56	19.4±0.68
3	6.4±0.32a	12.2±0.58	7.4±0.51b	11.8±0.40c	8.6±0.37b	14.4±0.68c	8.2±0.37b	14.6±0.80c	8.8±0.40b	13.6±0.93	11.2±0.51	12.8±0.51	10.3±0.32	11.6±0.40	10.4±0.45	11.2±0.45
4	7.27	16.51	7.53	17.54	7.68	21.28	7.46	22.55	7.64	21.46	12.34	18.60	10.58	18.89	11.29	18.14
5	46.8±0.71b	46.6±1.16c	47.2±0.78b	45.9±2.90c	49.1±1.18b	45.3±2.50c	49.5±0.83b	44.4±1.00c	54.8±0.67	45.6±1.53b	53.2±1.62	51.1±0.96	55.5±1.64	42.3±0.64	51.7±1.41	44.0±0.62
6	246±8.2	408±13.6b	229±7.6c	417±10.8a	242±7.5c	322±11.1c	236±6.7	386±8.1	278±9.7b	436±8.9a	259±12.0	306±9.3	246±5.5	272±9.8	229±7.8	317±10.1
7	8.3±0.47a	9.4±0.49	9.2±0.83a	9.6±0.82	5.7±0.36	8.3±0.21	5.6±0.37	8.8±0.77a	7.4±0.73a	9.1±0.54a	6.2±0.72	8.4±0.62	6.4±0.71	8.3±0.36	6.4±0.59	7.4±0.31
8	24.0±6.38c	39.0±3.45	21.0±2.97b	45.0±2.27c	19.0±4.04b	38.0±3.95	16.0±4.26b	30.0±2.74b	13.0±3.24a	26.0±3.03c	18.0±3.25	41.0±2.30	23.0±4.32	37.0±3.17	26.0±5.49	39.0±3.26
9	0.47±0.13a	1.3±0.15b	0.54±0.08a	1.02±0.10a	0.65±0.14b	1.23±0.17a	0.66±0.17b	1.05±0.09a	0.31±0.08a	0.95±0.12a	0.63±0.12	1.42+0.13	0.79±0.15	1.31+0.10	0.76±0.16	1.12+0.18
10	115.6±2.90a	518.2±3.66a	123.7±3.08a	425.3±4.85b	157.3±4.12a	396.1±5.63b	155.8±4.01c	405.3±5.70b	86.2±4.83a	414.2±6.05	163.3±4.56	424.5±4.07	194.3±5.63	356.3±3.68	174.0±6.71	355.0±4.86

Примечание. T — trait: 1 — plant height, cm, 2 — flag-leaf length, cm, 3 — flag-leaf wifth, ммм, 4 — flag-leaf area, cm^2, 5 — chlorophyll content, Spad units, 6 — productive stem number per m^2, 7 — ear length, cm, 8 — grain number per ear, 9 — grain weight per war, g, 10 — yield, g/m^2. For sample size, see the Materials and methods section.

a, b, c The differences are statistically significant ($p < 0.05$) when comparing with three standards, with two standards and with one standard, respectively.

According to our data, the lack of moisture, combined with elevated air temperatures, had a limiting effect on the growth of plants in height and the development of the assimilation surface, which we observed in 2021. Other researchers have also reported growth limitation in wheat during drought, which affects plant height, leaf area, dry mass, and other growth functions [25, 26].

Drought-tolerant genotypes have been found to maintain high chlorophyll content in leaves, necessary for photosynthesis [27]. In addition, chlorophyll is an indicator of photosynthetic activity and biosynthesis of assimilates [28], which makes it possible to use its content for the selection of drought-resistant forms of wheat [29]. Our data indicate an increase in the chlorophyll content in flag leaf cells under the influence of water and temperature stress on average for the collection of mutants to 49.2±2.0 units Spad vs. 45.1±0.64 units Spad in relatively favorable weather conditions. Thus, the differences in the chlorophyll content in leaves in 2021 compared to 2022 were most pronounced in three samples promising for further use in breeding, the No. 17 P_2 (0.002%) Skant 3 with 8.3%; No. 20 P_2 (0.002%) Skant 3 with 11.5%; No. 32 F_4 (0.002%) Cara × Skant 3 with 20.2% (see Table 5). It can be assumed that sample No. 32 F_4 (0.002%) Cara × Skant 3 is a tolerant genotype, since it contains the largest amount of chlorophyll (54.8±0.67 units Spad) compared to other genotypes. In the leaves of sample No. 4 F_4 (0.01%) Cara × Skant 3, the amount of chlorophyll was almost the same over the years, 46.8±0.71 and 46.6±1.16 units Spad in 2021 and 2022, respectively.

The optimum temperature for photosynthesis in wheat plants is approximately 25 °C [30]. It has been shown that an increase in air temperature by 1 °C during the grain filling period reduces yield by 3-4% [31]. According to S.S. Bhullar et al. [32], if heat stress occurs during the post-flowering period (grain filling period), it negatively affects photosynthesis and inhibits starch synthesis, which leads to a decrease in grain weight and yield. According to our average data, in the studied samples under stress conditions, 2 times fewer grains were formed in the ear, their weight decreased by 2.5 times, and their yield decreased by 2 times. High air temperatures (30 °C or more) during the flowering and pollination period turned out to be critical for the grain formation in the ear. In some cases, only single grains were formed in the ear. Since selection of drought-resistant forms based only on yield is not always effective [33], it is recommended to use morphological and physiological traits that play an important role in plant adaptation for a relatively quick assessment of genotypes [34]. The integrated approach allowed us to select valuable spring bread wheat genotypes, starting from early mutant generations [35]. Therefore, theoretical foundations of chemical mutagenesis proposed by I.A. Rapoport [36], continue to be relevant for increasing the efficiency of mutation breeding of plants.

It should be noted that the mutants selected for a number of traits (M_5, M_6) had identical alleles. Thus, samples No. 4 F_4 (0.01%) Cara × Skant 3, No. 5 F_4 (0.01%) Cara × Skant 3, No. 32 F_4 (0.002%) Cara × Skant 3 carried the same alleles at the gliadin *B1*, *D1* and *A2* loci. The *Gli-A1o* allele was common for samples No. 4 F_4 (0.01%) Cara × Skant 3, No. 17 P_2 (0.002%) Skant 3, and No. 20 P_2 (0.002%) Skant 3, *Gli-B2p* for mutants No. 5 F_4 (0.01%) Cara × Skant 3, No. 32 F_4 (0.002%) Cara × Skant 3, No. 17 P_2 (0.002%) Skant 3, and No. 20 P_2 (0.002%) Skant 3. Note that for alleles *Gli-B1e* and *Gli-B1f*, found in the isolated mutants, the number and relative electrophoretic mobility of gliadins were similar, with the exception of one component. Therefore, it is acceptable to assume that the effect that the *Gli-B1e* and *Gli-B1f* alleles may have on morphological characters will be similar.

Thus, the adaptation potential of spring soft wheat in the extreme conditions of the Northern Trans-Urals can be increased by using the genetic variability

of mutant populations. The chemical mutagen phosphemide has been proven to induce beneficial mutations in *Triticum aestivum*. The integrated use of mutational and recombination variability is effective to increase genetic diversity. Under the influence of water and temperature stress, a number of mutants showed an increase in chlorophyll content in flag leaf cells compared to that in favorable weather conditions. The identified differences can be used to select forms resistant to unfavorable environmental factors. Mutant accessions, characterized by diverse phenotypic and genotypic variations, are useful for improving economically valuable traits in wheat. Based on field trials of mutant generations M_5 and M_6, the best of them are of interest for breeding.

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THE QUALITATIVE COMPOSITION AND CONTENT OF PHENOLIC COMPOUNDS IN SHOOTS OF *Casuarina equisetifolia* L.

T.A. KROL[1] ✉, D.N. BALEEV[1], V.I. OSSIPOV[1, 2]

[1]*All-Russian Research Institute of Medicinal and Aromatic Plants,* 7, ul. Grina, Moscow, 117216 Russia, e-mail tatianakroll1@gmail.com (✉ corresponding author), dbaleev@gmail.com, ossipov@utu.fi;
[2]*Department of Chemistry, University of Turku*, Turku, Vatselankatu 2, 20014 Finland
ORCID:
Krol T.A. orcid.org/0003-4642-651X
Ossipov V.I. orcid.org/0000-0002-8383-9965
Baleev D.N. orcid.org/0000-0002-1228-0594
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Abstract

The species *Casuarina equisetifolia* L. is widely used in forestry in many countries with a tropical climate. Extracts from the shoots of *C. equisetifolia* are known to be rich in phenolic compounds which play an important role in plant growth and development, as well as in adaptation to abiotic and biotic environmental factors. Additionally, they exhibit antiviral, antibacterial, anti-inflammatory, anti-tumor, neuroprotective, and other activities. In this study, the composition of phenolic compounds primarily consisting of monomeric ellagitannins was comprehensively investigated for the first time in the shoots of *C. equisetifolia*. The aim of this study was to investigate the composition and content of phenolic compounds in *C. equisetifolia* shoots using ultra performance liquid chromatography coupled with photodiode and mass spectrometric detectors (UHPLC-PDA-MS). The study focused on the green one-year-old photosynthetic shoots of the *C. equisetifolia* tree grown in the greenhouse of the All-Russian Institute of Medicinal and Aromatic Plants (VILAR, Moscow). Samples were collected in the first decade of July 2019. The shoots were frozen, lyophilized, and ground. A 15 mg specimens were extracted with 1 ml of 80 % acetone for 60 min at room temperature with constant stirring. The extract was centrifuged for 20 min at 14000 rpm and evaporated to dryness at 45 °C. The extraction was repeated two more times. The resulting dry extract was dissolved in 1 ml of deionized water for 60 min, centrifuged for 20 min at 14000 rpm, diluted five times with deionized water, and filtered. An ultra-high performance liquid chromatographic system (UHPLC, Acquity UPLC® 2.9.0, Waters Corporation, USA) with a photodiode array detector (190-500 nm) and triple quadrupole mass spectrometer (Xevo TQ, Waters Corporation, USA) was used for the analysis of phenolic compounds. Separation was carried out in an Acquity UPLC® BEH Phenyl column (2.1×100 mm, 1.7 μm, Waters Corporation, Ireland). Data analysis was performed using the DataAnalysis 4.0 software. Phenolic compounds were identified based on mass spectrometry data by determining the m/z value of the [M-H] ion and its m/z fragments. The content of different classes of phenolic compounds such as gallolyl-glucoses, ellagitannins, condensed tannins, flavonoids (quercetin and kaempferol derivatives) was determined using multiple reaction monitoring. The extract was found to contain 16 phenolic compounds, with 14 belonging to the class of hydrolyzable tannins and 2 to the class of flavan-3-ols. It was discovered that *C. equisetifolia* shoots accumulate monomeric ellagitannins with molecular masses ranging from 784 to 1068 Da, containing glucose as a polyol in either cyclic or linear form. Among the ellagitannins of *C. equisetifolia*, casuarinin, two isomers of pedunculagin, stachyurin, chebulic acid, casuarininin, and casuarictin were identified for the first time. Two compounds with a molecular mass of 1068 Da were preliminarily identified as isomers of pterocarinin A. Ellagic acid and its derivatives, ellagic arabinoside and ellagic rhamnoside, were also identified in shoots. The total content of phenolic compounds was 55 mg/g dry weight, with ellagitannins being the main phenolic compounds. Their content reached 42 mg/g, or 76 % of the total amount of all phenolic compounds. Galloyl-glucose and condensed tannins each accounted for 10 % of the total amount of all phenolic compounds. These findings suggest the potential use of *C. equisetifolia* shoots as a raw material for obtaining individual ellagitannins and studying their antiviral, anti-inflammatory, and anti-tumor activities.

Keywords: *Casuarina equisetifolia* L., *Casuarinaceae*, liquid chromatography, mass spectrometry,

phenolic compounds, hydrolysable tannins, ellagitannins

Casuarina equisetifolia L. is a fast-growing evergreen woody plant of the family *Casuarinaceae* [1] with highly reduced scale-like leaves in whorls on long thin shoots, reminiscent of the needles of *Pinaceae* plants [2]. Unlike other species of the genus, *C. equisetifolia* has the largest natural area [3]. In many countries with tropical climates, the plant is used to restore degraded ecosystems, prevent erosion and stabilize sand, when planting coastal windbreaks, and in forestry in dry areas [4, 5].

C. equisetifolia can accumulate large amounts of phenolic compounds. For example, in the bark extract their total content varies from 43 to 76 mg/g [6, 7], and in the shoots it reaches 100 mg/g [9]. Among the phenolic compounds of *C. equisetifolia*, there are flavonoids, condensed and hydrolyzed tannins [9]. The main flavonoids are rutin, hesperetin, and the aglycones quercetin, narenginin, and kaempferol [10].

Condensed tannins are polymers of procyanidin, prodelphinidin and propelargonidin with a degree of polymerization of up to 30 [11]. In addition, monomeric precursors of condensed tannins, epicatechin and catechin, have been identified [10]. The composition of hydrolyzable tannins in *C. equisetifoli*a has not been studied in detail, but a study of the related species *C. stricta* shows that ellagitannins are the main phenolic compounds [12]. Pedunculagin, casuarinine, stachyurin, tellimagrandin I, strictinin, casuariin, casuarictin, 2,3-hexahydroxydiphenoyl-glucose and 4,6-hexahydroxydiphenoyl-glucose have been isolated and identified [12].

Phenolic compounds in plants perform a variety of physiological and environmental functions. They play an important role in growth, development, and adaptation to abiotic and biotic environmental factors, such as UV radiation, low temperatures, plant pathogens, and phytophagous insects [13, 14]. Currently, the mechanism of *C. equisetifolia* resistance to salinity and drought is being actively studied at the transcriptome and metabolome levels [15, 16]. Drought tolerance in *C. equisetifolia* is associated with changes in phenylpropanoid biosynthesis and an increase in condensed tannin content [15, 17]. In addition, many phenolic compounds have pharmacological activity, antioxidant, antimicrobial, anti-inflammatory, antitumor, and other beneficial properties [18-21]. Therefore, *C. equisetifolia* is of significant interest with regard to the efficient isolation of individual phenolic compounds and the study of their pharmacological activity.

In this work, the composition of phenolic compounds, which were mainly represented by monomeric ellagitannins, was studied for the first time in detail in the shoots of *C. equisetifolia*.

Our goal was to assay the composition and content of phenolic compounds in the shoots of *Casuarina equisetifolia* using ultra-performance liquid chromatography combined with photodiode and mass spectrometric detectors (UPLC-DAD-MS).

Materials and methods. Green annual photosynthetic shoots of the tree *C. equisetifolia* grown in the greenhouse of the Botanical Garden of the All-Russian Institute of Medicinal and Aromatic Plants (VILAR, Moscow) were collected in the first ten days of July in 2019. The shoots are articulated, about 1 mm thick, with reduced, fused scale-like leaves, collected in whorls of 6-8. Samples.

The shoots were frozen, freeze-dried (FreeZone 2.5 L, Labconco Corporation, USA) and ground (MM 400, Retsch GmbH, Germany). A sample of dry crushed shoot weighing 15 mg (CPA 225D, Sartorius AG, Germany) was extracted with 1 ml of 80% acetone for chromatography (Component-Reaktiv, Russia) for 60 min at room temperature and constant stirring (VORTEX Genie 2 , Scientific

Industries, Inc., USA). The extract was centrifuged for 20 min at 14,000 rpm (5430R, Eppendorf AG, Germany) and evaporated to dryness at 45°C (CentriVap concentrator, Labconco Corporation, USA). Sample extraction was repeated 2 more times. The resulting dry extract was dissolved in 1 ml of deionized water (Direct-Q3, Merck KGaA, Germany) for 60 min, centrifuged for 20 min at 14,000 rpm, diluted 1:5 with deionized water and filtered (PTFE filter Clean 2 , 0.45 rm, Thermo Fisher Scientific, Inc., USA).

To analyze phenolic compounds, an ultra-performance liquid chromatography system (UPLC, Acquity UPLC® 2.9.0, Waters Corporation, USA) witha photodiode detector (190-500 nm) and a triple quadrupole mass spectrometer (Xevo TQ, Waters Corporation, USA). Separation was carried out on an Acquity UPLC® BEH Phenyl column (2.1×100 mm, 1.7 μm, Waters Corporation, Ireland) in a gradient of 0.1% formic acid (A) and acetonitrile (B) according to the program: 0-0.5 min, 0.1% B in A; 0.5-5.0 min, 0.1-30.0% B in A (linear gradient); 5.0-6.0 min, 30-35% B in A (linear gradient). The eluent flow rate was 0.5 ml/min, and the injected sample volume was 5 μl [22]. To register phenolic compounds, the mass spectrometer operated in negative ionization mode. The obtained data were analyzed using the DataAnalysis 4.0 program.

When identifying phenolic compounds, we used mass spectrometry data, determining the m/z value of the $[M-H]^-$ ion and its fragments, and comparing the results with those published in the literature [12, 23] and in the mass spectrometric database The Human Metabolome Database (HMDB) [24].

The content of various classes of phenolic compounds, the galloyl glucose, ellagitannins, condensed tannins, and flavonoids (quercetin and kaempferol derivatives) was measured by the multiple reaction monitoring method [22] and expressed as mg/g shoot dry weight. We used calibration graphs for standards of phenolic compounds: 1,2,3,4,6-pentagalloylglucose, ellagic acid, gallic acid, (+)-catechin, quercetin and kaempferol (Sigma-Aldrich, USA). Total content was expresses as the sum of all classes of phenolic compounds.

Results. UPLC-DAD-MS analysis revealed 16 phenolic compounds in the shoot extract of *C. equisetifolia*. Based on the UV spectra, 14 phenolic compounds were classified as hydrolyzable tannins or their precursors and derivatives, and 2 compounds were classified as flavan-3-ols (Fig. 1).

Compound 1 with a retention time of 1.26 min showed a UV spectrum with two absorption maxima at 218 and 274 nm, which is characteristic of galloyl glucose (Fig. 2). The deprotonated ion m/z 331 $[M-H]^-$ and its fragment m/z 169 [gallic acid-H]$^-$ were identified in the mass spectrum (Table). Based on this, compound 1 was identified as monogalloyl-glucose, a precursor for hydrolyzable tannins.

Compounds 2, 3, 4 with a retention time of 2.14; 2.55 and 2.86 min had a UV spectrum with an absorption maximum at 228-229 nm and a small shoulder in the region of 260-280 nm, which is typical for ellagitannins, the structure of which does not contain galloyl groups (see Fig. 2). These compounds had a deprotonated ion m/z 783 $[M-H]^-$ and its fragment m/z 301 [ellagic acid-H]$^-$ (see Table). As a result, compound 2 was identified as casuariin, and compounds 3 and 4 as isomers of pedunculagin (see Table). All three ellagitannins have the same mass, but differ structurally. In the casuariin molecule, glucose has a linear form, while in pedunculagin it has a cyclic form [12]. Therefore, the retention time of casuariin in reverse-phase HPLC analysis is shorter than that of pedunculagin [25].

Compound 5 was identified as (+)-catechin based on the UV spectrum characteristic of flavan-3-ols with two absorption maxima at 226 and 278 nm (see Fig. 2), the deprotonated ion m/z 289 $[M-H]^-$, fragment m/z 245 $[M-H-CO_2]^-$

and ion m/z 579 $[2M-H]^-$ (see Table).

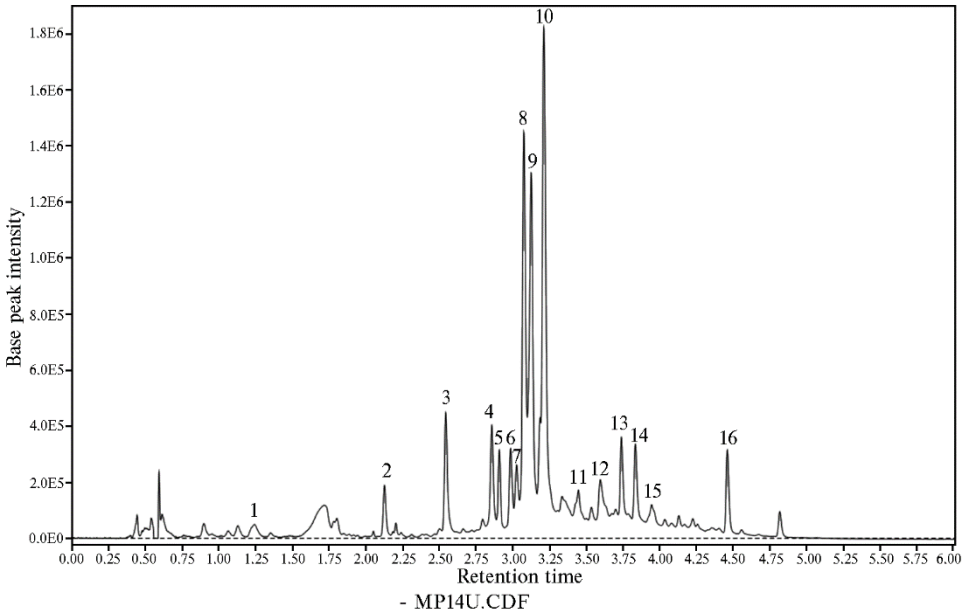

Fig. 1. Profile of phenolic compounds from the *Casuarina equisetifolia* L. shoot extract: 1 — monogalloyl-glucose, 2 — casuariin, 3 — pedunculagin (isomer 1), 4 — pedunculagin (isomer 2), 5 — catechin, 6 — pterocarinin A (isomer 1), 7 — pterocarinin A (isomer 2), 8 — stachyurin, 9 — chebulagic acid, 10 — casuarinine, 11 — catechin derivative, 12 — ellagitannin, 13 — ellagic acid arabinoside, 14 — casuarictin, 15 — ellagic acid, 16 — ellagic acid rhamnoside. (ultra-performance liquid chromatography with a photodiode detector (280 nm).

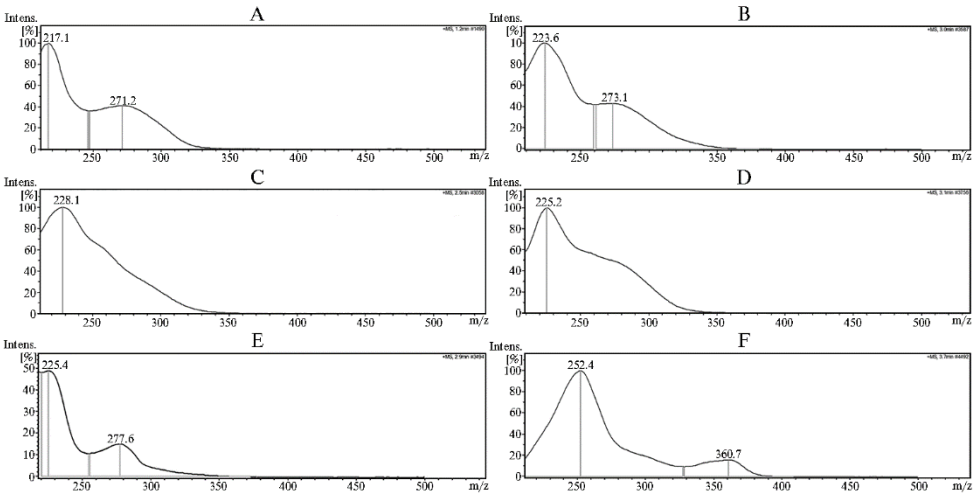

Fig. 2. Examples of UV spectra of various phenolic compounds identified by ultraperformance liquid chromatography in the *Casuarina equisetifolia* L. shoot extracts: A — monogalloyl-glucose, B — pterocarinin A (isomer 1), C — pedunculagin (isomer 1), D — casuarinine, E — catechin, F — ellagic acid arabinoside.

Compounds 6 and 7 had a UV spectrum with two absorption maxima at 224 and 271-273 nm (see Fig. 2), which is typical for ellagitannins containing galloyl and hexahydroxydiphenoyl groups. Examination of the mass spectrum of these compounds showed the presence of a deprotonated ion m/z 1067 $[M-H]^-$ and characteristic fragments m/z 169 [gallic acid-H]$^-$, 275 [decarboxylated hexahydroxydiphenic acid monolactone-H]$^-$ and 533 $[M-2H]^{2-}$ (see Table). As a result,

compounds 6 and 7 were tentatively identified as isomers of pterocarinin A, a monomeric ellagitannins with a C-glycosidic bond [26]. More accurate identification necessitates additional research.

Compounds 8, 10 and 14 also had a UV spectrum characteristic of ellagitannins. The mass spectrum contained a deprotonated ion m/z 935 $[M-H]^-$, an ion m/z 467 $[M-2H]^{2-}$, and a characteristic fragment m/z 301 [ellagic acid-H]$^-$ (see Table). These compounds were identified as stachyurin, casuarinine and casuarictin, respectively. Stachyurin and casuarinine are C-linked ellagitannins in which open-chain glucose forms ester bonds with two hexahydroxydiphenoyl groups, while casuarictin is a simple monomeric ellagitannin [12].

Identification of phenolic compounds in *Casuarina equisetifolia* L. shoots (ultra-performance liquid chromatography coupled with photodiode and mass spectrometric detectors)

No	Time, min	UV_{max}, nm	m/z of adduct or fragment			Compounds
			$[M-H]^-$	$[2M-H]^-$	fragments	
1	1.26	218; 274	331	–	169	Monogalloyl glucose
2	2.14	229	783	–	301; 391; 603	Kazuriin
3	2.55	228	783	–	275; 301; 391;	Pedunculagin (isomer 1)
4	2.86	229	783	–	275; 301; 375; 391; 483	Pedunculagin (isomer 2)
5	2.91	226; 278	289	579	245	(+)-Catechin
6	2.99	224; 273	1067	–	169; 275; 533	Pterocarinin A (isomer 1)
7	3.03	224; 271	1067	–	169; 275; 533	Pterocarinin A (isomer 2)
8	3.08	227; 273	935	–	169; 275; 467	Stachyurin
9	3.13	225; 270	953	–	169; 275; 301; 476; 633	Chebulagic acid
10	3.21	232; 274	935	–	179; 275; 301; 467	Casuarina
11	3.45	226; 275	458	917	289	Catechin derivative
12	3.60	225; 272	–	–	169; 275; 301; 633; 785; 917; 1063	Unidentified ellagitannin
13	3.74	252; 300pl; 361	433	867	301	Ellagic acid arabinoside
14	3.84	224; 273	935	–	301; 467	Casuarictin
15	3.95	252; 300pl; 365	301	–	-	Ellagic acid
16	4.46	249, 300pl; 365	447	895	301	Ellagic acid rhamnoside

N o t e. Dashes indicate that the ion or fragments of the ion were not present in the mass spectrum; pl — shoulder.

Compound 9 was identified as chebulagic acid or its isomer by the presence of a deprotonated ion with m/z 953 $[M-H]^-$ and characteristic fragments with m/z 169 [gallic acid-H]$^-$, m/z 275 [decarboxylated hexahydroxydiphenic acid monolactone-H]$^-$, m/z 301 [ellagic acid-H]$^-$, m/z 476 $[M-2H]^{2-}$ and m/z 633 [corilagin-H]$^-$ (see Table).

Compound 11 had a UV spectrum with absorption maxima atof 226 and 275 nm with the presence of ions with m/z 458 and 917, which corresponded to the $[M-H]^-$ and $[2M-H]^-$ ions, and the fragment of parent ion with m/z 289 [catechol-H]$^-$. Compound 11 could not be accurately identified, but based on the data obtained, we can assume that it is a catechin derivative.

For compound 12 with a retention time of 3.60 min, it was not possible to detect the deprotonated ion in the mass spectrum and to identify it. However, the UV spectrum characteristic of ellagitannins and the presence in the mass spectrum of ions with m/z 169, 275, 633, 785, 917 indicate that this compound belongs to ellagitannins.

Compounds 13, 15 and 16 had UV spectra characteristic of ellagic acid (see Fig. 2). Analysis of the mass spectra showed the presence of a fragment with m/z 301 [ellagic acid-H]$^-$. In the mass spectrum of compound 13, ions with m/z 433 and 867 were observed, which correspond to $[M-H]^-$ and $[2M-H]^-$ (see Table). Therefore, compound 13 was identified as ellagic acid arabinoside and compound 15 as ellagic acid. Compound 16 showed the presence of ions with m/z 447 $[M-H]^-$ and 895 $[2M-H]^-$ and was identified as ellagic acid rhamnoside. Although previous studies indicate the hydrolyzable tannins in *C. equisetifolia*, their composition has not been studied in detail.

Pedunculagin, casuarinin, and casuarictin were previously isolated from plants of another species of the *Casuarinaceae* family, the *C. stricta* [12]. Chebulagic acid is found in *C. glauca* [27]. Pterocarinin A has been identified in members of the family *Juglandaceae* which belongs to the same order as *Casuarinaceae* [23]. The presence of catechin and ellagic acid in *C. equisetifolia* has also been reported previously [28].

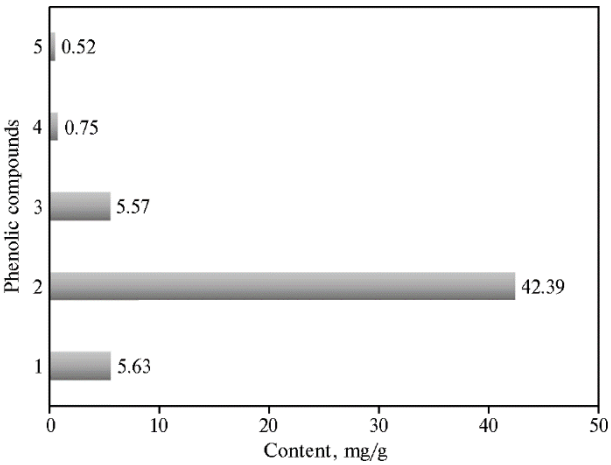

Fig. 3. Various classes of phenolic compounds in the *Casuarina equisetifolia* L. shoots: 1 — galloyl-glucose, 2 — ellagitannins, 3 — condensed tannins, 4 — kaempferol derivatives, 5 — quercetin derivatives.

The total content of phenolic compounds in the shoots of *C. equisetifolia* was 55 mg/g dry weight (Fig. 3). The total content of ellagitannins reached 42 mg/g dry weight of the shoot, or 76% of the sum of all classes of phenolic compounds, which basically corresponds to the data of other studies [6-8].

In the composition of ellagitannins, we revealed casuariin, two isomers of pedunculagin, two isomers of pterocarinin A, stachyurin, chebulagic acid, casuarinin, unidentified ellagitannin and casuarictin (see Table). Quantitatively, stachyurin, chebulagic acid and casuarinine predominated (see Fig. 1).

Ellagotannins play an important role in plant physiology [29], participating in growth, development and reproduction [30], and in protection from phytophagous insects and pathogens [31, 32]. The synthesis of these compounds occurs in plant cells via the shikimate pathway [33]. The composition and content of hydrolyzable tannins, including ellagitannins, depends both on the plant species and on the stage of plant development [34]. For example, at the beginning of the growing season, galloyl-glucoses predominate, which are then oxidized to ellagitannins [35].

Ellagotannins found in the shoots of *C. equisetifolia* have a variety of pharmacological activities. For example, chebulagic acid, pedunculagin and casuarinine have high antioxidant [36)] and antiviral [37, 38] activity. In addition, pedunculagin exhibits antitumor properties against cancer cell cultures [39)] and casuarinine is able to induce cell apoptosis [40]. The anti-inflammatory and anticoagulant effects of casuarinine have been established [41, 42]. Casuarictin, stachyurin and casuarinine have antibacterial activity [43].

Thus, using the method of ultra-performance liquid chromatography in combination with photodiode and mass spectrometric detectors, the composition and content of phenolic compounds in the shoots of the plant *Casuarina equisetifolia*, growing in the VILAR greenhouse complex was assayed. It has been shown that the main phenolic compounds in its shoots are ellagitannins. Casuariin, two isomers of pedunculagin, two isomers of pterocarinin A, strachiurin, chebulagic acid, casuarinin and casuarictin have been identified. The total content of ellagitannins was 42 mg/g, or 76% of the total phenolic compounds. The results obtained indicate the important role of ellagitannins in the vital activity of *C. equisetifolia*. Its shoots may be used for the preparative isolation of individual ellagitannins (stachyurin, chebulagic acid and casuarinin) in order to study their

pharmacological activity.

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BIOMARKERS FOR ALUMOTOLERANCE OF WINTER-HARDY FORMS OF *Triticum aestivum* L. FROM THE VIR COLLECTION

N.S. LYSENKO[1] ✉, L.L. MALYSHEV[1], R.K. PUZANSKY[2], A.L. SHAVARDA[2], T.V. SHELENGA[1]

[1]*Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources,* 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail leonid-lmalyshev@yandex.ru, t.shelenga@vir.nw.ru, N-Lysenko@yandex.ru (✉ corresponding author);
[2]*Komarov Botanical Institute RAS*, 2, ul. Professora Popova, St. Petersburg, 197022 Russia, e-mail Puzansky@binran.ru, shavarda@binran.ru
ORCID:
Lysenko N.S. orcid.org/0000-0002-5976-8634
Malyshev L.L. orcid.org/0000-0002-8595-1336
Puzansky R.K. orcid.org/0000-0002-5862-2676
Shavarda A.L. orcid.org/0000-0003-1778-2814
Shelenga T.V. orcid.org/0000-0003-3992-5353
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Abstract

All cultivated land in the world is approximately 50 % acidic soil, in Russia it is approximately 30 %. This limits the production of economically significant crops. The area of highly acidic farmland increases annually. The main stressor of acidic soils are aluminum ions (Al^{3+}). One of the most economically significant crops for the Russian Federation is wheat. Therefore, the search for alumotolerant wheat forms remains relevant. The largest number of Al^{3+} resistant genotypes is found among hexaploid wheat species with genome D, which include *Triticum aestivum* L. A distinctive feature of this culture is the combination of low temperatures resistance with good baking quality of the flour, therefore, the search for aluminum-tolerant forms among *T. aestivum* genotypes is economically justified. Hexaploid wheats are well represented in the collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR), the main part of which (44 thousand samples) is *T. aestivum*. In this paper, we for the first time compared the metabolomic profiles (MP) of *T. aestivum* accessions of different ecogeographical origins adapted to the conditions of the North-West of the Russian Federation, and identified the MP features in aluminum tolerant forms to detect putative metabolic markers for resistance to aluminum ions. Nonspecific metabolomic profiling of 7-day seedling rootlets of 20 *T. aestivum* accessions varying in degree of sensitivity to Al^{3+} was performed using gas chromatography coupled with mass spectrometry. Polyols, nucleosides, lactone forms of organic acids, free fatty acids and their derivatives, trioses, pentoses, hexoses, oligosaccharides, phenol-containing substances, terpenes, phytosterols were better represented in MP genotypes with low sensitivity to Al^{3+}. Dispersion analysis revealed significant differences of the MP of the accessions with a more expressed resistance to Al^{3+}. Exposure to a stressor presumably causes changes in the Krebs cycle, the synthesis of carbohydrates, plant hormones, other protective factors, glycerolipids and triglycerides of the membrane complex. Classical discriminant analysis followed by canonical analysis allowed us to identify eleven components with 100 % confidence separating *T. aestivum* samples with varying degrees of aluminum tolerance. Phosphoric, malic, succinic acids, tetra (RI = 1537) and pentaatomic (RI = 1735) alcohols, and linoleic acid methyl ester, which are statistically confirmed as aluminum tolerance markers, were the most informatively significant factors characterizing resistant forms of *T. aestivum*. The established biomarkers can be used to search for forms of *T. aestivum* resistant to Al^{3+}. These forms will be involved in breeding for highly productive *T. aestivum* varieties with complex resistance to stress factors and adapted to production in the conditions of the North-West of the Russian Federation.

Keywords: *Triticum aestivum*, aluminum resistance, non-specific metabolomic profiling, biomarkers, gas chromatography-mass spectrometry

Acidic soils occupies up to half of the world's cropland [1], andthe largest areas with excessive soil acidity are located in Russia [2-4], which limits the production of agricultural crops [5-7]. The main edaphic stressor in acid soddy-

podzolic soils is Al^{3+} cations, or the so-called mobile aluminum [8, 9]. The acidic reaction of soil in the presence of hydrogen and aluminum ions leads to a deterioration of its physical properties and increases the solubility of toxic compounds [4]. In these conditions, macro- and microelements pass into a form that is inaccessible to plants [9-15]. The main symptom of the toxic effect of aluminum ions is inhibition of root growth, leading to impaired plant development [6, 9-11].

The area of agricultural land with high acidity is growing every year [1, 5], which is due to the high migration ability of aluminum ions. This is especially important for regions with high air humidity, where even with moderate precipitation, excess water can accumulate in the soil [6, 11]. Such territories include the north-west of the Russian Federation.

The bulk of crops in Russia are winter and spring wheats [16]. H. Raman et al. [17] showed that Al^{3+}-resistant wheat genotypes are most common among hexaploid species with the D genome.

In the collection of the Federal Research Center the Vavilov All-Russian Institute of Plant Genetic Resources (VIR collection) hexaploid wheat species are widely represented, the main part of which belongs to *T. aestivum* (44 thousand samples). It should be noted that a distinctive feature of winter soft wheat is the combination of resistance to low temperatures with good baking properties of flour, which determines the frequent use of this species in breeding programs. It can be expected that studying the genetic potential of *T. aestivum* samples from the VIR collection will provide identification of new donors of aluminum tolerance among forms that have other economically significant traits. Such forms can subsequently be used in the selection of highly productive varieties of *T. aestivum* with complex stress resistance.

Al^{3+} ions act on the permeability of cell membranes, affecting the solubility of lipid and protein membrane complexes. As a result, intercellular transport is inhibited, the functioning of ion channels is disrupted, up to their complete blocking [18, 19]. This leads to disruption of DNA synthesis and cell division, slowing down the growth of roots and aboveground parts of the plant [20].

It was found that the neutralization of aluminum ions can occur either in the apoplast [6, 8, 21-25], where organic acids are excreted, or in the cytosol, followed by isolation of the resulting chelates in the vacuole [6, 8]. The entry of organic acids into the intercellular space occurs with the participation of an anion channel, which is activated by the action of Al^{3+} on the TaALMT1 protein, tha aluminium-stimulated malate transporter in *T. aestivum* [5, 26].

Organic acids, oligosaccharides, and root mucilage, which acts as a diffusion barrier, participate in the binding of aluminum ions [6]. A number of publications [6, 26-28] revealed an increase in the content of organic acids (especially malic and fumaric), glucose, sucrose and a decrease in the amount of fructose in the roots of aluminum-resistant corn samples. Increased levels of free fatty acids (including linoleic and linolenic acids) and flavonoid glycosides (rutin, kaempferol-3-O-glycoside, luteolin-6-C-hexosyl-hexoside) have been reported in aluminum-tolerant wheat varieties [29].

Thus, aluminum tolerance of economically important plants remains important for intensive study, including comparison of metabolomic profiles. But despite the fact that metabolomic research is being carried out quite actively, in the available literature, we have not found any work on the analysis of aluminum tolerance in winter-hardy forms that are resistant to other abiotic stresses.

This report presents for the first time data on the features of metabolomic profiles in seedlings of winter-hardy *T. aestivum* samples with low susceptivility to the effects of aluminum ions. Here, we revealed metabolites that are potential markers of resistance to aluminum ions.

The purpose of the work is to identify biomarkers of aluminum resistance in winter-hardy *T. aestivum* samples by nonspecific metabolomic profiling using gas chromatography coupled with mass spectrometry.

Materials and methods. The study involved 20 winter-hardy *T. aestivum* samples from the VIR collection [30-33]. Seedlings were grown from seeds of different years of reproduction (VIR collection) in a growing season of 2019 in the conditions of the VIR Pushkin laboratories as described [30]. For each treatment variant, 16 to 35 seeds of each sample were used,

Lab tests to assess embryonic root susceptibility to Al^{3+} ions was carried out according to the A. Aniol method in modification [33, 34] without adding eriochrome cyanine dye to the medium. Aluminum susceptibility in samples was determined at the early stages of plant development by root regrowth after damage [34]. The degree of susceptibility to the toxic effects of aluminum ions was determined by the difference in the average lengths of the roots of 7-day-old seedlings upon treatment and in control. For measurements, the seedlings of the sample were visually selected that had the longest roots.

For metabolomic profiling (MP) using gas-liquid chromatography coupled with mass spectrometry (GLC-MS), five 7-day-old seedlings of each sample with an average root length of 2 cm were selected. The roots were separated with a scalpel, weighed, placed in 2 ml plastic tubes and filled with liquid nitrogen to disrupt plant cells. The specimens were added with chilled methanol (+4 °C) until the biomaterial was completely immersed in the solvent and incubated for 24 h at +4 °C. The resulting extract was centrifuged, the supernatant was transferred into glass tubes for gas chromatography, placed in a CentriVap Labconco vacuum concentrator (Labconco, USA) and evaporated to dryness. To obtain volatile thermostable trimethylsilyl derivatives, 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane was added to the dry residue and heated for 15 min at 100 °C on a Digi-Block unit (Laboratory Devices, Inc., USA). As an internal standard, 20 μl of a solution of tricosane in pyridine (retention index RI = 2288, concentration 1 μg/μl) was added to each specimen. For each specimen, measurements were carried out in three analytical replicates. A mixture of trimethylsilyl ethers was separated using an Agilent HP-5MS capillary column (30 m; 0.25 mm in diameter; stationary phase 5% diphenyl, 95% dimethylpolyoxane with a film thickness of 0.25 μm, Agilent Technologies, USA) on a gas Agilent 6850A chromatograph coupled to an Agilent 5975 mass selective detector (Agilent Technologies, USA), according to protocol [35]. The initial temperature of the capillary column was 70 °C, the final temperature was 320 °C at a heating rate of 6 °C/min. The flow rate of the carrier gas (helium) was 1.5 ml/min. The injector temperature was 300 °C. The injected volume is 1 μl, the injection mode is “without reset”. Electron impact ionization was carried out at 70 eV and an ion source temperature of 230 °C. The chromatogram recording began after 4 min (solvent release time) and continued for 62 min in the ion scanning mode from 70 to 600 atomic mass units with registration of the total ion current. Scanning speed was 2 spectra/s. Metabolites (trimethylsilylyl derivatives) were identified by mass spectra and Kovacs retention indices (RI) using the AMDIS program (Automated Mass Spectral Deconvolution and Identification System, National Institute of Standards and Technology, USA, version 2.69, http://www.amdis.net), NIST 2010 mass spectral library (National Institute of Standards and Technology, USA, http://www.nist.gov) and in-house libraries of the Science Park of St. Petersburg University and the Komarov Botanical Institute RAS [27, 36]. Retention indices were assessed using calibration of normal aliphatic hydrocarbons with carbon chain length C_{10}-C_{40}. A metabolite was considered identified if the match factor of the obtained and library mass spectrum was more than 800. Semi-quantitative analysis of the obtained metabolite

profiles for total ion current given the internal standard was performed using the AMDIS program. Data are presented in mV.

Statistical processing was crried out in the Statistica 12 program (StatSoft, Inc., USA; 2019) (http://www.statsoft.com). When assessing the growth and development of roots as an indicator of susceptibility to aluminum chloride, the minimum and maximum values (min-max) were recorded, and the means (*M*) and standard errors of the means (±SEM) were calculated. The reliability of the influence of seedling root mass and the difference in the root length in the presence and absence of aluminum chloride in the medium (the treatment and control variants) on the distribution of *T. aestivum* samples by susceptibility to aluminum ions was assessed using one-way analysis of variance with Fisher's *F*-test. To identify components that reliably differentiate *T. aestivum* samples into groups with different degrees of aluminum tolerance, classical discriminant analysis was used, followed by canonical correlation analysis. Metabolites for which it was reliably established that the samples belonged to the group of susceptible to aluminum ions were considered informationally significant, which was confirmed by Fisher's *F* test (p not less than 0.05).

Results. For our study, we selected the following *T. aestivum* **a**ccessions from the VIR collection (Table 1):

1. Winter-hardy *Triticum aestivum* L. accessions from he Vavilov All-Russian Institute of Plant Genetic Resources (VIR) collection selected for the study (2019)

VIR Catalogue No. (k)	Variety	Origin	Year of reproduction
29466	RPG 27/36	Russia, Saratov Province	2019
32715	Untitle	Russia, Vladimir Province	2019
45885	Mironovskaya yubileinaya	Ukraine (until 1991)	2019
57573	Belosnezhnaya	Russia, Rostov Province	2019
58321	Stremnina	Russia, Samara Province	2019
59261	Severnaya zarya	Russia, Omsk Province	2019
59269	Nemchinovskaya 52	Russia, Moscow Province	2019
62431	Kazanskaya 84	Russia, Republic of Tatarstan	2019
63040	Zimorodok	Russia, Krasnodar Krai	2019
63353	Majoral	France	2019
63401	Rufa	Russia, Krasnodar Krai	2019
63521	Agassir	USA	2019
63523	Vista	USA	2019
63562	S89-142	Canada	2019
63568	Rodnic tarasovskii	Russia, Rostov Province	2019
63930	Arfa	Russia, Rostov Province	2019
64032	Fazit	Germany	2019
64163	CDC Harrier	Canada	2019
64180	Fatima	Hungary	2019
64278	Bezenchukskaya 616	Russia, Samara Province	2019

These are winter-hardy samples that were isolated as a result of field tests from 2007 to 2019 in the North-West region of the Russian Federation (Pushkin, Leningrad Province, 59°41′N 30°20′ E) [31, 32]. The degree of winter hardiness was determined using a scale developed at VIR [30] as a decrese in seedling dencity on plots in October before going into winter and in April after wintering. Complete death of plants was assessed as 0 points, very low winter hardiness (< 30% of seedlings survived) as 1 point, low winter hardiness (31-50%) as 3 points, medium winter hardiness (51-70%) as 5 points, high winter hardiness (71-90 %) as 7 points and very high winter hardiness (> 90%) as 9 points [21]). Samples with score that was higher than 7 points were considered winter-hardy [32]. In our experiment we used accessions medium, high and very high degrees of winter hardiness.

The length and weight of the roots are among the most indicative signs of the toxic effect of aluminum ions on *T. aestivum* seedlings [9, 23]. By seedling root susceptibility to aluminum ions, we divided the *T. aestivum* accessions into three groups (Table 2). In the first group, there were 2 samples with the smallest

difference between root length in the test and the control (2.2-3.0 cm) and low susceptibility to Al^{3+}. In the second group, there were 13 samples with medium difference (3.1-3.9 cm) and medium susceptibility to Al^{3+}. In the third group, there were 5 samples with a maximum difference (4.0-4.5 cm) and high susceptibility to Al^{3+}.

2. Distribution of the studied winter-hardy accessions of *Triticum aestivum* (VIR collection) according to the root length and root weight in 7-day-old seedlings in the presence of Al^{3+} (lab test, 2022)

VIR Catalogue No. (k)	Group	*n*	Root length, cm; min-max, *M*±SEM			Root weight, g (treatment)
			control	treatment	Δ	
57573	1	24	2.2-8.3 5.9±0.4	2.0-5.7 3.6±0.2	2.3	0.109
63523	1	27	2.4-7.0 5.3±0.5	1.2-4.4 2.8±0.3	2.5	0.133
29466	2	30	7.2-10.2 8.8±0.2	3.4-6.8 5.1±0.1	3.7	0.131
32715	2	30	6.7-10.5 8.0±0.2	3.6-5.5 4.3±0.1	3.8	0.118
45885	2	27	3.7-9.2 7.0±0.3	2.2-5.4 3.6±0.1	3.4	0.140
58321	2	24	6.7-10.8 8.4±0.2	2.5-6.9 5.0±0.2	3.4	0.106
59261	2	32	6.7-10.0 8.4±0.2	3.0-6.1 4.8±0.2	3.6	0.102
59269	2	24	3.7-9.3 7.4±0.4	2.1-5.2 3.6±0.2	3.8	0.139
63040	2	28	5.1-10.0 8.1±0.3	3.2-5.8 4.5±0.1	3.5	0.151
63401	2	30	4.5-9.0 7.7±0.2	3.1-10.0 4.3±0.3	3.4	0.153
63521	2	29	3.7-9.3 7.4±0.4	2.1-5.2 3.6±0.2	3.8	0.154
63568	2	27	4.4-8.2 7.0±0.3	2.1-5.2 3.4±0.1	3.7	0.144
63930	2	28	6.3-15.2 9.2±0.5	4.0-7.3 5.8±0.2	3.5	0.156
64032	2	16	5.0-11.5 8.7±0.3	3.0-8.3 5.6±0.3	3.1	0.076
64163	2	29	5.0-9.3 7.7±0.2	3.0-8.3 4.3±0.2	3.4	0.123
64180	3	30	6.6-11.0 8.4±0.2	2.7-5.5 3.9±0.1	4.5	0.234
64278	3	28	6.9-11.2 9.2±0.2	2.5-7.5 4.7±0.2	4.5	0.190
62431	3	20	4.4-9.0 7.3±0.2	1.0-4.2 3.3±0.2	4.1	0.121
63353	3	29	5.0-11.1 8.7±0.3	2.8-6.3 4.6±0.2	4.2	0.165
63562	3	25	5.5-9.5 8.0±0.2	2.2-4.5 3.7±0.1	4.3	0.160

N o t e. *T. aestivum* accessions were divided into $AlCl_3$ sensitivity groups based on differences in seedling root length as an indicator of sensitivity to aluminum chloride; group 1 — samples with low susceptibility; group 2 — samples with medium sens susceptibility itivity, group 3 — samples with high susceptibility to $Al3^{+}$. The mass of roots (5 per sample in the experiment) used for extraction is indicated.

The reliability of the influence of the parameters given in Table 2 on the distribution of *T. aestivum* samples in accordance with susceptibility to aluminum ions was checked by analysis of variance (Fig. 1, A, B). It turned out that the distribution of *T. aestivum* accessions into Al^{3+} resistance groups was significantly affected only by the difference in root lengths between the treaatment and control (see Fig. 1, A, $F = 68.68$; $p = 0.05$), while the effect of root mass was not significant (see Fig. 1, B, $F = 0.17$; $p = 0.847$). However, it should be taken into account that with an increase in the size of the analyzed sample, both for the influence of the "resistance group" factor on the root mass of seedlings and for the influence of root mass on the grouping of samples according their resistance to Al^{3+}, the reliability may be higher.

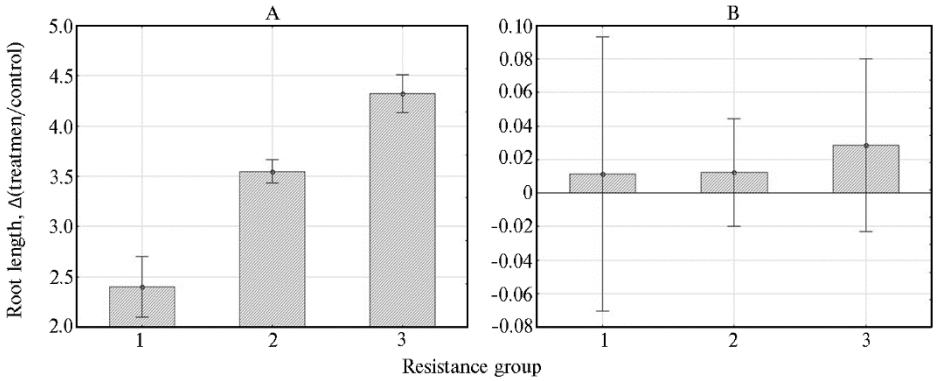

Fig. 1. Analysis of variance of the root length (A, cm) **and root weight** (B, g) **differences in 7-day-old winter-hardy seedlings of *Triticum aestivum* (VIR collection) in the presence and absence of Al^{3+}** (treatment and control, respectively) (lab test, 2022). Sample sizes correspond to those indicated in Table 2.

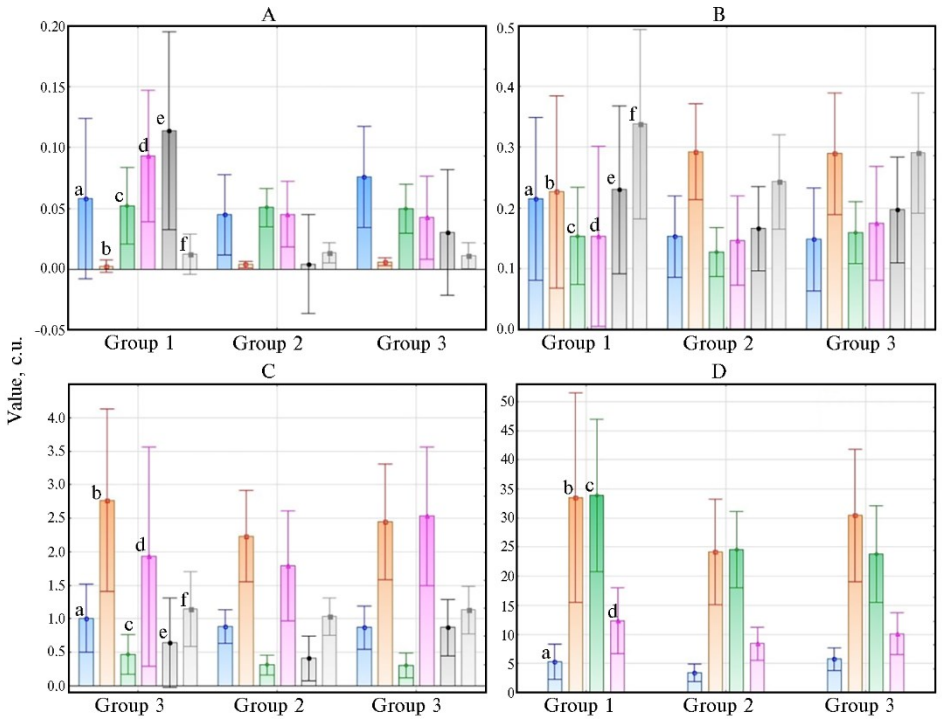

Fig. 2. The main compounds identified in the root metabolomic profiles of the *Triticum aestivum* (VIR collection) 7-day-old winter-hardy seedlings from groups differing in susceptibility to aluminum ions: A — pyridines (a), cyclic amide (b), alkanes (c), trioses (d), terpenes (e), amines and amides (f); B — lactones (a), derivatives (esters of organic acids and phosphoric acid) (b), monoacylglycerols (d), pentoses (e), phenol-containing compounds (f); C — polyols and their derivatives (a), free fatty acids and their derivatives (b), nucleosides (c), free amino acids (d), derivatives of monosaccharides (e), phytosterols (f); D — organic acids (a), hexoses (b), oligosaccharides (c), unidentified components (d), Agilent 6850A gas chromatograph coupled with an Agilent 5975 mass selective detector (Agilent Technologies, USA), lab tests, 2022. The results are presented based on the mass of five roots for each accession with averaging for the group. Sample sizes correspond to those indicated in Table 1. Measurements were performed in 3 analytical replicates.

Based on the results of nonspecific metabolomic profiling, we identified approximately 500 peaks in MP for accessions from different groups of resistance to Al^{3+}. Almost 250 substances were identified to the class, 120 to the final compound (Table 1 of the Appendix, see http://www.agrobiology.ru). The identified substances were divided into 19 main groups (Table 1 of the Appendix, see

http://www.agrobiology.ru), i.e., 33 acids, 13 polyols and their derivatives, 2 pyridines, 22 free fatty acids and their derivatives, 2 monoacylglycerol, 12 free amino acids, cyclic amide (2-pyrrolidinone), 7 methyl derivatives of monosaccharides, 38 monosaccharides (triose, pentose, hexose), 90 oligosugars, 10 phenol-containing compounds, 2 terpenes, 11 phytosterols, amides and amines (decylamine, urea), 4 lactone forms of organic acids, 6 derivatives of organic acids and phosphoric acid which included organic esters and esters of phosphoric acids, 2 alkanes, 3 nucleosides and unidentified components.

In the MP of accessions with low susceptibility to aluminum ions, polyols, nucleosides, lactone forms of organic acids, free fatty acids and their derivatives, monosaccharides, oligosaccharides, phenol-containing substances, terpenes, phytosterols dominated; with high susceptibility, organic acids, pyridines, monoacylglycerols, free amino acids, and monosaccharide derivatives dominated. The MPs of accessions with medium susceptibility to Al^{3+} did not have pronounced features (Fig. 2, A-D). Organic acid methyl esters, phosphoric acid esters, and alkanes were equally represented in all groups of *T. aestivum* accessions.

Despite the noted differences, the MPs of accesions with different resistance to aluminum ions were generally similar. A clear exception was the group of lactones. MPs of the accessions with medium and low resistance to Al^{3+} were characterized by a more pronounced presence of arabic acid lactone (RI = 1657) and 1,5-lactone gluconic acid (RI = 1696), while for MPs for the accessions with high resistance to Al^{3+}, only arabic acid lactone (RI = 1657) was characteristic. It can be assumed that lactones of sugar acids are involved in plant protection from Al^{3+}, which is mostly associated with the formation of chelate complexes [6, 8] between aluminum ions and organic acids, according to our examination, with lactone forms of sugar acids. In addition, the stressor intensify formation of root mucilage which may contain these compounds thus leading to an increase in their concetrations [6, 8, 21-24].

Dispersion analysis of all metabolites, except for unidentified ones, showed that in seedlings with more pronounced resistance to Al^{3+} (group 1), the MPs of roots significantly differs from the MPs of other groups in values for succinic, caprylic, stearic, oleic, linoleic acids, methyl esters of phosphoric acids, ethyl esters of palmitic and linolenic acids, while in seedling with medium susceptibility (group 2) in values for a number of oligosugars (RI = 2730, RI = 294, RI = 3625, RI = 3189). In seedings susceptible to aluminum ions (group 3), we did not identify any significant features of the MPs. However, given the differences that are close to significant ($0.1 > \rho > 0.05$), it can be noted that MPs of acceions from group 3 were characterized by more pronounced changes in the amount of terpenes, including lupeol (Table 2 of the Appendix, http://www.agrobiology.ru).

In resistant accessions, an increase in the concentration of succinic and phosphoric acids in MPs may be associated with their accumulation to neutralize Al^{3+}. There is an opinion that, in addition to organic acids, root mucilage secreted by the outer layers of the root cap is involved in the binding of aluminum ions [6]. Changes in the composition of oligosugars in *T. aestivum* acessionss with medium susceptibility to Al^{3+} may be due to this defense mechanism. Oligosugars can be part of root mucilage which acts as a diffusion barrier, limiting the entry of Al^{3+} into plant root cells [6]. In addition, accumulation of oligosugars occurres due to the destruction of cell walls by hydrolases in response to stressors [37]. Lupeol, like other terpenoids, also acts as a plant protector from salt stress [38], so it can be assumed that the accumulation of terpenes in the roots of *T. aestivum* seedlings which are susceptible to aluminum ions, is initiated by the stressor.

Thus, under the influence of aluminum ions, *T. aestivum* seedlings exhibit changes in carbohydrate, energy, lipid metabolism and the biosynthesis of secondary

metabolites. The synthesis of both some organic acids and their entire pool can be induced by a stressor, e.g., aluminum ions, and by activation of the Al^{3+} ion neutralization through the chelating mechanisms [5, 6, 8, 26, 39]. The accumulation of free fatty acids and their esters is also a response to stress that may reflect a modification of the membrane complex, namely glycerolipids and triglycerides, and activated production of anti-stress plant hormones jasmonic acid and nitroalkenesthat the precursors of which these compouns are [40-43]. To summarize, we can assume that in our study, aluminum ions had the most significant effect on the Krebs cycle, the synthesis of carbohydrates, plant hormones, other protective factors, glycerolipids and triglycerides of the membrane complex.

Classical discriminant analysis followed by canonical analysis made it possible to identify 11 components that, with 100% confidence, separate *T. aestivum* accessions differing in aluminum tolerance (Table 3 of the Appendix, http://www.agrobiology.ru). These are phosphoric, malic, 2-deoxyribonic, succinic, caprylic acids, tetraatomic (RI = 1537) and pentaatomic (RI = 1735) alcohols, methyl esters of oleic, linoleic acids, monoacylglycerol [16:0/0:0/0:0], and oligosugar (RI = 2749). Of these substances, the most informationally significant ($p < 0.05$) were phosphoric, malic, succinic acids, tetrahydric (RI = 1537) and pentahydric (RI = 1735) alcohols, and methyl ester of linoleic acid.

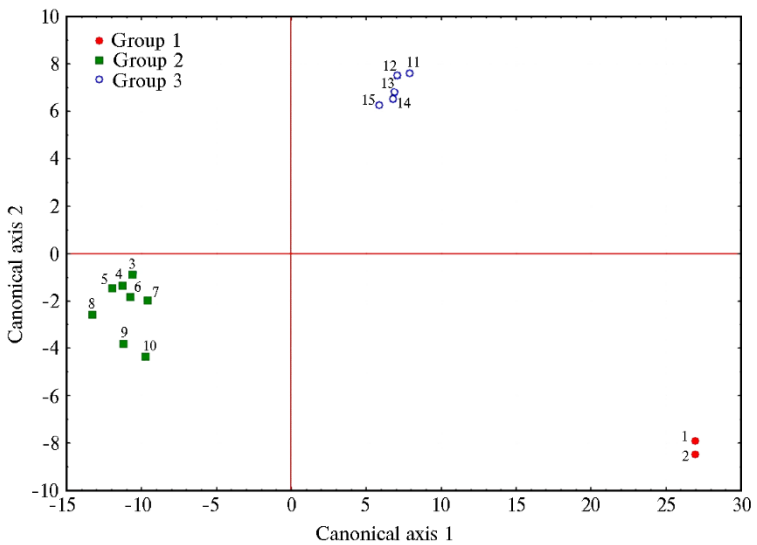

Fig. 3. Canonical distribution of 15 acccessions of *Triticum aestivum* (VIR collection) from groups differing in susceptibility to aluminum ions: group 1 — with high aluminum tolerance, group 2 — with average, group 3 — with low aluminum tolerance; 1 — k-45885, 2 — k-64032, 3 — k-64163, 4 — k-59261, 5 — k-32715, 6 — k-59269, 7 — k-63568, 8 — k-63930, 9 — k-63401, 10 — k-29466, 11 — k-62431, 12 — k-61180, 13 — k-63562, 14 — k-62521, 15 — k-64278 (lab test, 2022). The analysis was performed based on the metabolomic profiling, see Fig. 2, Table 4 of the Appendix).

Figure 3 shows canonical distribution of *T. aestivum* accessions that differ in suseptinility to aluminum ions (groups 1-3). The variables are canonical functions (axis 1 and axis 2, Table 4 of the Appendix, http://www.agrobiology.ru) for 11 parameterw included in the model in classical step-by-step discriminant analysis. Along the canonical axis 1 (or Root 1), covering 86.5% of the variance, accessions of group 2 with a medium level of aluminum resistance are separated from groups 1 and 3. Axis 2 (or Root 2, 13.5% of the variance) separates groups 1 and 3. Moreover, groups 1 and 3 form compact clusters, in contrast to group 2. The most significant discriminating factors were six metabolites, the phosphoric, malic, succinic acids, tetrahydric (RI = 1537) and pentaatomic (RI = 1735) alcohols and methyl ester of linoleic acid (see Table 3 of the Appendix, http://www.agrobiology.ru).

These compounds reliably ($p < 0.05$) characterize the Al^{3+} resistant forms of *T. aestivum* we studied in this work, and are statistically confirmed markers of aluminum resistance.

Discussing our results, it should be noted that metabolomic studies of economically important plants with varying degrees of resistance to aluminum ions and work on identifying markers of aluminum tolerance are few, although aluminum tolerance is subject to intensive study.

It was reported [6, 27, 28] that the content of organic acids (especially malic and fumaric), glucose, and sucrose increased, and the amount of fructose decreased in the roots of aluminum-tolerant maize accessions. In the aluminum-tolerant *T. aestivum* plants we studied, MPs differed from other MPs in the quantitative paameters of succinic and caprylic acids, methyl esters of phosphoric acid and esters of free fatty acids, including precursors of plant hormones [40-42]. Malic acid was not included in the list of reliable characteristics to distinguish forms of *T. aestivum* resistant to aluminum ions from susceptble ones, although the predominantly secretion of this acid is a feature of the protective response of wheat to contact with Al^{3+} [26, 37, 44]. Therefore, malic acid can still be considered a metabolite that marks aluminum resistance of *T. aestivum* seedlings. Oligosugars turned out to be characteristic of MPs of accessions with medium resistance to aluminum ions. That is, when exposed to aluminum ions, the accumulation of carbohydrates, the synthesis and transport of anti-stress substances, energy metabolism, and the metabolism of starch and sucrose change [27], which is generally consistent with our data. Studies on the mechanisms of aluminum resistance indicate different activities of organic acids when binding aluminum ions, Malonic acid belongs to the group with medium activity, succinic and lactic acids to the group with low activity [4]. According to our data, in *T. aestivum* seedlings the main acid capable of neutralizing Al^{3+} is succinic, which on this basis we assigned to the group with medium activity of binding aluminum ions. However, we can assume that other acids that are not among those we listed are also involved in protection against this stressor. Thus, we found that organic acids in general, as well as lactone forms of sugar acids, were better represented in the MPs of resistant *T. aestivum* forms Our assumption is consistent with the results of other studies [2, 3, 6, 8]. The authors point to a wide range of acids involved in protecting plants from the toxic effects of Al^{3+} [13, 15, 45]. According to published data [4, 15, 46], amino acids and phenolic compounds, like organic acids, are capable of chelating Al^{3+} ions that are not dangerous for the plant. The amount of such substances tends to increase after exposure to a stressor [19], which we also confirmed in the study. As we noted, phenol-containing compounds predominated in the MPs of resistant *T. aestivum* seedlings, and free amino acids predominated in the MPs of susceptable forms. The enhanced accumulation of phosphoric acid and methyl phosphate in the roots of seedlings that we observed in accesstions susceptable to Al^{3+}, is also associated with inactivation of aluminum ions [3, 29].

The increase in the proportion of nucleosides, in particular adenosine, in the MPs of aluminum-resistant forms that we detected may be caused by an increase in the activity of S-adenosylmethionine synthetase, which is associated with changes in the cell wall under the influence of Al^{3+}, as reported by M.W. Oh et al. [37]. The decrease in the amount of nucleosides we noted in forms susceptable to Al^{3+} corresponds to the mechanisms of aluminum tolerance discussed in the review by N.V. Amosova et al. [15].

A.L. Garcia-Oliveira et al. [6] found a relationship between the amount of root mucilage and Al^{3+} resistance in wheat. Among the compounds that provide stabilization of macromolecules of plant cells under abiotic stress, R.K. Sairam et al. [47] indicated high sugars (fructose and glucose), polyols (glycerol, mannitol,

sorbitol, methylated inositol, myoinositol, ononitol), oligosugars (trehalose, raffinose, sucrose and fructans), proline, and ascorbic acid. In our study, a feature of MPs of *T. aestivum* seedlings medium resistant to aluminum ions was an increased content of some oligosaccharides, which may be part of the mucilage that protects the roots. In addition, we considere tetraatomic (RI = 1537) and pentaatomic (RI = 1735) alcohols as markers of aluminum resistance, the increased synthesis of which is most likely associated with the action of a stressor. This is also consistent with the above publications.

M.D. Mashabela et al. [29] showed that aluminum-tolerant wheat varieties are characterized by a higher content of free fatty acids (including linoleic and linolenic), flavonoid glycosides (rutin, kaempferol-3-O-glycoside, luteolin-6-C-hexosyl-hexoside), and for susceptible ones, hydroxycinnamic acids are characteristic. A number of other works confirm the participation of secondary metabolites in plant protection from Al^{3+} [6, 48-50]. In the MPs of the Al^{3+}-resistant accessons we studied, the amount of free fatty acids also increased, with a predominance of linoleic acid and phenol-containing compounds, mostly quinic and syringic acids. Flavonoid glycosides in Al^{3+}-resistant *T. aestivum* accessons were represented by arbutin, and phenol-containing compounds in susceptible samples were predominantly quinic acid and arbutin. With the negative impact of aluminum ions on plant tissue, the synthesis of unsaturated fatty acids is activated. They are involved in maintaining the cell membrane integrity and serve as precursors in the synthesis of stress hormones jasmonates [39-41, 51]. As already noted, in the MPs of resistant accessions, the concerations of free fatty acids and their derivatives were higher. According to our data, an increase in the content of methyl and ethyl esters of unsaturated fatty acids, the precursors of stress hormones, is a reliable biochemical marker of the studied winter-hardy aluminum-resistant accessions of *T. aestivum*.

We revealed a more intense accumulation of phosphoric acid and methyl phosphate in *T. aestivum* seedlings susceptible to Al3+, which is apparently also associated with the mechanism of ion inactivation [3, 29]. It was noted [51] that the aluminum ions affects the main metabolic processes, the tricarboxylic acid cycle, glycolysis and the formation of secondary metabolites, which is consistent with our data.

One of the objectives of our study was to determine in MPs of *T. aestivum* markers for resistance to Al^{3+}. In the work of M.D. Mashabela et al. [29] markers of aluminum-resistant forms of *T. aestivum* are quinic, linolenic, 9,12,13-trihydroxy-10,15-octadienoic acids, valine, and the flavone isoorientin. This does not coincide with our results. Based on nonspecific metabolomic profiling, we identify phosphoric, malic, succinic acids, tetrahydric (RI = 1537) and pentahydric (RI = 1735) alcohols and methyl ester of linoleic acid as markers of aluminum tolerance in *T. aestivum* seedlings.

The *T. aestivum* accessions we revealed can be used in breeding of both Al^{3+}-resistant wheat and triticale varieties [26].

Thus, our results and data from other studies are largely consistent. The existing discrepancies are most likely related to the properties of the biomaterial taken for study and the specifics of the methods chosen for the analysis.

So, we have identified the features of metabolomic profiles (MPs) in winter-hardy samples of *Triticum aestivum* with varying susceptibility to aluminum ions and confirmed the influence of Al^{3+} on both primary metabolism (Krebs cycle, synthesis of carbohydrates, glycerolipids and triglycerides of the membrane complex) and secondary metabolism (synthesis of plant hormones and other protective factors, e.g., glycosides and terpenes). Metabolites were identified that, with a high confidence ($p < 0.05$), distinguish aluminum-tolerant *T. aestivum*

froms from susceptible forms. These are phosphoric, malic, succinic acids, tetraatomic (RI = 1537) and pentahydric (RI = 1735) alcohols and methyl ester of linoleic acid the contents of which change under the influence of aluminum ions. These compounds can be used as biochemical markers of winter bread wheat resistance to Al^{3+} in the search for aluminum-tolerant forms and selection of highly productive *T. aestivum* varieties adapted to the conditions of northwestern Russia with complex resistance to stress factors. In addition, isolated samples of *T. aestivum* can be the parent forms in bfeeding resistant triticale varieties.

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DROUGHT TOLERANCE OF WHEAT *Triticum aestivum* L. PLANTS DIFFERING IN THE DROUGHT ADAPTATION STRATEGIES DURING EARLY ONTOGENESIS

A.M. AVALBAEV✉, R.A. YULDASHEV, Ch.R. ALLAGULOVA, A.A. PLOTNIKOV, O.V. LASTOCHKINA

Institute of Biochemistry and Genetics — Subdivision of the Ufa Federal Research Center RAS, 71, pr. Oktyabrya, Ufa, Russia 450054, e-mail avalbaev@yahoo.com (✉ corresponding author), yuldashevra@gmail.com, allagulova-chulpan@rambler.ru, plotnikow87@mail.ru, oksana.lastochkina@ufaras.ru
ORCID:
Avalbaev A.M. orcid.org/0000-0002-4001-0700
Yuldashev R.A. orcid.org/0000-0001-9033-6867
Allagulova Ch.R. orcid.org/0000-0002-7547-8215
Plotnikov A.A. orcid.org/0009-0006-2968-2262
Lastochkina O.V. orcid.org/0000-0003-3398-1493
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Abstract

The early stages of ontogenesis during which plants are most sensitive to water deficit, is one of the most crucial in the plant development. Soft wheat (*Triticum aestivum* L.) is one of the most widespread valuable crops in the world and in the Russian Federation. In the course of natural and artificial selection on the territory of Russia, two ecological groups of wheat were formed, the West Siberian forest-steppe and the Volga steppe ecotypes which differ significantly in the strategy of adaptation to drought. The spring drought is typical for Western Siberia, and, therefore, wheat plants of the forest-steppe West Siberian ecotype grow slowly at the initial stage of ontogenesis. In the south-eastern regions of the European Russia, in particular, in the Volga region, drought occurs later, and cultivars of the steppe Volga ecotype, on the contrary, grow intensively at the beginning of the growing season in order to develop an extensive root network by the time of the onset of summer drought. This work has revealed for the first time the role of drought-induced changes in the hormonal balance and in the content of the amino acid proline on the degree of drought tolerance of wheat ecotypes that differ in their adaptation strategy to drought at the initial stage of ontogenesis. The goal of the work was to evaluate various physiological and biochemical parameters of wheat plants of Zauralskaya Zhemchuzhina (forest-steppe West Siberian ecotype) and Ekada 70 (steppe Volga ecotype) cultivars during early ontogenesis under normal and drought conditions. In the first series of experiments, seeds were germinated in Petri dishes (15 seeds per dish) on filter paper moistened with 5 ml of 4 %, 8 % and 12 % sucrose solutions to simulate drought. Petri dishes were placed in a climate chamber and the seeds were germinated in the dark at 22 °C for 3 days. Seeds germinated in distilled water served as a control. On day 3, the energy of seed germination was calculated as the ratio (%) of the number of germinated seeds to the total number of seeds used. The length of the main root of the seedlings was measured. In the second series of tests, pot experiments were carried out under controlled conditions. Seeds were sown in 15 liter pots (30 plants per pot, sowing depth was 4-5 cm, distance between rows was 2.5 cm, and distance between plants in a row was 2.5 cm). In the control pots, the soil moisture was maintained at 70 % of the total water retention capacity of soil. Other plants were subjected to early soil drought by not watering until the soil moisture dropped to 30 % of the total water retention capacity of soil. On days 7, 8 and 9, the fresh and dry weight of control and drought-stressed seedlings were measured. The contents of cytokinins (CK), indoleacetic acid (IAA) and abscisic acid (ABA) in 10 seedlings with fresh weight of 0.9-1.0 g on day 7, 8 and 9 were determined by the enzyme-linked immunosorbent assay (ELISA) using hormone specific rabbit antibodies and peroxidase-labeled anti-rabbit antibodies. Extraction of free proline and its quantification were also carried out. It was revealed that concentrated solutions of sucrose simulating drought reduced the energy of seed germination and inhibited the main root growth of 3-day-old seedlings of both wheat ecotypes. It should be noted that the inhibition of both seed germination and main root growth was more pronounced in the Zauralskaya

Zhemchuzhina cultivar. Soil drought also suppressed the growth of 7-9-day-old seedlings, and the suppression was also more pronounced in the Zauralskaya Zhemchuzhina plants. We revealed an imbalance in phytohormone levels in 7-9-day-old seedlings of both cultivars associated with the drought-induced accumulation of ABA and a decrease in the contents of CK and especially auxins. However, in Ekada 70 seedlings the range of drought-induced changes in hormonal balance was noticeably smaller compared to Zauralskaya Zhemchuzhina plants. It was also found that the soil drought led to an increase in the content of proline, an important osmoprotectant, while a higher concentration of proline was characteristic of 7-9-day-old seedlings of Ekada 70 cultivar both under normal and stress conditions. To summarize, our findings indicate a higher drought tolerance during early ontogenesis of the steppe Volga ecotype plants compared to the forest-steppe West Siberian ecotype plants. This is probably due to less altered phytohormone profiles and the increased osmoprotectant proline accumulation in the steppe Volga ecotype plants under drought.

Keywords: *Triticum aestivum*, wheat, ecotypes, drought, hormonal system, proline

Drought is one of the most common stresses that results in disruption of metabolism and significant crop yield loss, including wheat [1-3]. Early ontogenesis when plants are especially sesusceptible to unfavorable factors is crucial for wheat development. Moisture deficiency causes a decrease in seed germination, inhibition of seedling emergence and growth, a lag in crop growth, which, in turn, leads to a significant decrease in yield [4, 5]. Therefore, early drought resistance is of particular interest in order to identify the most effective mechanisms of plant protection and crop management under unfovorable water regime [5-7].

During natural and artificial selection under various drought conditions, two groups of wheat significantly different in adaptation strategy appeared, the forest-steppe West Siberian ecotype and steppe Volga ecotype [8-10)]. Distinquishing features of the West Siberian ecotype plants are slow germination and a rather long tillering stage due to the spring drought characteristic of the Western Siberia regions. Plants of this ecotype use abundant summer rains typical of the Western Siberian climate zone for rapid growth and development. Varieties of the steppe Volga ecotype intensively grow in spring using the soil moisture reserves, therefore, by the time of summer drought, the plants form an extensive network of roots providing a good harvest. That is, differences in adaptation to drought in wheat varieties of the forest-steppe West Siberian and steppe Volga ecotypes are especially pronounced at early plant development. Therefore, studying the features of early protective mechanisms of these ecotypes during moisture deficiency will allow progress in understanding the mechanisms of their drought resistance.

In this work, drought-induced changes in the hormonal balance and the content of the amino acid proline were revealed for the first time in the forest-steppe West Siberian and steppe Volga ecotypes of wheat at the initial stage of ontogenesis. Seedlings of the steppe Volga ecotype showed more pronounced resistance to dehydration icompared to plants of the forest-steppe West Siberian ecotype, which is due to significantly lower amplitude of stress-induced rearrangements in the content of phytohormones and higher accumulation of proline.

The purpose of the work was to assess the physiological and biochemical parameters of wheat plants of the varieties Zauralskaya Zhemchuzhina (forest-steppe West Siberian ecotype) and Ekada 70 (steppe Volga ecotype) in the initial stage of their ontogenesis under normal and drought conditions.

Materials and methods. Seeds of soft spring wheat (*Triticum aestivum* L.) varieties Ekada 70 (the steppe Volga ecotype) and Zauralskaya Zhemchuzhina (forest-steppe West Siberian ecotype) provided by the Chishminsky breeding center of the Bashkir Research Institute of Agriculture (Republic of Bashkortostan, Chishmy) were pre-sterilized with 96% ethanol and used in two experiments.

In the first experiment, 15 seeds per Petri dish were laid out on filter paper moistened with 5 ml of 4%, 8% and 12% sucrose solutions to simulate drought [11, 12]. The Petri dishes were placed in a TSO-1/80 SPU thermostat (JSC Smolensk

Special Design and Technology Bureau of Programmed Control Systems, Russia) and the seeds were germinated in the dark at 22 °C for 3 days. Control seeds were germinated in distilled water. On day 3, the germination energy was calculated by the ratio (%) of the sprouted seeds to the total number of seeds intended for germination. Seeds that produced a root of minimal length were considered germinated. In sprouted seeds, the length of the main root was measured [13].

In the second experimetn, pot trials were carried out under controlled conditions. Seeds were sown in 25×25×25 cm/15 l container, sowing depth of 4-5 cm, distance between rows 2.5 cm, distance between plants in a row 2.5 cm, 30 plants per container. Agrotechnical expanded clay (2-3 cm layer, Terra Master LLC, Krasnoyarsk) was placed at the bottom of each container. Soil with an optimal NPK ratio (pH 6.5, humidity 65%; Veltorf LLC, Russia) was poured on top of the expanded clay. Plants were grown at 21-23 °C, 16-hour photoperiod, illumination of 360 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and 60% air humidity. Some plants were subjected to early soil drought. During its modeling, watering was not carried out until the moisture content reached 30% of the soil's total moisture capacity (TMC). In the control, humidity was maintained at 70% of the TMC.

Physiological and biochemical parameters were assessed at the germination stage on 7-9-day old seedlings. The stages of ontogenesis were as proposed for cereals by T.B. Batygina [14]. On days 7, 8 and 9 of growth, the wet and dry weights were assessed in control and in seedlings exposed to soil drought. Experiments to assess growth parameters were carried out in three biological replicates, each variant included at least 30 plants.

The contents of cytokinins (CK), indolylacetic acid (IAA) and abscisic acid (ABA) in 10 wheat seedlings (fresh weight 0.9-1.0 g) on days 7, 8 and 9 were determined by enzyme immunoassay (ELISA) test using specific rabbit antibodies and peroxidase-labeled anti-rabbit antibodies [15]. The seedlings were homogenized in 80% ethanol at a weight:volume ratio of 1:10, followed by incubation of the samples at 4 °C for 16 h. After centrifugation at 10,000 g for 20 mins (Avanti J-E centrifuge, Beckman Coulter, Inc., USA) the supernatant was evaporated in a stream of air to an aqueous residue, in an aliquot of which the total content of free cytokinins was determined. From the remaining aqueous residue, IAA and ABA were extracted with sulfuric ether, methylated with diazomethane, after evaporation, the dry residue was dissolved in 80% ethanol, and the IAA and ABA were quantigied in an aliquot. In details the procedure for purification and extraction of IAA and ABA, and the steps for phytohormone immunoassays have been described [15].

Extraction and quantification of free proline were carried out according to L.S. Bates et al. [16]. Samples of plant material (2 g) were poured with boiling distilled water (2.5 ml) and cooled. To 2 ml of a cold sample, 2 ml of ninhydrin reagent and 2 ml of glacial acetic acid were added. The mixture was boiled for 1 h and cooled. The color intensity of the proline complex with ninhydrin was measured at $\lambda = 522$ nm. The proline concetration was calculated using a calibration curve, which was constructed using chemically pure proline (Sigma Aldrich, USA).

The figures and tables present the arithmetic mean values (M) for three independent repetitions of an experiment, each carried out in three biological replicates, and their standard errors (±SEM). The results were processed statistically by ANOVA analysis of variance using SPSS 13.0 for Windows (SPSS, Inc., USA). The significance of the difference between the means was assessed by the LSD test at $p \leq 0.05$.

Results. Drought, as one of the most common unfavorable abiotic environmental factors, significantly reduces functional activity of cells in wheat plants, especially in the early development [17-19]. During this period, seeds and young seedlings are most susceptible to disturbances in the water regime.

In Russia, natural and artificial selection have produced two ecological groups of wheat, significantly different in drought adaptation strategies, the West Siberian forest-steppe ecotype and the Volga steppe ecotype. The regions of Western Siberia are characterized by spring drought, and therefore wheat plants of the forest-steppe West Siberian ecotype grow slowly at the initial stage of ontogenesis. In the southeastern regions of the European Russia, in particular, in the Volga region, drought occurs later, and varieties of the steppe Volga ecotype grow intensively at the beginning of the growing season to maximize root development.

1. Seed germination energy (%) **of wheat *Triticum aestivum* L. spring varieties Ekada 70 (steppe Volga ecotype) and Zauralskaya Zhemchuzhina (forest-steppe West Siberian ecotype) under different concentrations of sucrose solutions** (M±SEM, lab test)

Variety	Control	Sucrose solution, %		
		4	8	12
Ekada 70	91.1 ± 3.9^{Aa}	80.2 ± 3.4^{Ab}	71.1 ± 3.1^{Ac}	40.7 ± 1.7^{Ad}
Zauralskaya Zhemchuzhina	85.3 ± 3.7^{Aa}	72.4 ± 3.2^{Bb}	60.8 ± 2.7^{Bc}	20.5 ± 0.9^{Bd}

N o t e. Three experiments were carried out in triplicate with 15 seeds each. Different letters indicate statistically significant differences between options at $p \le 0.05$ (ANOVA, LSD test). Large letters indicate differences in columns,

2. Length of the main root (cm) **in 3-day-old seedlings of wheat *Triticum aestivum* L. spring varieties Ekada 70 (steppe Volga ecotype) and Zauralskaya Zhemchuzhina (forest-steppe West Siberian ecotype) under different concentrations of sucrose solutions** (M±SEM, lab test)

Variety	Control	Sucrose solution, %		
		4	8	12
Ekada 70	2.95 ± 0.13^{Aa}	1.91 ± 0.08^{Ab}	0.86 ± 0.04^{Ac}	0.23 ± 0.01^{Ad}
Zauralskaya Zhemchuzhina	2.44 ± 0.11^{Ba}	1.26 ± 0.06^{Bb}	0.49 ± 0.02^{Bc}	0.05 ± 0.002^{Bd}

N o t e. Three experiments were carried out in triplicate (from 25 to 125 seedlings per variant). Different letters indicate statistically significant differences between options at p 0.05 (ANOVA, LSD test). Large letters indicate.

In the first series of our experiments, the drought resistance of plants was assessed by the seed germination energy (Table 1) and the length of the main root of 3-day-old seedlings (Table 2) in sucrose solutions of different concentrations (4, 8 and 12%), simulating drought. It was found that sucrose solutions simulating drought suppressed the germination of seeds of both wheat ecotypes, and germination energy decreased with increasing sucrose concentration.

The works of other researchers have shown that at early ontogenesis, the effect of dehydration leads to inhibition of seed germination and seedling growth, which subsequently significantly reduces wheat grain yield [20, 21]. It should be noted that the percentage of germinated seeds indicates the ability of plants to use hard-to-reach moisture and characterizes relative drought resistance of a certain variety. Thus, seeds of the Ekada 70 variety expressed a higher germination energy in concentrated sucrose solutions compared to the Zauralskaya Zhemchuzhina variety (see Table 1). These results confirm the previously reported data on the higher drought resistance of wheat of the steppe Volga ecotype at early ontogenesis [22].

Under dehydration conditions, the growth of the main root was inhibited in 3-day-old wheat seedlings of both ecotypes (see Table 2). However, on sucrose solutions, in the steppe Volga ecotype Ekada 70 variety the growth of the main root was inhibited significantly less than in seedlings of the Zauralskaya Zhemchuzhina variety.

Lab tests can only give an idea of the potential drought resistance of plants, while field trials or data obtained under conditions close to them are of practical importance. In this regard, we studied the effect of soil drought, which was modeled by the absence of irrigation, on growth in seedlings of different wheat ecotypes in a growing season. It was found that plants of the Ekada 70 variety in the control were

characterized by an increased seedling mass throughout the entire period compared to the less drought-resistant variety of West Siberian selection (Fig. 1).

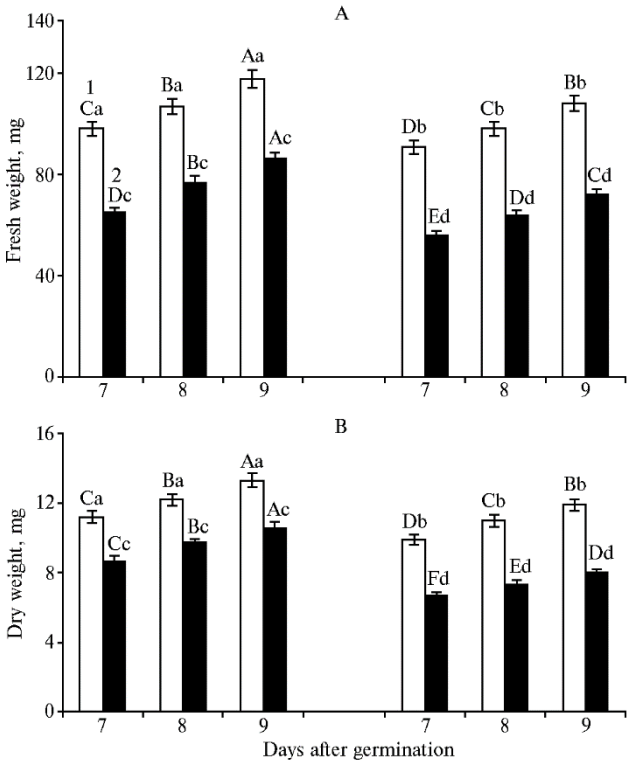

Fig. 1. Fresh (А) **and dry** (Б) **weight of 7-9-old seedlings in wheat *Triticum aestivum* L. spring varieties Ekada 70** (steppe Volga ecotype, left) **and Zauralskaya Zhemchuzhina** (forest-steppe West Siberian ecotype, right) **under simulated soil drought: 1** — control, **2** — treatment ($M \pm$SEM, pot trials). Three experiments were carried out in triplicate, each with 30 seedlings. Different lowercase letters indicate statistically significant differences between treatment variants for seedlings of the same age ($p \leq 0.05$, ANOVA, LSD test). Different capital letters indicate statistically significant differences between seedlings of different ages in the same treatment variant ($p \leq 0.05$, ANOVA, LSD test).

Soil drought led to a noticeable inhibition of growth rates in seedlings of both varieties, but in the Zauralskaya Zhemchuzhina variety it was more pronounced (see Fig. 1). It can be stated that wheat ecotypes with different strategies for adaptation to drought differ in their resistance to moisture deficiency at the initial stage of development. Plants of the steppe Volga ecotype are more drought-resistant compared to forest-steppe West Siberian ecotype. The changes that we observed in the studied ecotypes are due to genetic and ecological-physiological factors [9, 10].

The hormonal system plays a key role in the regulation of plant growth and development [23-25], and therefore it was of interest to trace changes in its state in the compared wheat varieties under soil drought conditions (Fig. 2). According to the results obtained, control plants of the resistant variety Ekada 70 accumulated a noticeably higher concentrations of auxin and cytokinins compared to the Zauralskaya Zhemchuzhina variety, while the concentration of ABA in both varieties was almost the same. Previously, many studies revealed a pronounced growth-stimulating effect of IAA and CK [15, 17]. That is is probably why in the Ekada 70 plants the growth rate parameters were highercompared to the Zauralskaya Zhemchuzhina (see Fig. 1). It is well known that under unfavorable conditions, in particular when the water regime is disturbed, significant changes occur in the plant hormonal balance with a sharp increase in the concentration of the stress hormone abscisic acid and a decrease in the level of growth-stimulating hormones auxins and

cytokinins [17, 23, 26].

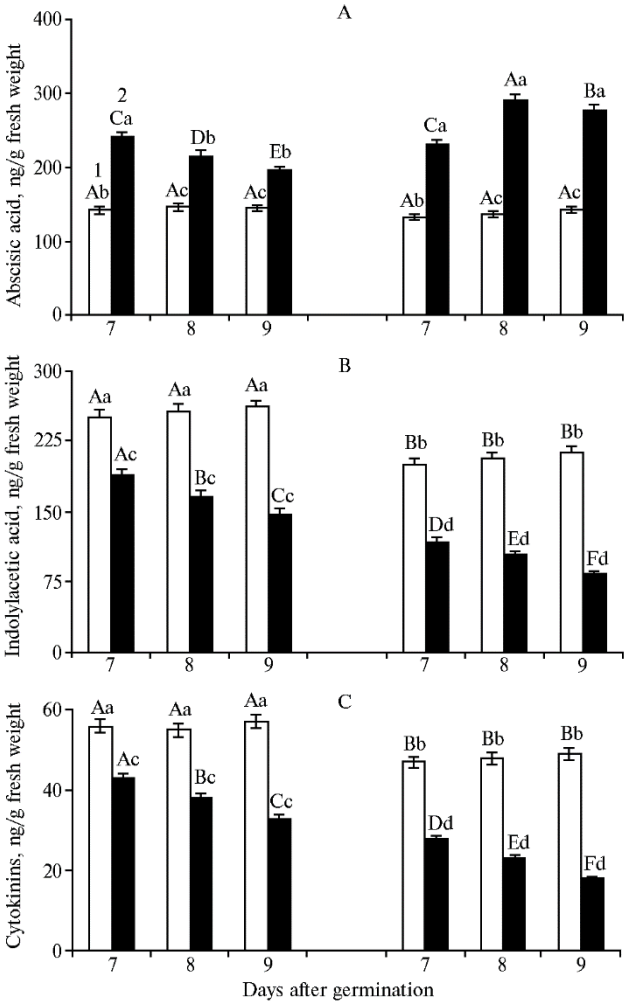

Fig. 2. Accumulation of abscisic acid (A)**, indolylacetic acid** (B) **and cytokinins** (C) **in 7-9-old seedlings of wheat *Triticum aestivum* L. spring varieties Ekada 70** (steppe Volga ecotype, left) **and Zauralskaya Zhemchuzhina** (forest-steppe West Siberian ecotype, right) **under simulated soil drought:** 1 — control, 2 — treatment (M±SEM, pot trials). Three experiments were carried out in triplicate, each with 10 seedlings. Different lowercase letters indicate statistically significant differences between treatment variants for seedlings of the same age ($p \le 0.05$, ANOVA, LSD test). Different capital letters indicate statistically significant differences between seedlings of different ages in the same treatment variant ($p \le 0.05$, ANOVA, LSD test).

Indeed, it can be seen (see Fig. 2) that growing wheat under simulated soil drought conditions also led to changes in the hormonal status of seedlings in both studied varieties. There was an accumulation of ABA and a noticeable decrease in the concentrations of IAA and CK, but the studied varieties differed in these indicators. Thus, in the drought-resistant variety Ekada 70, the maximum 2-fold accumulation of ABA in seedlings occurred only on day 7, followed by its gradual decrease. In the variety Zauralskaya Zhemchuzhina, more than 2-fold stress-induced accumulation of ABA in seedlings remained until the end of the test. The impact of soil drought led to a progressive decrease in the concentration of IAA and cytokinins in seedlings of both varieties, but in Zauralskaya Zhemchuzhina variety, by the end of the experiment (9 days), the content of IAA and cytokinins was almost 2.5 times lower than the control values (see Fig. 2). That is, when the water regime was disrupted, a sharp imbalance of phytohormones occurred in plants of the Zauralskaya

Zhemchuzhina variety which led to a pronounced growth inhibition. Plants of the Ekada 70 variety showed resistance to moisture deficiency due to significantly lower stress-induced changes in the hormonal system.

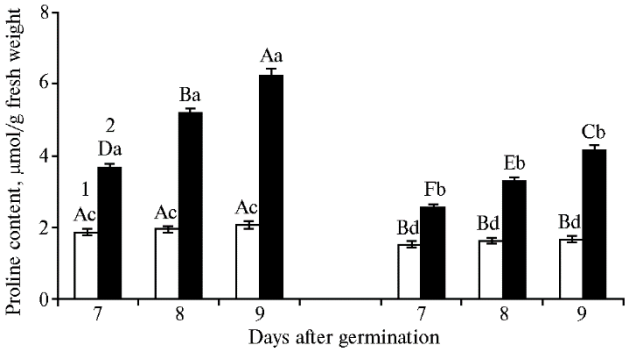

Fig. 3. Proline content in 7-9-old seedlings of wheat *Triticum aestivum* L. spring varieties Ekada 70 (steppe Volga ecotype, left) **and Zauralskaya Zhemchuzhina** (forest-steppe West Siberian ecotype, right) **under simulated soil drought:** 1 — control, 2 — treatment ($M \pm$SEM, pot trials). Three experiments were carried out in triplicate, each with 20 seedlings. Different lowercase letters indicate statistically significant differences between treatment variants for seedlings of the same age ($p \leq 0.05$, ANOVA, LSD test). Different capital letters indicate statistically significant differences between seedlings of different ages in the same treatment variant ($p \leq 0.05$, ANOVA, LSD test).

Since the moisture deficiency causes both a violation of the water regime and osmotic stress, we compared the levels of proline as the effective osmoprotector in wheat seedlings of two varieties (Fig. 3). In controls, the proline content remained relatively stable throughout the experiment, although in the roots of the Zauralskaya Zhemchuzhina plants were distinguished by a slightly lower proline content (see Fig. 3). In both varieties, in seedlings under soil drought, a significant progressive accumulation of proline occurred compared to control (see Fig. 3), which indicates the participation of this amino acid in protecting plants from dehydration. Thus, in the Ekada 70 variety, in stressed seedlings by day 9 of the experiment, the proline content increased more than three times compared to control. In the Zauralskaya Zhemchuzhina variety, stress-induced proline accumulation in seedlings was also found but its absolute values were inferior to those in the Ekada 70 (see Fig. 3).

As is known, drought causes a decrease in the osmotic potential of plants, which induces the accumulation of proline as one of the key osmolytes [27, 28]. Many studies have shown that an increase in proline content provided an increase in plant stress resistance [29, 30]. Moreover, the proline accumulation helps reduce the concentration of reactive oxygen species and significantly reduces membrane damage from adverse factors, including drought [31]. There is a positive correlation between increased proline levels and plant resistance to extreme conditions. We found that normally the seedlings of the drought-resistant Ekada 70 variety contained 25-30% more of this osmoprotector than the Zauralskaya Zhemchuzhina variety (see Fig. 3). Under simulated soil drought, in both varieties there was a progressive accumulation of proline in the seedlings, being although more than 1.5 times higher in the drought-resistant Ekada 70 compared to Zauralskaya Zhemchuzhina, which probably contributes to a more pronounced resistance of steppe Volga ecotype to drought during early ontogenesis.

Thus, our findings indicate that at the initial stage of plant development the varieties Zauralskaya Zhemchuzhina (forest-steppe West Siberian ecotype) and Ekada 70 (steppe Volga ecotype) differ in their resistance to moisture deficiency. Under drought conditions, the Ekada 70 had higher seed germination energy and seedling growth rates, that is, this variety showed greater drought resistance

compared to the Zauralskaya Zhemchuzhina variety. Differences in drought resistance of the ecotypes may be due to different responses of the hormonal system to moisture deficiency. Plants of the Zauralskaya Zhemchuzhina variety show much more pronounced stress-induced changes in hormonal balance, which led to a greater growth inhibition. The ability of the Ekada 70 variety to induce a significant accumulation of the amino acid proline, an important plant defender, significantly contributs to a higher drought resistance.

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LAB TESTS ON EFFICIENCY OF A BIOLOGICAL FERTILIZER BASED ON NITROGEN-FIXING AND PHOSPHATE-MOBILIZING BACTERIA

V.A. BABAK ✉, E.Zh. ZHAKUPOV, I.A. PUNTUS, A.N. FOMINA

«BIOTRON Progress» LLT, 021500 Republic of Kazakhstan, Akmola Province, Stepnogorsk, Industrial zone 4, complex 7, e-mail vgavm2003@mail.ru (✉ corresponding author), zhakupov_bio@mail.ru, puntusira@mail.ru, vgavm2003@mail.ru
ORCID:
Babak V.A. orcid.org/0000-0003-1860-1210
Zhakupov E.Zh. orcid.org/0000-0002-7294-257X
Puntus I.A. orcid.org/0009-0004-0628-5999
Fomina A.N. orcid.org/0000-0001-8655-0879
The authors declare no conflict of interests
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Abstract

Soil microbiota has a direct impact on ыoil fertility and composition and, as a consequence, on plant productivity. Currently, agricultural production requirements focus on biological farming the essence of which is to use the potential capabilities of natural ecosystems, in particular soil microorganisms. The most extensive and diverse group of soil microorganisms in properties are free-living and symbiotic nitrogen-fixing bacteria. Another group, phosphate-mobilizing soil microorganisms, participate in the conversion of hard-to-reach inorganic and organic phosphates into water-soluble forms assimilated by plants. Environmentally friendly and safe complex biological fertilizers based on isolates of microorganisms from local natural biogeocenoses are important to increase crop yields and improve soil fertility in the conditions of Kazakhstan. The purpose of the submitted work was a lab assessment of the effectiveness of a combined biological fertilizer based on phosphate-mobilizing and nitrogen-fixing bacteria and its compatibility with some fungicidal and herbicide preparations used in the Republic of Kazakhstan. In pot trials, the biological fertilizer BioAzoPhosfit based on nitrogen-fixing bacteria *Raoultella oxytoca* MS and phosphate-mobilizing bacteria *Serratia plymuthica* MS was tested on cucumber seedlings of the Meva variety and spring wheat seeds of the Akmola 2 variety. Tests confirmed the effectiveness of the combined biological fertilizer on cucumber plants according to the main biometric indicators (e.g., length of stem and internodes, number and size of fruits). It was revealed that the average fruit length in the treatment was 12.4 % ($p < 0.05$) greater than in the control, and the total weight of harvested fruits in the treatment was 20.3 % ($p < 0.05$) greater than in control. Biofertilizer BioAzoPhosfit showed effectiveness on spring wheat after pre-sowing seed treatment. The treated seeds produced more vigorous shoots (the proportion of seedlings that sprouted by the set date was 5.2 % higher), and the plants from the treated seeds were significantly ($p < 0.05$) ahead of the control ones in growth and development throughout the entire observation period (by 12-31 %). In lab tests with the main fungicidal (Flamingo, Dividend Extreme) and herbicide (Assoluta, Tribune, Glyphosate and Smerch) preparations used on wheat in Kazakhstan, the combined biofertilizer BioAzoPhosfit showed a decrease in the viability of living microorganisms under the influence of broad-spectrum herbicides Glyphosate and Smerch. All other fungicides and herbicides tested slightly suppressed microbial cultures. Therefore, we recommend not using biofertilizers based on live microorganisms with broad-spectrum herbicides. The results of the tests allow us to recommend the growth and productivity parameters we used in this work for laboratory express testing the quality of microbiological preparation batches.

Keywords: rhizobacteria, nitrogen-fixing bacteria, phosphate-mobilizing bacteria, biological fertilizer, fertility, fungicide, herbicide

The economic and environmental crisis, the decline in the quality of crop products, and the deterioration of natural soil fertility stimulate attention to biological farming, the essence of which is to use the potential of natural ecosystems, in particular soil microorganisms [1, 2].

Soil microbiota has a direct impact on the soil fertility and composition and, thereby, on plant productivity. Soil microorganisms improve the structure of the soil, accumulate nutrients in it, and mineralize various organic compounds, transforming them into forms easily digestible by plants. To stimulate these processes, various bacterial fertilizers are used based on beneficial microorganisms that enrich the rhizosphere layer of soil with nutrients [3, 4]. Microorganisms used for preparation biologicals improve the supply of plants with mineral nutrition elements, e.g., nitrogen and phosphorus, and physiologically active substances, the phytohormones, vitamins, etc. The positive effect of many biologicals is also due to their phytosanitary function by displacing pathogenic soil microorganisms and inhibiting their reproduction [5, 6].

Free-living and symbiotic nitrogen-fixing bacteria that assimilate the nitrogen of the air are the largest and most diverse group of soil microorganisms. They are associated with the root system or the aboveground vegetative part of plants in cereals, nightshades, oilseeds and other plant families (7, 8).

It has been experimentally revealed that nitrogen fixed by microorganisms is 100% absorbed by plants, while nitrogen from mineral fertilizers is only 50% absorbed. In addition, since bacteria use the energy of organic substances synthesized by plants during photosynthesis to carry out nitrogen fixation, biological nitrogen is practically 'unpayable' [9, 10].

Phosphorus is a biogenic element that participates in the synthesis of nucleic acids, proteins, carbohydrates and energy metabolism in animals and plants. In addition, phosphorus is the main limiting nutritional element for plants in the soil. The lack of phosphorus during early development cannot be compensated by abundant phosphorus nutrition in subsequent growth stages [11-13]. Phosphate-mobilizing soil microorganisms are involved in the conversion of hard-to-reach inorganic and organic phosphates into water-soluble forms assimilable by plants [14, 15].

The various biochemical processes occurring in the soil are caused by the activity of microorganisms. Evolutionally, between soil microbiota and plants both symbiotic and antagonistic complex relationships have been esyablished. The symbiosis between plants and soil bacteria is mainly trophic. The vital activity of microorganisms, in turn, largely determines the regime of root nutrition, plant resistance to diseases and unfavorable environmental conditions, and ultimately, productivity [16-18].

Every year, researchers discovered new strains of soil microorganisms from various families with useful phosphate-mobilizing and nitrogen-fixing properties and, based on these microorganisms, develop monocomponent and complex biological fertilizers. Biological fertilizers have a pronounced effect as growth stimulants and protectants for different plant families, mainly grain and leguminous crops, e,g,, wheat, barley, and soybeans [19-22].

In the conditions of Kazakhstan, to increase crop yields and improve soil fertility, the creation and practical use of environmentally friendly and safe complex biological fertilizers based on isolates from local biogeocenoses is relevant. Therefore, assessment of effectiveness and quality of such biologicals, as well as the developemt of practical regulations for their use are in the focuse.

This work submits the first lab test data on the efficacy of combined biological fertilizer BioAzoPhosfit in cucumbers, variety Meva and spring wheat, variety Akmola 2 and the susceptabilty of the microorganisms making the base of BioAzoPhosfit to a number of fungicides and herbicides. The used biometric parameters we propose as indicators to rapidly assess in lab tests the effectiveness of microbiologicals for grain and vegetable crops, and the quality of factory batches of such preparations.

The purpose of our research was to evaluate the effectiveness of a com-

bined biological fertilizer based on phosphate-mobilizing and nitrogen-fixing bacteria and its compatibility with some fungicidal and herbicidal preparations applicable in the Republic of Kazakhstan.

Materials and methods. Complex biological fertilizer BioAzoPhosfit based on bacterial strains *Raoultella oxytoca* MS (nitrogen-fixing component, NFS) and *Serratia plymuthica* MS (phosphate mobilizing strain, FMS) (23) (series Nos. 2, 3 and 4) were produced by BIOTRON GROUP LLP (Republic of Kazakhstan). Commercial strains *R. oxytoca* and *S. plymuthica* were isolated in 2017 from dark chestnut soils in the Akmola region (Northern Kazakhstan), identified (BIOTRON Progress LLP), and in 2018 deposited under the numbers B-RKM 0833 and B-RKM 0832, respectively in the Republican State Enterprise State Collection of Microorganisms. Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan.

For biofertilizer BioAzoPhosfit series No. 2, the strains were grown in a mixed culture, the total drug titer was 3.0×10^9 CFU/ml. Strains for series No. 3 and No. 4 were cultured separately and then combined into a two-component preparation of NFS and PMS. For No. 3, the titer of the strains was 1.15×10^{10} and 1.48×10^9 CFU/ml, respectively, for No. 4, 3.65×10^9 and 2.18×10^9 CFU/ml.

Biofertilizer BioAzoPhosfit No. 2 was used in lab tests to increase seed germination of spring wheat (*Triticum aestivum* L.) variety Akmola 2 (TNK Agrofirm LLP, Akmola region, Republic of Kazakhstan) and stimulate the growth of seedlings in cucumber (*Cucumis sativus* L.) variety Meva (Greenhouse Technologies of Kazakhstan LLP, Stepnogorsk, Republic of Kazakhstan) at the first true leaf stage. The research was carried out at BIOTRON Progress LLP (Akmola region, Republic of Kazakhstan). In experiments with wheat, artificial beds were pre-formed, each bed was a 90×50×15 cm pallet. The soil is dark chestnut with humus content from 3 to 4%, pH 7.46-7.49 (slightly alkaline), 0.15-0.21% of total nitrogen in horizon A, 2.6-4.2 mg-eq. freely hydrolyzable nitrogen, and up to 0.10-0.13% of gross phosphoric acid. The amount of total phosphorus in the soil-forming rock reached 0.18%, the content of total potassium along the horizon profile ranged within 1.6-2.2%.

Cucumber were planted from a shipping container into the ground 15 days after germination (2021), 3 plants per each of 6 pots in the control and in the test (n = 18 each). In the test variant, plants were treated only with biofertilizer during the growing season. The instructions for using the biologicals on vegetable crops provide the following regulations: a dosage of 1.0 l/t for pre-sowing seed treatment and 1.0 l/ha for treatment during the growing season; an aqueous solution of the biologicals was prepared before use at the rate of 5-7 l/t of seeds and 200-250 l/ha. In the control, the plants did not receive any fertilizers in the form of chemical monocomponents, mineral (NPK) or biological fertilizers. The regimes of insolation and soil watering (as the soil dries out) were the same in the test and control variants. During the experiment (70 days), growth and development indicators were recorded weekly. Statistical assessment of the results was based on biometric indicators (number of fruits, fruit length, length of internodes, leaf area, and fruit weight) on days 50 and 62 during active fruiting.

On spring wheat variety Akmola 2, pre-sowing seed treatment with biofertilizer was carried out for 4 h, followed by a single treatment during the growing season. The instructions for using the biologicals product on grain crops provide the following regulations: a dosage of 1.5 l/t for pre-sowing seed treatment and 1.5 l/ha for treatment during the growing season; an aqueous solution of the biological product was prepared before use at the rate of 10-15 l/t of seeds and 250-300 l/ha. In the experiment, only biofertilizer was used when growing wheat.

In the control, tap water was used to soak seeds for 4 h and to treat vegetative plants. Seeds were sown on artificial beds on April 1, 2021 with the same seeding rate in the test and control. Control plants did not receive any fertilizing in the form of chemical monocomponents, mineral (NPK) or biological fertilizers. The regimes of insolation and soil watering (as the soil dried out) were the same in the test and control variants. From sowing the seeds to day 10 of growth, visual observation and biometric measurements (uniformity of germination, leaf length) were performed dayly from day 4. Measurements were carried out twice, randomly, in different parts of the bed, according to the envelope design at five points, 4 measurements in each. In statistical processing, for 20 plants in each of two adjacent beds, the measurement of the leaf having the maximum length was used.

The survival and compatibility of phosphate-mobilizing and nitrogen-fixing bacteria of the BioAzoPhosfit preparation was assessed with the fungicides Flamingo, Dividend Extreme (Syngenta LLC, Russia), herbicides Assoluta, Tribune (Agro Expert Group LLC, Russia), Glyphosate (AFD Chemicals, USA) and Smerch (Astana-Nan LLP, Republic of Kazakhstan). The two-component fungicide Flamingo is used at the rate of 0.4 l/t of seeds in 10 l of water (working solution 1:25). Fungicide protectant Dividend Extreme is used at the rate of 0.5 l/t of seeds in 10 l of water (working solution 1:20). Assoluta preparation is used at 0.5 l/ha of crops in 150 l of water (working solution 1:300). Post-emergence herbicide Tribune with the active ingredient tribenuron-methyl is used at 20 g/ha of crops in 150 l of water (working solution 1:7500). Systemic herbicide Glyphosate (glyphosate, isopropylamine salt) is used at 5 l/ha of crops in 150 l of water (working solution 1:30). Systemic herbicide Smerch (glyphosate, isopropylamine salt) was dissolved in 150 l of water providing 5 l/ha of crops (working solution 1:30).

Biofertilizers No. 3 and No. 4 were mixed with the drugs in their working concentration (1:1, exposure in one test up to 4 h). The titers of the *R. oxytoca* MS and *S. plymuthica* MS strains were determined by serial dilutions, from 10^{-6} to 10^{-9}, on Ashby agar and MPA (meat peptone agar) based on counting colonies in 48-h cultures grown at 29 °C. The counting was carried out in triplicate, 2 Petri dishes each, an average of 18 dishes per preparation. The viable cell number per 1 cm^3 suspension was calculated according to OFS.1.7.2.0008.15 "Determination of the concentration of microbial cells" as (n6 + n7)/1.1, where C is the number of viable cells per 1 cm^3 of the drug, $\times 10^6$; *n*6 and *n*7 are the mean numbers of colonies derived from the 10^{-6} and 10^{-7} dilutions, respectively; and 1.1 is a constant coefficient. A decrease in the titer (ΔC) of NFS and PMS by no more than 1.0×10^1 CFU/ml ($\Delta C < 1.0$ lg) was taken as a positive compatibility indicator; a negative result was a decrease in the titer of strains by more than one order of magnitude ($\Delta C > 1.0$ lg).

Statistical processing was carried out in Microsoft Office Excel 2016 using a statistical package for data analysis and in the STATISTICA 8.0 program (StatSoft, Inc., USA). The significance level of all presented values was $p < 0.05$. We used generally accepted statistical processing methods for biotechnological research. Variance, means (*M*), standard deviations (±SD), confidence intervals of means were determined, and a two-sample *t*-test with equal variances was performed [24].

Results. The *R. oxytoca* MS strain belongs to the associative soil nitrogen fixers of the *Enterobacteriaceae* family. Amplification of a gene region with primers nifH-1F and nifH-1R we previously performed showed that a specific ~ 430 bp PCR product is present in *R. oxytoca* MS, confirming thah the strain is a nitrogen fixer (Report on the research work "Isolate and select strains of phos-

phate-mobilizing and nitrogen-fixing bacteria in order to obtain a complex biofertilizer based on them" within the framework of the EurAsEC Interstate Target Program, 2014). The bacteria *S. plymuthica* MS belong to free-living soil microorganisms of the *Enterobacteriaceae* family. Phosphate-mobilizing *S. plymuthica* MS is capable of dissolving inorganic and organic phosphates into digestible water-soluble compounds, which was studied on a selective glucose-aspartic agar medium containing inorganic phosphorus and on PSM medium with calcium phytate. The capability of phosphate mobilizer strains to dissolve inorganic phosphates ranged as 35-52 mg P/l. Based on the gene bank data, *S. plymuthica* has been identified as a phosphate-mobilizing bacterium [23].

In cucumbers on day 50 after germination (Table 1), the average fruit length upon BioAzoPhosphit applicatiton was 17.25% greater than in the control, the average length of internodes was 5.6% greater than in the control. The average leaf area (LA), an indicator of photosynthetic biomass) was 2183 cm^2 upon the treatment vs. 1872 cm^2 in the control, or 16.6% greater. The average number of fruits on day 50 was 16.8±0.65 vs. 14.7±1.36 in the control, exceeding the indicator without the use of biofertilizer by 14.28%.

1. Plant biometric parameters of cucumber (*Cucumis sativus* L.) Meva variety upon reatment with the combined biofertilizer BioAzoPhosphit based on *Raoultella oxytoca* MS and *Serratia plymuthica* MS on days 50 and 62 of growth (n = 18)

Parameter	Treatment	Control	To control, %
	Day 50		
Main stem length, cm:			
M±SD	131.5±6.54	127.3±5.20	+3.30
Sample variance, D[X]	40.2	23.4	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0330 < given significance level p = 0.05			
Fruit number:			
M±SD	16.8±0.65	14.7±1.36	+14.28
Sample variance, D[X]	0.85	1.62	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0029 < given significance level p = 0.05			
Fruit length, cm:			
M±SD	13.3±0.91	12.6±0.82	+5.50
Sample variance, D[X]	1.15	0.94	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0160 < given significance level p = 0.05			
Leaf area, cm^2:			
M±SD	2183.0±34.70	1872.0±29.11	+16.60
Sample variance, D[X]	765.7	573.7	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0311 < given significance level p = 0.05			
	Day 62		
Main stem length, cm:			
M±SD	142.5±7.40	134.4±4.50	+6.00
Sample variance, D[X]	46.7	21.2	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0337 < given significance level p = 0.05			
Fruit number:			
M±SD	18.5±0.55	15.7±1.36	+17.80
Sample variance, D[X]	0.92	1.86	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0025 < given significance level p = 0.05			
Fruit length, cm:			
M±SD	16.3±0.86	14.4±0.78	+13.20
Sample variance, D[X]	0.74	0.62	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.010 < given significance level p = 0.05			
Leaf area, cm^2:			
M±SD	2586.0±42.18	2130.0±31.45	+21.40
Sample variance, D[X]	996.2	747.6	
Two-sample *t*-test with equal variances, p(T ≤ *t*) two-tailed 0.0241 < given significance level p = 0.05			

Note. For each parameter in the table, the data upon treatment are statistically significantly different from the control at p < 0.05. Statistical processing included testing hypotheses of the difference in the mean values of two distributions with equal variances and their two-tailed comparison at a given significance level of 5 %.

On day 62 (see Table 1), the average length of the fruits was 16.3 cm vs. 14.4 cm in the control, that is, 13.2% longer, the number of fruits was on average 17.8% greater than in the control, the average leaf area was 2586 cm^2 vs. 2130 cm^2 in the control. In general, the complex biofertilizer significantly in-

creases the plant biometric parameters (see Table 1), which was confirmed in a two-sample *t*-test by the *t*-critical two-tailed at p(T ≤ *t*) two-tailed < a given significance level p = 0.05. The total weight of the harvested fruits in the experiment was 20.3% (p < 0.05) greater vs. the control.

Cucumber plants treated with complex biofertilizer, even upon visual inspection, were ahead of the control plants in terms of development (plant height, leaf area, length and number of fruits) (Fig. 1). That is, the yield potential per plant turned out to be greater when treated with BioAzoPhosfit biofertilizer.

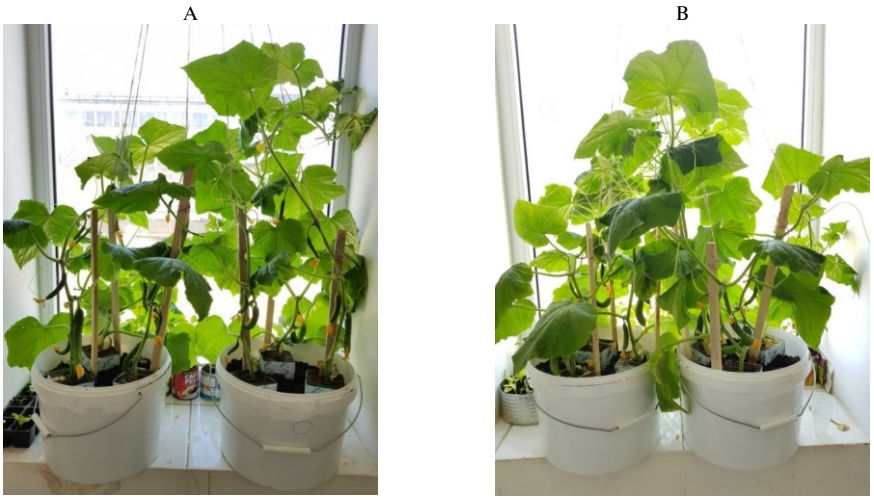

Fig. 1. Cucumber (*Cucumis sativus* L.) Meva variety plants treated (A) **and untreated** (B) **with the combined biological fertilizer BioAzoPhosfit based on the bacterial strains *Raoultella oxytoca* MS and *Serratia plymuthica* MS, 40 days after germination.**

On spring wheat variety Akmola 2, seed germination on days 1-2 was 96.7% vs. 91.5% in the control. In terms of growth intensity, already from 4-5 days after germination, the treated plants were statistically significantly ahead of the control ones (p < 0.05) (Table 2).

2. Leaf length (cm) **in spring wheat (*Triticum aestivum* L.) variety Akmola 2 upon the treatment with the combined biological fertilizer BioAzoPhosfit based on the bacterial strains *Raoultella oxytoca* MS and *Serratia plymuthica* MS** (*n* = 40, *M*±SD)

Variant	Days						
	4	5	6	7	8	9	10
Treatment	4.83±1.011	7.75±2.085	11.14±2.056	14.32±2.562	16.18±1.822	18.65±1.134	21.04±1.215
Control	3.68±0.838	6.29±1.874	9.94±2.206	12.22±3.034	14.14±1.681	16.63±1.573	18.54±1.299
To control, %	+31.25	+23.31	+12.10	+17.23	+14.43	+12.15	+13.49

N o t e. For each parameter in the table, the data upon treatment are statistically significantly different from the control at p < 0.05. Calculation data are given for 20 leaves for 20 plants from each of two adjacent beds (see section Materials and methods).

The average leaf length on day 4 upon the treatment was 4.83 cm, which was 31.25% longer than in the control; on day 7, the indicator was 17.23% higher (see Table 2). The excess of the average leaf length upon the treatment vs. the control remained at approximately the same level and by the end of observation (day 10) amounted to 13.49%. The biofertilizer BioAzoPhosfit statistically significantly increased the green mass growth of wheat sprouts by 12.1-31.25% (*t*-statistics 3.58610 > *t*-critical two-tailed 2.64691, p(T ≤ *t*) two-tailed 0.0241 < given level significance p = 0.05). In addition, spring wheat plants, when pre-sowing seeds were treated with biofertilizer, produced more vigorous shoots, the number of sprouted seeds by the due date was 5.2% higher.

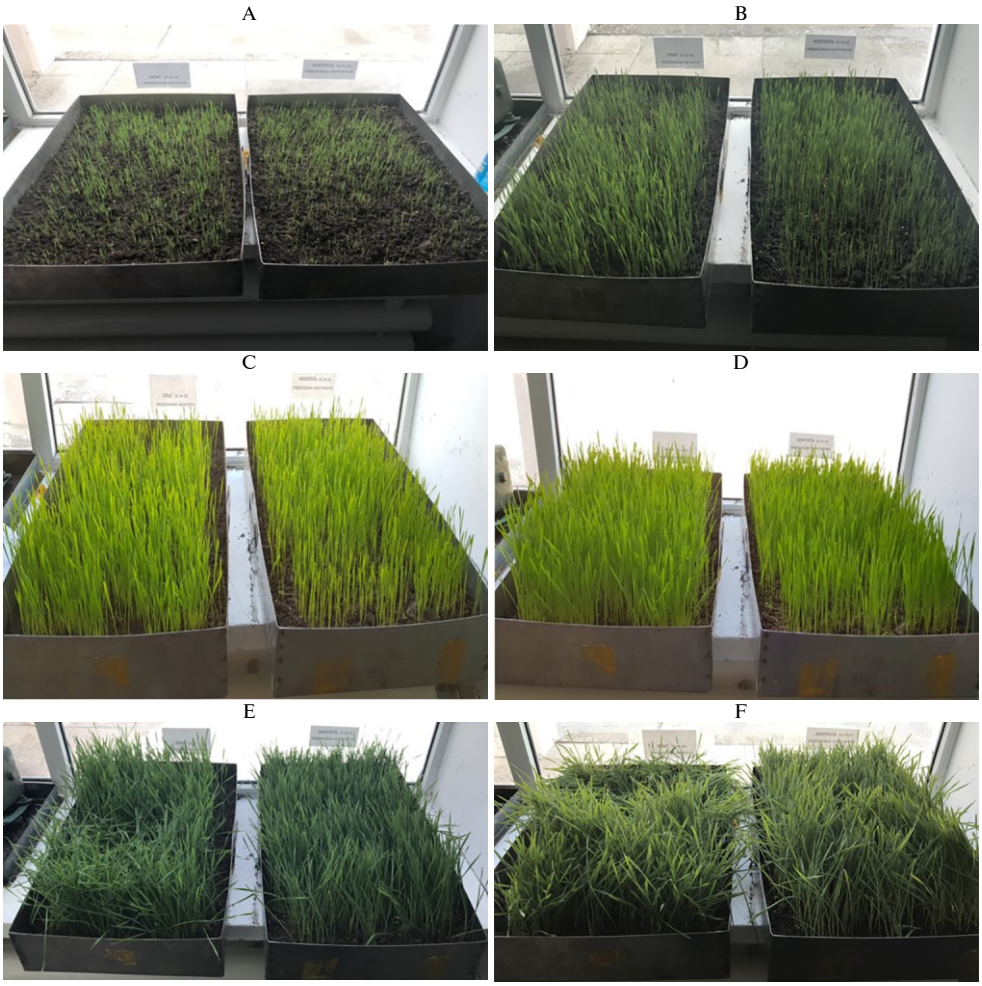

Fig. 2. Spring wheat (*Triticum aestivum* L.) variety Akmola 2 plants treated (left) **and untreated** (right) **with the combined biological fertilizer BioAzoPhosfit based on the bacterial strains Raoultella oxytoca MS and Serratia plymuthica MS:** A — 3-day old, B — 6 day old, C — 8-day old, D — 9-day old, E — 10-day old, F — 11-day old.

It is known that the release of bacterial auxins by nitrogen-fixing strains has a positive effect on the initiation and elongation of roots, the development of lateral roots and root hairs. This accelerates growth, improves nutrient uptake, and increases plant resistance to stress [5, 16, 17]. Visual observations (Fig. 2) confirmed our results of assessing the advanced growth of treated wheat plants. From day 10, massive lodging of wheat seedlings occurred, to a greater extent in the control, probably due to the low depth of seed placement in the soil. Further observation and recording were difficult and therefore stopped.

In general, the results of pot test showed that pre-sowing treatment of seeds and spraying of vegetative wheat plants with complex biofertilizer BioAzo-Phosfit stimulated the leaf growth. The data we obtained on the influence of the nitrogen and phosphate components of the biofertilizer BioAzoPhosfit on the formation of the photosynthetic surface of plants (vegetative biomass) are consistent with the conclusions of other authors [9, 14, 17] for biologicals Bioplant-K (NPO Bioprom LLC, Russia) and Extrasol (Bisolbi Plus LLC, Russia) used on various varieties of spring soft wheat. V.S. Kursakova et al. [17] established the effect of nitrogen biofertilizer and a direct relationship between the development of the photosynthetic surface and increased yield in different wheat varie-

ties. In addition, the assessment of biological fertilizer effectiveness by vegetative mass and average plant height, number and weight of seeds has been reported [3, 25], which is generally consistent with the results of lab tests we performed.

Thus, we believe that the lab test we used on different crops (cucumbers and wheat) to stafy the effectiveness of biofertilizers is quite informative as a rapid assessment of their effect.

In studies on grain crops of two-component biologicals and their various combinations [21], the combination of biofertilizers Azotobacterin and Phosphatobacterin (Innovative Company Bioinvest-Agro, Ukraine) was found the most effective, as well as the addition of organic fertilizer Gumat K (Chemistry and Technology LLP, Kazakhstan) which had qualitative and quantitative positive effects on grain crops. These results confirm the correctness of the strategy we chosed for the development of a complex biological fertilizer BioAzoPhosfit base on nitrogen-fixing and phosphate-mobilizing components.

When developing regulations for the use of microbiological preparations, their compatibility with the fungicides and herbicides is critical. Thus, the two-component fungicide Flamingo of systemic contact action based on tebuconazole and prochloraz is used to protect grain seeds from a complex of diseases transmitted through seeds and soil (hard, dusty, stone smut, helminthosporium and fusarium root rots, seed molding). The fungicide-protectant Dividend Extreme based on difenoconazole and mefenoxam is active against smut and loose smut, fusarium root rot, helminthosporium root rot, seed mold and other pathogens. Assoluta is a systemic herbicide active against annual dicotyledons, including those resistant to 2,4-D (dichlorophenoxyacetic acid) and MCPA (2-methyl-4-chlorophenoxyacetic acid), and some perennial weeds in wheat, barley and corn crops. Tribune post-emergence herbicide is designed to control a wide range of dicotyledonous weeds in cereal crops. Glyphosate is a continuous-action systemic herbicide for the control of annual and perennial cereal and dicotyledonous weeds, as well as for desiccation; Smerch is a continuous-action systemic herbicide for the destruction of vegetative plants.

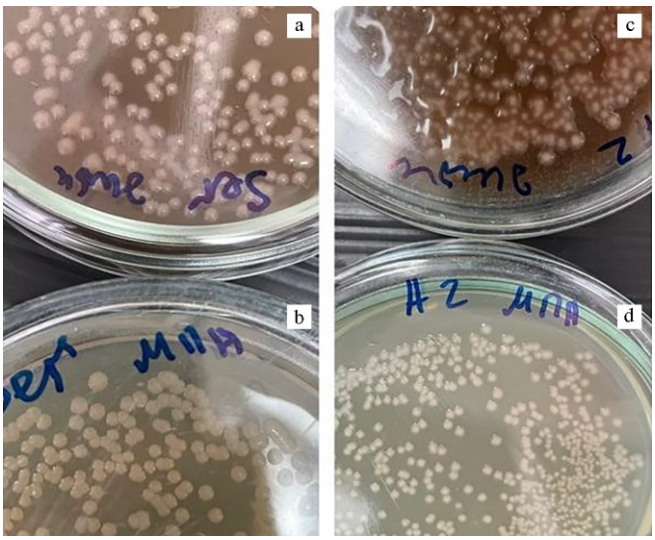

Fig. 3. Growth of *Serratia plymuthica* MS (a, b) **and *Raoultella oxytoca* MS** (c, d) **on Ashby's medium** (a, c) **and meat peptone agar** (b, d)**.**

Testing the compatibility of the BioAzoPhosfit biofertilizer with the fungicides Flamingo and Dividend Extreme revealed that these drugs did not have a significant effect on the strains (Table 3). Native biological products of the same series served as a control (Fig. 3) with the addition of equal volumes of sterile

distilled water. The activity of each component was above the minimum threshold of compliance with the manufacturer's standard (STO) (1.0×10^8 CFU/ml). Accordingly, they can be freely used in the tank mixture during pre-sowing seed treatment.

3. Tests on the compatibility of BioAzoPhosfit biofertilizer with systemic contact fungicides (n = 18 per preparation, $M\pm$SD)

Preparation	Activity, CFU/ml	Preparation	Activity, CFU/ml
Raoultella oxytoca, control (batch No. 4)	$(3.65\pm0.650)\times10^9$	*Serratia plymuthica,* контроль (batch No. 4)	$(2.18\pm0.522)\times10^9$
Raoultella oxytoca (batch No. 4) + Flamingo	$(1.48\pm0.315)\times10^9$	*Serratia plymuthica* (batch No. 4) + Flamingo	$(1.40\pm0.248)\times10^9$
Raoultella oxytoca (batch No. 4) + Dividend Extream	$(1.83\pm0.288)\times10^9$	*Serratia plymuthica* (batch No. 4) + Dividend Extream	$(1.25\pm0.154)\times10^9$
Raoultella oxytoca (batch No. 3) + Flamingo	$(6.35\pm0.731)\times10^9$	*Serratia plymuthica* (batch No. 3) + Flamingo	$(7.42\pm0.436)\times10^8$
Raoultella oxytoca, control (batch No. 3)	$(1.15\pm0.347)\times10^{10}$	*Serratia plymuthica,* контроль (batch No. 3)	$(1.48\pm0.271)\times10^9$

N o t e. For each parameter in the table, the data upon treatment are statistically significantly different from the corresponding control at $p < 0.05$.

4. Tests on the compatibility of BioAzoPhosfit biofertilizer (No. 3) with systemic fungicides and continuous action herbicides (n = 18 per preparation, $M\pm$SD)

Preparation	Activity, CFU/ml	Preparation	Activity, CFU/ml
Raoultella oxytoca, control	$(1.15\pm0.170)\times10^{10}$	*Serratia plymuthica,* control	$(1.48\pm0.125)\times10^9$
Raoultella oxytoca + Tribune	$(5.20\pm0.286)\times10^9$	*Serratia plymuthica* + Tribune	$(8.46\pm0.228)\times10^8$
Raoultella oxytoca + Assoluta	$(3.26\pm0.130)\times10^9$	*Serratia plymuthica* + Assoluta	$(1.21\pm0.147)\times10^9$
Raoultella oxytoca + Glyphosate	$(1.63\pm0.181)\times10^8$	*Serratia plymuthica* + Glyphosate	$(1.00\pm0.092)\times10^7$
Raoultella oxytoca + Smerch	$(2.37\pm0.272)\times10^7$	*Serratia plymuthica* + Smerch	$(1.84\pm0.426)\times10^7$
Raoultella oxytoca + Smerch (duble)	$(6.00\pm0.321)\times10^7$	*Serratia plymuthica* + Smerch (duble)	$(3.72\pm0.361)\times10^6$

N o t e. For each parameter in the table, the data upon treatment are statistically significantly different from the corresponding control at $p < 0.05$.

Systemic herbicides Tribune and Assoluta also did not have a significant effect on the BioAzoPhosfit strains (Table 4). The activity of each component was above the minimum threshold for compliance with the manufacturer's STO (1.0×10^8 CFU/ml), theregore, their use in a tank mixture is possible for treating seedlings by irrigation. Continuous action herbicides Glyphosate and Smerch caused a significant decrease in strain titers, by 2-3 lg, and the residual cell concentration was low for the effective use of biofertilizer. Therefore, we do not recommend biofertilizers based on live microorganisms for use with broad-spectrum herbicides.

Many authors [26-30] confirm the weak toxic effect of herbicides and fungicides on microbiologicals and recommend their combined use. An assessment in planta of the compatibility of biological and chemical means on cucumber showed a slight suppressive effect of the drugs on the culture of microorganisms [26]. The effectiveness and feasibility of combining all used plant protection products of both chemical and biological origin has been shown. It has been established [27, 28] that it is acceptable to combine herbicides of different chemical composition groups and their tank mixtures with biological preparations that are resistant to chemical attack. The use of herbicides with the anti-stress biologicals Fitosporin M [29] affects the biological effectiveness of the preparations themselves, the phytotoxicity of the herbicides does not manifest itself, and as a result, the yield and quality of grain significantly increases. However, some chemicals notably reduce the activity of microbiological fertilizers, which must be accounted when preparing a tank mixture or when spraying seedlings [30, 31].

Thus, in lab tests we reliably ($p < 0.05$) confirmed the effectiveness of the combined biological fertilizer BioAzoPhosfit based on phosphate-mobilizing bacteria *Serratia plymuthica* MS and nitrogen-fixing bacteria *Raoultella oxytoca*

MS on cucumber seedlings of the Meva variety and seeds and sprouts of spring wheat variety Akmola 2. In cucumber, we measured the length of the stem and internodes, the number and size of fruits, and the leaf area as biometric indicators. Our data indicate that the average length of cucmver fruits was 13.2% greater vs. the control, and the total weight of fruits collected from plants was 20.3% greater. Spring wheat seeds, upon a pre-sowing treatment with the biofertilizer BioAzoPhosfit, produced more vigorous shoots, the proportion of germinated seeds within the prescribed period was 5.2% higher, and in terms of growth and development, the treated plants were 12-31% ahead of the control ones over the entire observation period. These biometric indicators can be used in lab express tests to assess the quality of microbiological preparations for grain and vegetable crops, and to control the quality of factory batches of the product. Regarding the compatibility of the biofertilizer BioAzoPhosfit with the main fungicidal and herbicide preparations used on cereal crops, it is not recommended to simultaneously use a biofertilizer based on living microorganisms and broad-spectrum herbicides, for example Glyphosate and Smerch (if necessary, they should be used separately). The remaining fungicides and herbicides that we tested had an insignificant suppressive effect on microbial cultures.

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BIOLOGICAL AND AGROCHEMICAL PROPERTIES OF THE MEADOW-CHERNOZEM SOIL OF OMSK IRTYSH REGION AND FODDER CROP PRODUCTIVITY AS INFLUENCED BY MINERAL FERTILIZERS

N.N. SHULIKO ✉, A.Yu. TIMOKHIN, O.F. KHAMOVA, V.S. BOIKO, E.V. TUKMACHEVA, I.A. KORCHAGINA, A.A. VEINBENDER

Omsk Agrarian Scientific Center», 26, prosp. Koroleva, Omsk, 644012 Russia, e-mail shuliko@anc55.ru (✉ corresponding author), timokhin@anc55.ru, olkhaa48@mail.ru, boicko.vasily2011@yandex.ru, tukmacheva@anc55.ru, korchagina@anc55.ru, veybender@anc55.ru
ORCID:
Shuliko N.N. orcid.org/0000-0001-5641-434X
Timokhin A.Yu. orcid.org/0000-0002-5120-4068
Khamova O.F. orcid.org/0000-0003-0757-7008
Boiko V.S. orcid.org/0000-0002-4871-231X
Tukmacheva E.V. orcid.org/0000-0002-1312-3881
Korchagina I.A. orcid.org/0000-0001-5188-7148
Veinbender A.A. orcid.org/0000-0002-5089-3703
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Abstract

The world experience indicates that both natural factors (soil fertility, plant biopotential, etc.) and agrogenic effects (fertilizers, farming systems, etc.) promote sustainable growing crops. Mineral fertilizers have an impact on the abundance, activity and diversity of soil microflora by increasing the productivity of the system, the return of plant residues and the content of organic matter in the soil. Here we show that as a result of the systematic application of mineral fertilizers, the biological state of meadow-chernozem soil remains favorable for a number of microbiological indicators and for an increase in fodder crop yields. Our purpose was to assess the effects and relationship of application of fertilizers on soil microbiological and agrochemical parameters and, as a result, on crop productivity. The tests were carried out in 2020-2022 in the forest-steppe zone of the South Western Siberia, Omsk region (55.04192°N, 73.46504°E) in a stationary field experiment. The content of mobile phosphorus in the soil according to Chirikov is medium. In eight-field grain-grass crop rotation, a mix of perennial grases *Dőctylis glomerőta* L. with *Onobrɔchis arenőria* (Kit.) DC. and annual sorghum-sudank hybrid grass *Sorghum* × *drummondii* (Steud.) Millsp. & Chase were grown. The number of different physiological groups of microorganisms, enzymatic activity, the content of nitrates, the nitrification ability of the arable soil layer, and crop yields were assessed. We revealed that the growth of agronomically valuable groups under annual grasses is more intensive than under perennial grasses. Optimized mineral nutrition ($N_{60}P_{60}$) of the mixed grass stand stimulated mostly the growth of the phosphate-mobilizing microorganisms and soil micromycetes (by 118 and 122 % compared to control), under the sorghum Sudangrass hybrid, the number of amylolytic and oligonitrophilic microbiota significantly increased by 57-90 % vs. control, respectively. The analysis of changes in the total microbial number showed the stimulating effect of mineral fertilizers on the soil microbocenosis under agricultural crops. The use of mineral fertilizers affected the total microbial numbers equally under perennial and annual grasses, with 51-52 % increase vs. the control without fertilizers. It was revealed that the long-term use of mineral fertilizers negatively affects the activity of catalase, the redox enzyme. The decrease vs. the control was up to 14 % depending on the culture. The activity of hydrolytic enzymes urease and invertase remained not significantly affected. Observations of the meadow-chernozem soil nitrate regime showed that mineral fertilizers used at a dosage of $N_{60}P_{60}$ in sorghum-Sudangrass hybrid and grass mixture crops increased the soil nitrate nitrogen content during the growing season by two times or more compared to variants without fertilizers. In our research, the yield of perennial grasses over the years was 3.84-4.57 t/ha DM in the control and 4.82-4.89 t/ha DM upon fertilization. The studied technology of mineral fertilizer application significantly increases the yield of sorghum-Sudangrass (by 1.65 t/ha DM, or by 39 % vs. control. Crop yields had the strongest direct correlation with the microbiota of the nitrogen cycle, the amylolytic and proteolytic microorganisms (r = 0.98 and r = 0.85, respectively, $p < 0,05$).

Keywords: soil microorganisms, enzymatic activity, mineral fertilizers, nitrate nitrogen,

nitrification ability, crop rotation, perennial grasses, sorghum-Sudangrass hybrid, yield

Sorghum crops are among the leaders in global agricultural production [1]. The area of their cultivation has a steady tendency to expand due to high and stable productivity, efficient use of insolation and photosynthetic resources, and drought resistance [2, 3]. Perennial grasses, in addition to their nutritional benefits, enrich the soil with organic matter and improve water-physical, agrochemical and biological properties of the soil [4].

The role of biological factors in maintaining the health and productivity of plants, as well as sustainable agrocenoses, is currently receiving much attention [5]. Microorganisms are the most numerous participans of of biology life of the soil, which directly affects the stability of the agroecosystem [6].

Numerous organisms with active metabolism inhabit the soil [7-9]. Microbial cenosis forms the basis of the fertility of all soil ecosystems [10, 11]. Counts and diversity of microorganisms are indicators, assessing the impact of various agricultural practices on ecosystems. Microbial activity is susceptible to different types of fertilizers and plant protection products, soil tillage methods, or exposure to root exudates of the cultivated plant [12, 13].

Studying the structure of ecological-trophic groups of soil microorganisms, which are indicative of the state of microbiocenosis, is necessary to determine the balance of mineralization and synthesis of organic substrates [14].

World experience indicates the need to use natural (soil fertility, climate, landscape, plant biopotential) and agrogenic (fertilizers and plant protection products, modern farming systems) factors in crop growing [15].

The effect of mineral fertilizers is not limited to the direct impact on the production process of the plant. They also influence the number, activity, and diversity of soil organisms. Mineral fertilizers can intensify processes in the soil biological system. Its productivity enhances, crop residue return increases, and soil organic matter content rises [16]. In moderate doses, fertilizers activate the vital activity of various groups of microorganisms. Populations of soil aerobic and anaerobic nitrogen fixers, ammonifiers, cellulose decomposers, actinomycetes and microfungi become more abundant [17, 18]. However, excessive activation of soil microbiota can lead to negative environmental consequences, e.g., deterioration of the biological and physicochemical properties of soils, humus mineralization, increased gaseous nitrogen losses due to denitrification and nitrification, accumulation of nitrates in soil, plants, groundwater, destruction of the ozone screen of the stratosphere [19, 20]. Long-term use of increased doses of mineral fertilizers can intensify growth of toxin-producing microbes [21]. A number of studies have shown the negative effect of mineral fertilizers on the microbiological activity of the soil, which is explained by acidification and a decrease in the reserves of biologically available carbon [22].

Changes in the enzymatic activity of soils are indicators of the influence of various agricultural practices on the soil biological processes [23, 24]. The relationship between soil fertility and enzymatic activity established in many studies allows us to compare the effectiveness of agricultural practices, soil fertility in general, and changes in soil properties during various anthropogenic and natural processes in the ecosystem by the soil enzymatic activity [25].

The formation of enzyme potential largely depends on the agrochemical properties of soils. Their optimal parameters provide favorable conditions for the development of microorganisms and plants, and as a result, the natural supply of the soil with enzymes increases due to the formation of a larger mass of microbes and their higher physiological activity [26].

It has been reported that the application of high doses of mineral nitrogen-phosphorus fertilizers has a positive effect on the soil agrochemical parameters soil

and increases enzyme activity [27]. However, other studies have established a negative effect of anions of mineral fertilizers on the activity of the redox enzyme catalase [28] and the inhibitory effect of chemicalization agents on the activity of urease in early development of wheat plants, while the phosphatase and invertase activity of chernozem practically did not change in dynamics [29].

In the microorganisms—soil—plants system, mineral fertilizers pose a direct positive effect on the productivity of agrocenoses and a stimulating effect on the microbial community, thus affecting soil fertility which, in turn, also affects plant productivity.

It can be stated that in the scientific literature there is data on the absence, positive and negative effects of fertilizers on the soil enzymatic activity. Little is known about the consequences of prolonged use of mineral fertilizers for enzymatic activity in the agricultural landscapes of the Omsk region, since in Siberia (and in Russia as a whole) a limited number of long-term stationary experiments have been preserved in which such information can be obtained.

Meadow-chernozem soil has been fertilized for a long time in field experiments in a crop rotation system, and in this regard, the tolerance of microbial communities to fertilizers and changes in the soil biochemical and agrochemical parameters, directly affecting crop productivity are of special interest. There is still little research into the biological activity of soil during long-term (more than 40 years) agricultural use in the conditions of the Omsk Irtysh region. [24, 30, 31].

The impact of mineral fertilizers on the activity of ecological trophic groups of soil microbiota and enzymatic activity still remains relevant and important problem that requires permanent monitoring.

In this work, we established that intensive crop cultivation technology in crop rotation increases activity of the microbial cenosis of meadow-chernozem soil. This has a positive effect on the yield of perennial grasses and sorghum-Sudangrass hybrid. There are no significant changes in the activity of hydrolytic enzymes, but a decrease in catalase activity occurs.

Here, we aimed to assess changes in the biological properties, namely, in microbial pool activity and enzymatic activity, and agrochemical properties of meadow-chernozem soil under application of mineral fertilizers in a long-term (more than 40 years) stationary experiment, and the impact of these changes on productivity of annual and perennial herbal agrocenoses.

Materials and methods. The research was carried out in 2020-2022 in an eight-field crop rotation based on a stationary experiment (established in 1978). Perennial grasses, the mix of *Dactylis glomerata* L. with *Onobrychis arenaria* (Kit.) DC., were sown in 2020. The choice of the mixture is due to the nutritional value of the crops. An annual grass was sorghum-Sudangrass hybrid *Sorghum* × *drummondii* (Steud.) Millsp. & Chase.

The experimental site is located in the southern forest-steppe zone (Omsk Province, Omsk District, 55.04192°N, 73.46504°E). The territory belongs to the Priomskaya Plain, part of the Barabinskaya Neogene Plain.

In the two-factor experiment design a crop (factor A) was perennial grasses or annual grass (sorghum-Sudangrass hybrid); nitrogen-phosphorus fertilizer (factor B) options were without fertilizers (control), annual pre-sowing application of $N_{60}P_{60}$. For fertilization, ammonium nitrate (grade B, KAO Azot, Russia) and ammophos (Balakovo branch of JSC Apatit, Russia) were used. Mineral fertilizers were applied with a disk seeder SZ-3.6 (Russia) before sowing, followed by pre-sowing soil cultivation and the sowing. From the second year of life of perennial grasses, fertilizers were applied with a seeder as top dressing in the spring. The seeding rate for sainfoin is 6 million seeds/ha, for cocksfoot grass are 8 million seeds/ha (sowing every 0.45 m), for sorghum-Sudangrass hybrid 20 kg/ha (1 million

seeds/ha), sowing time is the second decade of May. In each variant, the plot area was 360 m^2, each year the experiment was carried out in triplicate.

The soil samples for microbiological studies were collected in sterile parchment bags 3 times in the main stage of plant development, for perennials cocksfoot grass at tillering, heading, filling, for perennials sainfoin at stemming, budding, flowering, for annual sorghum-Sudangrass hybrid at tillering, stemming, and milky ripeness. Each bulk soil sample was formed from four drilling to a 0-20 cm depth. Identification and quantification of microbial groups were carried out on meat peptone agar (MPA) for proteolytic bacteria, on starch-ammonia agar (SAA) for amylolytics, on Mishustina's medium for oligonitrophils, on Muromtsev's medium for phosphate-mobilizers, on Hutchinson's medium for cellulose decomposers, on aqueous leached agar added with double ammonium-magnesium salt of phosphoric acid for nitrifiers, on acidified Czapek medium for soil fungi [32]. The counts of soil bacteria, actinomycetes and micromycetes was expressed in colony-forming units (CFU) per 1 g of absolutely dry soil. Micromycetes were identified to genus based on morphological and cultural properties [33].

The soil enzymatic activity analysis was carried out in air-dry samples, for invertase according to Kuprevich, for urease according to Hoffmann, for catalase gasometrically [34].

Nitrate nitrogen in the soil was determined by the Grandval-Lage method/ To evaluate the results, scales of soil nitrate nitrogen supply generally accepted in agrochemistry were used [35, 36]. The nitrification capacity of the soil was determined according to Kravkov with 21-day incubation [37].

Accounting for green mass yield, calculated for DM separately for each cutting according to the botanical composition, was carried out manually from 1 m^2 area with 3 repetitions [38].

Statistical processing was performed in Microsoft Excel (Statistica 10.0, StatSoft, Inc., USA). The influence of factors was assessed by analysis of variance at $p < 0.05$ [39]. Mean values (*M*), standard error of means (±SEM), and Pearson correlation coefficients (*r*) were calculated. The least significant differences at a 5% significance level (LSD_{05}) were assessed [39] using the Microsoft Excel statistical software package.

Results. The soil of the experimental plot is meadow-chernozem, medium-thick, medium-humic, heavy loamy, with a humus content in the 0-0.2 m layer of approximately 7.0%, pHaq. 7.2; the thickness of the humus horizon A is 0.45 m. The surface soil is heavy loam with 40-46% physical clay. The profile has a 4-5-membered structure and is typical for the southern forest-steppe soil-climatic zone. Water permeability, determined 1 month after flat-cut loosening in the fall, is characterized as good. The density of the arable layer is 1.07-1.14 g/cm^3. The phosphorus content in the 0-0.2 m layer in the control is medium (less than 100 mg/kg), the supply of exchangeable potassium is very high (more than 180 mg/kg) regardless of the agricultural background [40].

The summer of 2020 was dry, with HTC = 0.69 from May to August vs. a norm of 1.0. In June, there were rains only in the third decade, 44 mm of precipitation vs. a norm of 51 mm. August was favorable in terms of precipitation. The 2021 season can generally be recognized dry and hot. Precipitation fell unevenly and was torrential in nature. May and the first half of June, the first and third decades of July, and the first half of August were particularly hot and dry, for May-August, HTC = 0.7. The growing season of 2022 was insufficient in moisture and unfavorable for the growth and development of crops in the experiment, on average for May-August, HTC = 0.81.

The aridity of the growing seasons resulted in small reserves of available moisture in the soil. Thus, in 2020, when sowing perennial grasses, June moisture

reserves did not exceed 73% of the lowest moisture capacity (LMC) in both the arable and meter layers of soil, decreasing in July to critical values close to wilting moisture. Similar changes occurred in sowings of the sorghum-Sudangrass hybrid. Due to autumn-winter precipitation, moisture was replenished and the next year in June it amounted to more than 80% of the moisture content, which is sufficient for normal growth and development of plants. However, reserves further decreased to 55% of LMC in both arable and meter layers, regardless of the crop. The year 2022 was no exception. Throughout the growing season, drying of the soil profile occurred except for the arable horizon in July after precipitation, which increased the level of moisture to 86% LMC.

Studies by Chinese colleagues conducted at the Shenyang experimental station (Northeast China) showed that long-term application of mineral fertilizers has no does not have a significant effect on the soil microbiological activity [41]. A significant body of information by American researchers revealed a negative effect of mineral fertilizers on growth in the microbial community, which is explained by their acidifying effect and the deficiency of available carbon sources after the initial increase in mineralization activity [42].

In our experiement, we used a dose of $N_{60}P_{60}$ fertilizer calculated to ensure a positive balance of nutrients. In our studies in 2020 on perennial grasses, the application of $N_{60}P_{60}$ statistically significantly ($p < 0.05$) stimulated an increase in the number of phosphate-mobilizing microorganisms by 122% compared to the control, cellulolytics and microfungi that destruct organic matter in the soil by 51 and 128%, respectively (Table 1).

1. Composition of microbial community (CFU/g) **of the meadow-chernozem soil as affected by the fertilizers and crops** (for each group, $n = 9$, $M \pm$SEM; Omsk ASC, Omsk, 2020-2022)

Microbial group	Perennial grasses		Sorghum-Sudangrass hybrid		LSD_{05}	LSD_{05}
	control	$N_{60}P_{60}$	control	$N_{60}P_{60}$	A, B	AB
			In 2020			
Proteolytics, $\times 10^6$	46.6±24.7	56.2±22.4	35.8±11.4	47.2±9.6	19.9	28.4
Amylolytics, $\times 10^6$	13.1±5.0	17.7±5.0	12.6±6.9	21.3±7.6*	5.8	8.2
Oligonitrophils, $\times 10^6$	221.5±8.1	199.4±13.2	167.9±5.6	340.4±38.4*	115.3	163.0
Phosphate mobilizing, $\times 10^6$	179.8±5.1	398.9±16.5*	223.7±14.5	229.3±36.3	77.1	109.1
Microfungi, $\times 10^3$	103.0±16.9	236.1±17.7*	144.4±32.2	103.7±27.2	44.4	62.7
Nitrifiers, $\times 10^3$	1.65±0.39	1.76±0.18	1.57±0.31	1.81±0.49	0.68	0.97
Cellulose decomposers, $\times 10^3$	85.2±31.3	128.3±25.4	72.8±3.2	129.2±9.0*	46.4	65.5
Total, $\times 10^6$	461.3±18.4	672.6±15.0*	440.2±29.2	638.5±89.3*	188.2	111.2
			In 2021			
Proteolytics, $\times 10^6$	22.8±4.0	28.0±4.4	27.8±4.7	39.1±8.9*	9.3	13.2
Amylolytics, $\times 10^6$	16.9±4.3	28.6±7.9*	24.3±8.5	34.8±5.1*	11.3	16.0
Oligonitrophils, $\times 10^6$	35.4±3.9	58.4±7.6*	58.6±16.5	119.3±24.3*	33.7	47.7
Phosphate mobilizing, $\times 10^6$	32.3±10.6	49.5±6.6	65.6±27.1	108.8±7.1*	27.4	38.8
Microfungi, $\times 10^3$	26.2±6.3	78.1±55.2*	25.2±3.9	39.2±1.7	32.7	39.8
Nitrifiers, $\times 10^3$	0.55±0.04	0.58±0.09	0.75±0.19	1.11±0.60	0.72	1.02
Cellulose decomposers, $\times 10^3$	61.5±21.9	88.7±34.8	71.1±20.6	80.4±28.31	38.2	54.0
Total, $\times 10^6$	107.5±17.2	164.8±11.6	176.4±50.4	302.2±36.1*	66.0	93.3
			In 2022			
Proteolytics, $\times 10^6$	25.0±7.1	33,0±7,1*	26,5±4,3	31,1±9,3	7,0	10,0
Amylolytics, $\times 10^6$	21.2±5.9	26,4±6,4	22,6±6,3	35,1±11,5*	10,8	15,1
Oligonitrophils, $\times 10^6$	63.9±23.6	92,2±34,8	70,4±30,9	100,2±54,2	62,5	88,3
Phosphate mobilizing, $\times 10^6$	39.2±11.7	97,5±32,1*	47,7±11,9	87,9±44,7	45,4	64,2
Microfungi, $\times 10^3$	35.0±10.3	46,8±7,5	31,5±3,3	49,2±8,3	21,6	30,5
Nitrifiers, $\times 10^3$	1.70±0.44	1,40±0,21	1,70±0,61	1,50±0,23	0,79	1,12
Cellulose decomposers, $\times 10^3$	63.7±14.5	116,1±10,8*	55,8±17,4	81,4±4,5*	20,6	29,2
Total, $\times 10^6$	149.3±47.6	249,3±79,3	167,27±49,4	254,47±89,5	118,5	167,5

N o t e. Perennial grasses are a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*. LSD_{05}A corresponds to the crop factor, LSD_{05}B to the fertilizer application, LSD_{05}AB to the interaction crop × fertilizers; n is the number of measurements.

* Differences with the corresponding control are statistically significant at $p < 0.05$.

The increase in the number of biota that decomposes organic compounds in the soil when fertilizers are used is associated with its enrichment with mineral

nutrition elements and an increase in plant mass during the growing season [43, 44]. Plant residues are a source of nutrition and energy for microorganisms and a comfortable space for colonization. Residues contain a complex nutrient and energy substrate for most microorganisms, the main source of soluble low molecular weight organic substances. Elements of mineral nutrition that enter the soil with fertilizers, in particular nitrogen, are especially important for the active life of microorganisms and the decomposition of organic matter [45-47].

Improving the mineral nutrition of sorghum-Sudangrass hybrid plants had a positive effect on the agronomically valuable microbiota. The number of copiotrophs (proteolytics and amylolytics) increased by 32 and 69%, respectively ($p < 0.05$) compared to the control. More than 52 and 87% effects, respectively, were due to the significant influence of fertilizers. Studies by Polish scientists have revealed a similar pattern of growth in the number of biota secreting amylase with the use of fertilizers [48].

In the microbial community we studied, oligonitrophils are the most common group. Oligonitrophilic microbiota that plays a major role in preserving and replenishing nitrogen reserves in the soil, and cellulose-degrading microorganisms responded to a greater extent to the $N_{60}P_{60}$ application by changing their numbers with statistically significant increase by 103 and 79%, respectively, compared to the control ($p < 0.05$). According to N. Roljević et al. [49], mineral fertilizers stimulated this microbiota with a 14.6 to 37.7% increase compared to the control. Differences in the absolute CFU values in our studies and those of our colleagues are due to different types of soils and, particularly, different enrichment in humus. It is known that richer and cultivated soils are highly biogenic [50], in our experiement, meadow-chernozem soil had a humus content of 7.0%.

The fertilizers provided a significant ($p < 0.05$) and almost equal increase in the total number of microbiota we found under annual and perennial crops, by 45 and 46%, respectively, compared to the control (see Table 1).

In 2021 which was characterized by a deficit of atmospheric precipitation coupled wirh extremely high air temperatures during the growing season, the increase in the number of soil microbiota was less than in the previous year 2020, due to unfavorable environmental factors.

The highest abundance of microorganisms in the control and treatment was found under annual grass. With a high number of bacteria upon applying fertilizers, the number of microfungi in the soil under the sorghum-Sudangrass hybrid decreased. Apparently, the growth of micromycetes was suppressed by the high number of bacteria. This may be due to the well-developed root system of the sorghum-Sudangrass hybrid and its specific root secretions (see Table 1).

The use of mineral fertilizers had a positive effect on various groups of microorganisms. Thus, in the soil under perennial and annual grasses, the number of proteolytic microorganisms increased by 23 and 41%, of bacteria exhibiting amylolytic activity by 69 and 43%, oligonitrophils which play an important role in the nitrogen cycle in nature, in particular in supplying plants with available nitrogen by 65 and 104%.

Microfungi in the soil play the role of saprophytes, reducers, and symbionts. Their contribution to the yield is enormous. They participate in decomposition of complex organic compounds, enter into symbiosis with plants, produce pigments, antibiotics, biologically active compounds and form soil structure [51].

In our studies, the number of soil microfungi under perennial grasses increased by almost 200%, that is, 3 times. Scientific publications on this issue indicate that the use of chemical agents does not always have a clear effect on this group of microorganisms. The authors of the studies point to both the inhibitory and stimulating effects of such agricultural practices on the growth of microfungi

[52, 53]. In addition, this effect may be due to less competition between these microorganisms and bacteria. It should be noted that more intensive growth of these microorganisms in the soil may be an unfavorable phenomenon posing a risk of proliferation of toxicogenic or pathogenic species [54].

It is worth noting a significant increase in the number of phosphate-mobilizing and nitrifying microorganisms involved in plant nutrition that we rebealed under sorghum-Sudangrass hybrid upon fertilization, by 66 and 48%, respectively, vs. the control.

The total number of detectable microorganisms, as influenced by the fertilizers, increased compared to control, in the soil under perennial grasses by 53%, under sorghum crops by 71%.

In 2022, the use of $N_{60}P_{60}$ contributed, depending on the type of crop, to an increase in the number of proteolytic bacteria decomposing organic nitrogen-containing compounds by 17 and 32%, amylolytic microflora by 24 and 55% ($p < 0.05$) vs. the control, with 67% influence of factor B (fertilizers) (see Table 1).

The number of oligonitrophilic microorganisms, as well as phosphate-mobilizing bacteria, statistically significantly ($p < 0.05$) increased under the sowing of perennial grasses by 46 and 148%, respectively, under the sorghum-Sudangrass hybrid by 43 and 85%, with 94 and 96% influence of factor B (fertilizers). Changes in the number of microorganisms decomposing organic residues under perennial and annual grasses were significant ($p < 0.05$). With the use of fertilizers, the number of cellulolytics increased by 84 and 47%, respectively, and soil micromycetes by 31 and 58%.

The use of fertilizers provides an increase in the total number of detectable microflora in the soil under perennial grasses by 67%, under sorghum crop by 52% vs. the control.

The growing seasons during the experiment differed in heat and moisture supply, which made it possible to more fully assess the impact of the studied agricultural practice. On average, over 3 years of research, long-term moderate use of mineral fertilizers ($N_{60}P_{60}$) in a stationary experiment stimulated several ecological trophic groups of soil microorganisms, that is, the trend in their activity remained over the years. In perennial grasses upon application of fertilizers, the number of phosphate-mobilizing microorganisms and soil micromycetes increased to the greatest extent, by 118 and 122% vs. the control ($p < 0.05$), with 77 and 44% influence of factor B (fertilizers). Under sorghum-Sudangrass hybrid, the counts of amylolytics and oligonitrophilics increased significantly by 57-90% compared to the control, wirh 77 and 48% influence of factor B (fertilizers) (Table 2).

2. Microbial abundance (CFU/g) **in the meadow-chernozem soil as affected by the fertilizers and crops** (for each group, $n = 9$, $M \pm$SEM; Omsk ASC, Omsk, 2020-2022)

Microbial group	Perennial grasses		Sorghum-Sudangrass hybrid		LSD_{05}	LSD_{05}
	control	$N_{60}P_{60}$	control	$N_{60}P_{60}$	A, B	AB
Proteolytics, $\times 10^6$	31.4±5.4	39.0±4.9	30.0±4.1	39.1±2.6*	7.8	11.0
Amylolytics, $\times 10^6$	17.0±4.3	24.2±6.3*	19.8±6.6	30.4±4.9*	3.4	4.7
Oligonitrophils, $\times 10^6$	106.9±54.1	116.6±28.1	98.9±38.4	186.6±64.8*	49.2	69.5
Phosphate mobilizing, $\times 10^6$	83.7±47.2	181.9±102.1*	112.3±65.9	142.0±36.0	77.1	109.0
Microfungi, $\times 10^3$	54.7±25.2	120.3±65.1*	66.9±25.6	64.0±15.9	44.4	62.8
Nitrifiers, $\times 10^3$	1.30±0.02	1.25±0.06	1.34±0.10	1.47±0.25	0.39	0.56
Cellulose dcomposers, $\times 10^3$	70.1±013.8	111.0±9.6*	66.5±4.7	97.0±5.5*	18.2	25.7
Total, $\times 10^6$	239.3±96.7	362.2±98.4*	261.2±103.4	398.3±98.3*	78.6	111.1

N o t e. Perennial grasses are a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*. LSD_{05}A corresponds to the crop factor, LSD_{05}B to the fertilizer application, LSD_{05}AB to the interaction crop × fertilizers; n is the number of measurements.

* Differences with the corresponding control are statistically significant at $p < 0.05$.

Research by A.V. Kurakova [44] found that an increase in the number of

soil microfungi may resulted from the soil acidification with salt anions during long-term application of fertilizers. Since the main function of soil microfungi is the decomposition of plant organic residues, it can be assumed that when using fertilizers this process occurs more intensely due to more substrate. We identified microscopic fungi belonging to six genera, the *Penicillium*, *Aspergillus*, *Mucor*, *Fusarium*, *Trichoderma* and *Alternaria* (Fig. 1).

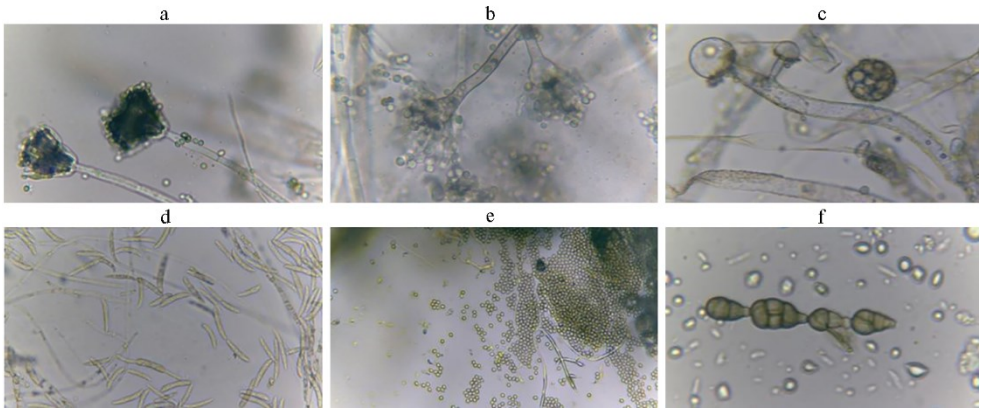

Fig. 1. Micromycetes isolated from the meadow-chernozem soil: a — *Penicillium* ssp., b — *Aspergillus* ssp., c — *Mucor* ssp., d — *Fuzarium* ssp., e — *Trichoderma* ssp., f — *Alternaria* ssp. (a microscope TS 2000, Biolab, Russia, ×200; Omsk ASC, Omsk, 2020-2022).

Facultative saprophytes which include representatives of the genera *Fusarium* and *Alternaria*, are widespread regardless of the precursor crop, they cause leaf damage and root rot and are able to survive in winter on plant debris. Members of the genera *Aspergillus* and *Penicillium*, according to the publication [55], are classified as epiphytes that use exclusively the waste products of the plant, without causing harm to it, but worsening the crop quality.

It should be noted that the lack of growth in the number of nitrifying bacteria under perennial grasses is possibly due to low soil moisture. This is consistent with the research [56] in which a negative effect of low soil moisture on this microbial group was also revealed.

Analysis of changes in the total number of detectable microflora showed the stimulating effect of mineral fertilizers on the state of soil microbiocenosis under agricultural crops. In terms of microbial populations, the effects during the years of observation, varied to a greater or lesser extent depending on the crop. However, on average for 2020-2022, optimization of mineral nutrition increased the total microbial pool in the soil under perennial and annual grasses equally, by 51-52% compared to the control. The highest abundance of soil microorganisms was found under sorghum-Sudangrass, from 261×10^6 to 398×10^6 CFU/g vs. 239×10^6 to 362×10^6 CFU/g under perennial grasses.

The activity of soil enzymes can be used to assess the intensity of biological processes. In our experiment, the use of mineral fertilizers reduced catalase activity under perennial grasses by 14% and under sorghum by 11%. Similar studies in stationary experiments showed that as the time of using mineral fertilizers and the soil $N\text{-}NO_3$ content increse, the activity of this enzyme decreases [23, 28]. F.H. Khaziev [26] reported a negative correlation between the activity of catalase and the content of nitrate nitrogen in the soil. We also found an inverse in correlation between the activity of catalase and the soil content of nitrate nitrogen ($r = \pm 0.85 \pm 0.21$, $p < 0.05$) due to which there was a decrease in enzyme activity when applying fertilizers. This is related to the duration of fertilizer application, as it was reported that during the first crop rotations this trend was not

observed (31).

On average, over the years of our research, when applying mineral fertilizers to perennial grasses and sorghum-Sudangrass hybrid, the activity of the hydrolytic enzymes ureased while activity of invertase did not change significantly and we did not find a negative effect of mineral fertilizers on the activity of these enzymes (Table 3). According to the D.G. Zvyagintsev scale [20] proposed to assess the enrichment of soils with enzymes, the studied meadow-chernozemic soil for catalase activity is classified as poor (1.0-3.0), for urease activity as very poor (< 3.0), and for invertase as medium enriched (15.0-50.0).

3. Enzimatic activity of the meadow-chernozem soil as affected by the fertilizers and crops (for each variant, $n = 9$, M±SEM; Omsk ASC, Omsk, 2020-2022)

Treatment	Catalase, O_2 $cm^3 \cdot min^{-1} \cdot g^{-1}$	Urease, mg NH_3/g	Invertase, mg invert sugar/g
Perennial grasses			
Control	2.026±0.099	0.665±0.012	17.89±0.99
$N_{60}P_{60}$	1.776±0.100	0.654±0.019	18.43±0.97
Sorghum-Sudangrass hybrid			
Control	1.880±0.090	0.678±0.059	18.47±0.33
$N_{60}P_{60}$	1.690±0.090	0.699±0.072	18.41±1.53
LSD_{05}A, B*	0.321	0.832	1.6
LSD_{05}AB	0.139	0.361	0.7

Note. Perennial grasses are a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*. LSD_{05}A corresponds to the crop factor, LSD_{05}B to the fertilizer application, LSD_{05}AB to the interaction crop × fertilizers; n is the number of measurements.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

In long-term studies on the regime of mineral nitrogen compounds in chernozems, the dependence of the supply of field crops with nitrogen on the amount of its nitrate form (N-NO_3) was established, which makes it possible to recognize this parameter as a diagnostic indicator [57, 58]. Our observations showed that during the tillering phase (June), under sorghum-Sudangrass hybrid and perennial grasses, the N-NO_3 content in the arable layer (0-20 cm) upon fertilization was high (> 20 mg/kg) according to the scale used [36], with 48% influence of factor B (fertilizers) (Table 4).

4. N-NO3 content (mg/kg) **in the meadow-chernozem soil as affected by the fertilizers and crops** (a 0-20 cm layer; for each variant, $n = 9$, M±SEM; Omsk ASC, Omsk, 2020-2022)

Crop (factor A)	$N_{60}P_{60}$ (factor B)	Initial level				Post-nitrification level			
		June	July	August	average	June	July	August	average
Perennial grasses	Control	12.0±5.6	2.3±1.72	0.6±0.4	5.0±3.5	54.0±13.9	30.1±1.8	19.1±4.6	34.4±10.3
	$N_{60}P_{60}$	27.4±9.9	10.0±5.8	4.0±3.5	13.8±7.0*	69.0±14.7	42.2±8.7	25.4±6.0	45.5±12.7
Sorghum-Sudangrass hybrid	Control	26.2±1.2	9.6±2.8	11.9±11.2	15.9±5.2	69.5±3.4	44.7±6.6	36.3±12.5	50.2±10.0
	$N_{60}P_{60}$	59.7±2.6*	37.2±20.4*	24.0±11.5*	40.3±10.4*	159.6*±31.9	71.0±16.2	53.4±10.0	94.7±32.9*
LSD_{05} A, B			8.0				27.3		
LSD_{05} AB			11.3				38.6		

Note. Perennial grasses are a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*. LSD_{05}A corresponds to the crop factor, LSD_{05}B to the fertilizer application, LSD_{05}AB to the interaction crop × fertilizers; n is the number of measurements.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

During the growing season, a decrease in the amount of nitrogen and nitrates in the soil occurred, mainly due to removal by crops. The use of mineral fertilizers in crops of perennial and annual grasses at a dose of $N_{60}P_{60}$ increased the content of the element on average during the growing season by two or more times vs. unfertilized sowings.

Soil microorganisms are also responsible for processes associated with the circulation of nitrogen, the most important nutrient for plant nutrition. Nitrification is one of the key microbiological processes affecting crop yields [59].

Quantitative changes in soil microbiota affected the mobilization of

nutrients. Differences in the abundance and activity of proteolytic and nitrifying microorganisms determine the ability to provide plants with nitrogen nutrition, which can be seen in the soil's ability to accumulate nitrogen under favorable conditions [60]. The accumulation of nitrogen and nitrates during soil composting in the experiment was highest in June-July. Under sowing of perennial grasses and sorghum upon fertilization, the N-NO_3 content increased by 130% with 37% influence of factor B (fertilizers). In August, this indicator decreased which was associated with a decrease in the amount of easily mobilized nitrogen-containing compounds in the soil towards the end of the plant growing season [61].

Based on the results of a laboratory assessmrnt of the nitrification activity of soils under favorable heat and moisture supply, the accumulation of nitrogen and nitrates in sorghum-Sudangrass hybrid crops was more intense compared to perennial grasses. On average, the nitrification capacity of soil over the years of research upon the use of fertilizers exceeded the control values under perennial grasses and sorghum-Sudangrass hybrid by 8 and 59%, respectively (at $p < 0.05$) due to increased mineralization. Polish scientists made similar conclusions. Their experiment was carried out at the University of Life Sciences in Lublin (Poland), and it was shown that nitrification was more intense when fertilizers were used [62].

During the growing season in July and August, the accumulation of nitrogen and nitrates slowed down which can be explained by the consumption of easily mobilized organic compounds in mineralization. During the years of research, the the sowing of sorghum crop stood out as having the greatest nitrification capacity (Fig. 2).

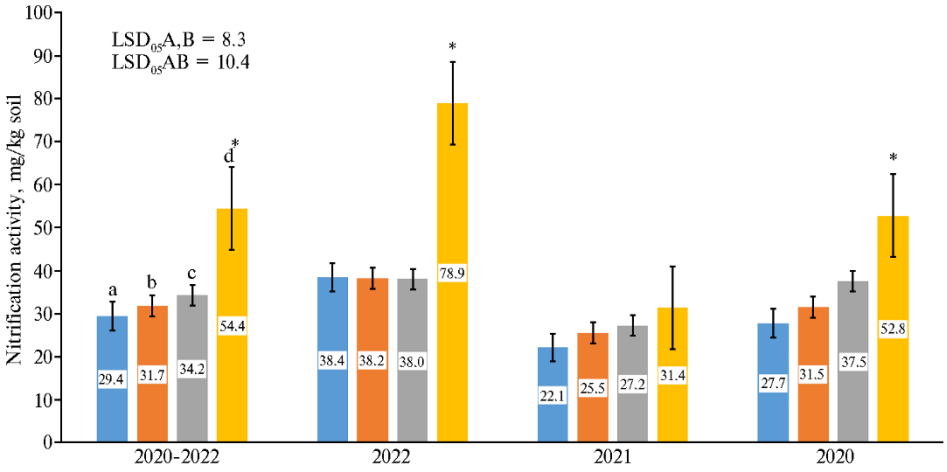

Fig. 2. Nitrification activity of the meadow-chernozem soil as affected by the fertilizers and crops: a — perennial grasses (control), b — perennial grasses ($N_{60}P_{60}$), c — Sorghum-Sudangrass hybrid (control), r — Sorghum-Sudangrass hybrid ($N_{60}P_{60}$) (for each variant, $n = 9$, $M \pm SEM$; Omsk ASC, Omsk, 2020-2022).

* Differences with the corresponding control are statistically significant at $p < 0.05$.

The prevailing weather conditions, moisture supply and mineral nutrition regimes during the years of observation had a positive effect on the productivity of agricultural crops. Perennial grasses were not mowed in the first year of life (in 2020). In subsequent years, the components of the grass mixture behaved differently. The cocksfoot grass component predominated in the grass mixture. V.I. Chernyavsky [4] noted that the loss of leguminous grasses occurs most quickly when grown with cocksfoot grass. In the future, the application of nitrogen fertilizers under the grass mixture can lead to the displacement of the legume crop and a significant reduction in its share in the botanical composition. Our studies

have established a strong negative correlation between the mass of orchard grass plants and legumes. The share of sainfoin in the second year of life was 41-50%, in the third up to 47%. The yield of cocksfoot grass mixed with sainfoin was 3.84-4.57 t/ha DM in the control and 4.82-4.89 t/ha when fertilizers were used (Table 5).

5. Crop yield (t/ha DM) **on the meadow-chernozem soil as affected by the fertilizers** (for each variant, $n = 9$, M±SEM; Omsk ASC, Omsk)

Crop (factor A)	$N_{60}P_{60}$ (factor B)	Year			
		2020	2021	2022	average
Perennial grasses	Control		4.57±0.04	3.84±0.19	4,20±0,18
	$N_{60}P_{60}$		4.82±0.10	4.89±0.38*	4,86±0,18*
Sorghum-Sudangrass hybrid	Control	6.55±0.80	2.96±0.28	3.10±0.49	4,21±0,66
	$N_{60}P_{60}$	7.24±0.86	6.67±0.32	3.64±0.34	5,85±0,62*
LSD_{05} A, B			0.52	0.85	0.59
LSD_{05} AB			0.74	1.19	0.84

N o t e. Perennial grasses are a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*. LSD_{05}A corresponds to the crop factor, LSD_{05}B to the fertilizer application, LSD_{05}AB to the interaction crop × fertilizers; n is the number of measurements.
* Differences with the corresponding control are statistically significant at $p < 0.05$.

The yield of sorghum-Sudangrass hybrid during the years of research varied from 2.96 to 6.65 t/ha DM in the control and from 3.64 to 7.24 t/ha DM when using nitrogen-phosphorus fertilizers, which is associated with better plant supply with elements of mineral nutrition. On average for 2020-2022, the use of fertilizer at a dose of $N_{60}P_{60}$ contributed to a significant increase in the yield of sorghum-Sudangrass hybrid by 1.65 t/ha DM, or by 39%, compared to the control. The results we obtained are generally consistent with the studies of African colleagues who noted an increase by 47-98% vs. the control in the yield of sorghum when using mineral fertilizers [63, 64].

The importance of microorganisms is demonstrated by their ability to improve soil composition, stimulate plant growth, and increase crop yields [65-68]. In our experiement, 2020 was generally dry. However, when during the critical period of plant development (the third decade of June) the precipitations was 78% higher than the long-term average, the number of soil microorganisms determined on agar media was the greatest compared to 2021 and 2022, and the yield of the sorghum-Sudangrass hybrid was 2.1-2.2 times higher than in 2021 and 2022.

6. Correlations (r) between the counts of ecological trophic microbial groups in the meadow-chernozem soil under crops (for each group, $n = 9$, M±SEM; Omsk ASC, Omsk, 2020-2022)

Microbial group	1	2	3	4	5	6	7
Proteolytics (1)		0.85	0.71	0.84	0.58	0.96	0.38
Amylolytics (2)	0.85		0.91	0.66	0.23	0.72	0.09
Oligonitrophils (3)	0.71	0.91		0.32	0.14	0.49	0.38
Phosphate mobilizing (4)	0.84	0.66	0.32		0.88	0.93	–0.65
Microfungi (5)	0.58	0.23	0.14	0.88		0.78	0.92
Nitrifiers (6)	0.96	0.72	0.49	0.93	0.78		–0.61
Cellulose decomposers (7)	0.38	0.09	0.38	–0.65	0.92	–0.61	

N o t e. For the crops of perennial grasses (a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*) and Sorghum-Sudangrass hybrid, n is the number of measurements; r values are statistically significant at $p < 0.05$.

We assessed both the relationship between the abundance of the studied groups of microorganisms and their relationship with the productivity of the crops. Examining patterns of interdependence between various ecological and trophic groups of soil microorganisms, we revealed a strong positive correlation between the number of saprotrophic bacteria isolated on meat peptone agar and amylolytics, oligonitrophilics, phosphate mobilizrs, and cellulose decomposers. This occurs since the main types of interaction between microorganisms in the biocenosis are reduced to trophic and metabolic connections via excretion of metabolic products, physiologically active substances, etc. (Table 6). Nitrifying bacteria occupy

their own ecological niche and are more dependent on environmental conditions.

The yield of forage crops had a strong correlation with the microbiota of the nitrogen cycle. These groups are amylolytic microorganisms that consume mineral forms of nitrogen (NH_3) and serve as an indicator of the intensity of mineralization processes in the soil, proteolytic microorganisms that assimilate organic nitrogen, and cellulose-degrading microorganisms acting as decomposers in the trophic chain of soil ecosystems (r 0.98 and –0.93; 0.83 and 0.94; 0.99 and 0.82, respectively, $p < 0.05$) (Table 7).

7. Crop yields correlations (r) with the counts of ecological trophic microbial groups, nitrification capacity and N-NO_3 accumulation (M±SEM) in the meadow-chernozem soil (for each group, n = 9,; Omsk ASC, Omsk, 2020-2022)

Parameter	Microbial counts, CFU/g	r	±Sr	t_o	t_t
Perennial grasses					
Proteolytic microbiota, $\times10^6$	39.0±4.9	0.83*	0.39	1.62	2.45
Amylolytic microbiota, $\times10^6$	24.2±6.3*	0.98*	0.10	9.85	2.45
Oligonitrophils, $\times10^6$	116.6±28.1	–0.16	0.49	–0.32	2.45
Phosphate mobilizing microbiota, $\times10^6$	181.9±102.1*	0.58	0.41	1.42	2.45
Microfungi, $\times10^3$	120.3±65.1*	0.38	0.46	0.82	2.45
Nitrifiers, $\times10^3$	1.25±0.06	0.23	0.49	0.47	2.45
Cellulose-destroying microbiota, $\times10^3$	111.0±9.6*	0.99*	0.07	14.04	2.45
Total counts, $\times10^6$	362.2±98.4*	0.53	0.42	1.25	2.45
Nitrification capacity, mg/kg dry soil	31.7±5.8	0.73	0.34	2.14	2.45
N-NO_3 conent, mg/kg dry soil	13.8±7.0*	0.99*	0.07	14.04	2.45
Sorghum-Sudangrass hybrid					
Proteolytic microbiota, $\times10^6$	39.1±2.6*	0.94*	0.17	5.51	2.45
Amylolytic microbiota, $\times10^6$	30.4±4.9*	–0.93*	0.50	–0.06	2.45
Oligonitrophils, $\times10^6$	186.6±64.8*	0.97*	0.12	7.98	2.45
Phosphate mobilizing microbiota, $\times10^6$	142.0±36.0	0.98*	0.10	9.85	2.45
Microfungi, $\times10^3$	64.0±15.9	0.58	0.41	1.42	2.45
Nitrifiers, $\times10^3$	1.47±0.25	0.10	0.50	0.20	2.45
Cellulose-destroying microbiota, $\times10^3$	97.0±5.5*	0.82*	0.35	2.08	2.45
Total counts, $\times10^6$	398.3±98.3*	0.98*	0.10	9.85	2.45
Nitrification capacity, mg/kg dry soil	54.4±22.8	–0.30	0.48	–0.63	2.45
N-NO_3 conent, mg/kg dry soil	40.3±10.4*	0.83*	0.25	3.33	2.45

N o t e. Perennial grasses are a cocksfoot grass *Dactylis glomerata* mix with sainfoin *Onobrychis arenaria*. Correlations were calculated for yield upon fertilizer application, 4.86 t/ha for perennial grasses and 5.85 t/ha for Sorghum-Sudangrass hybrid (with statistically significant difference vs. the corresponding control), n is the number of measurements.

* The r-values are statistically significant at $p < 0.05$.

For the abundance of phosphate-mobilizing microflora, medium correlation was found with the productivity of perennial grasses (r = 0.58) and strong correlation with the productivity of sorghum-Sudangrass hybrid (r = 0.98, $p < 0.05$) which is explained by the participation of this microbial group directly in providing plants phosphorus. The content of available soil nitrogen which characterizes the potential reserves of its organic form, turning into mineral compounds under favorable conditions, is important for the formation of productivity (r = 0.99 and r = 0.83, respectively; $p < 0.05$).

Thus, fertilizers at a dose of $N_{60}P_{60}$ applied to meadow-chernozemic soil under perennial grasses mostly stimulated reproduction of phosphate-mobilizing microorganisms and soil micromycetes, by 118 and 122% ($p < 0.05$). Under the sorghum-Sudangrass hybrid, the abundance of amylolytic and oligonitrophilic microbiota significantly increased, by 57 and 90% ($p < 0.05$). The total number of microorganisms increased equally under perennial grasses and under sorghum-Sudangrass hybrid, by 51-52% ($p < 0.05$) tthat enhanced mineralization of plant residues and had a positive effect on soil fertility. The use of mineral fertilizers reduced the activity of catalase in the soil by 14% ($p < 0.05$), but did not have a significant effect on the hydrolytic enzymes urease and invertase. Under the influence of fertilizers, the content of nitrate nitrogen during the growing season increased on average by 2 times or more. The high biological activity of the soil dus to addition of $N_{60}P_{60}$ positively influenced productivity of perennial grasses

and sorghum-Sudangrass hybrid, the yields were 4.82-4.89 and 3.64-7.24 t/ha DM, respectively, vs. 3.84-4.57 and 2.96-6.65 t/ha DM in control. We revealed a close correlation between the yield of crops and the abundance of soil amylolytic, proteolytic and cellulose-degrading microbiota, *r* values were 0.98 and -0.93, 0.83 and 0.94, 0.99 and 0.82, respectively ($p < 0.05$). Our results confirm the importance of mineral fertilizers in optimizing soil health and stimulating the growth of beneficial microorganisms, which ultimately slows down the decomposition of soil organic matter.

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