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

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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 eco-geographical 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], and the 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  $\text{Al}^{3+}$  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  $\text{Al}^{3+}$ -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.

$\text{Al}^{3+}$  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  $\text{Al}^{3+}$  on the TaALMT1 protein, the 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 susceptibility 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  $\mu$ l 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  $\mu$ l of a solution of tricosane in pyridine (retention index RI = 2288, concentration 1  $\mu$ g/ $\mu$ l) 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  $\mu$ m, 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  $\mu$ l, 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<sub>10</sub>-C<sub>40</sub>. 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 carried 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 ( $\pm$ 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* accessions from the VIR collection (Table 1):

**1. Winter-hardy *Triticum aestivum* L. accessions from the 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	Untitled	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 decrease in seedling density 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

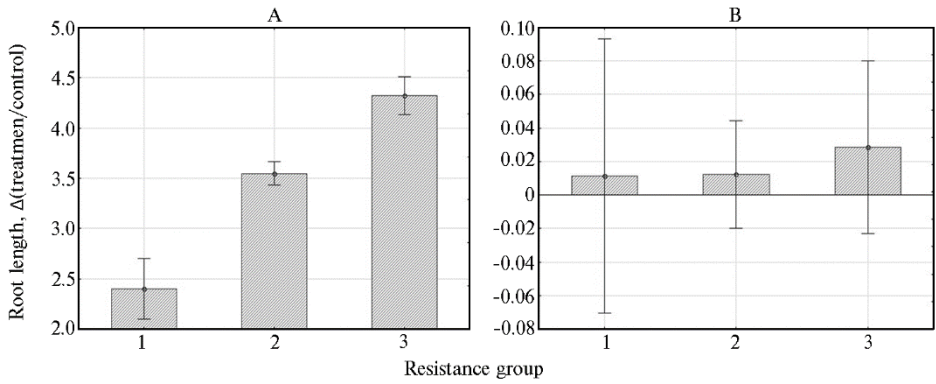
difference between root length in the test and the control (2.2-3.0 cm) and low susceptibility to  $Al^{3+}$ . In the second group, there were 13 samples with medium difference (3.1-3.9 cm) and medium susceptibility to  $Al^{3+}$ . In the third group, there were 5 samples with a maximum difference (4.0-4.5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

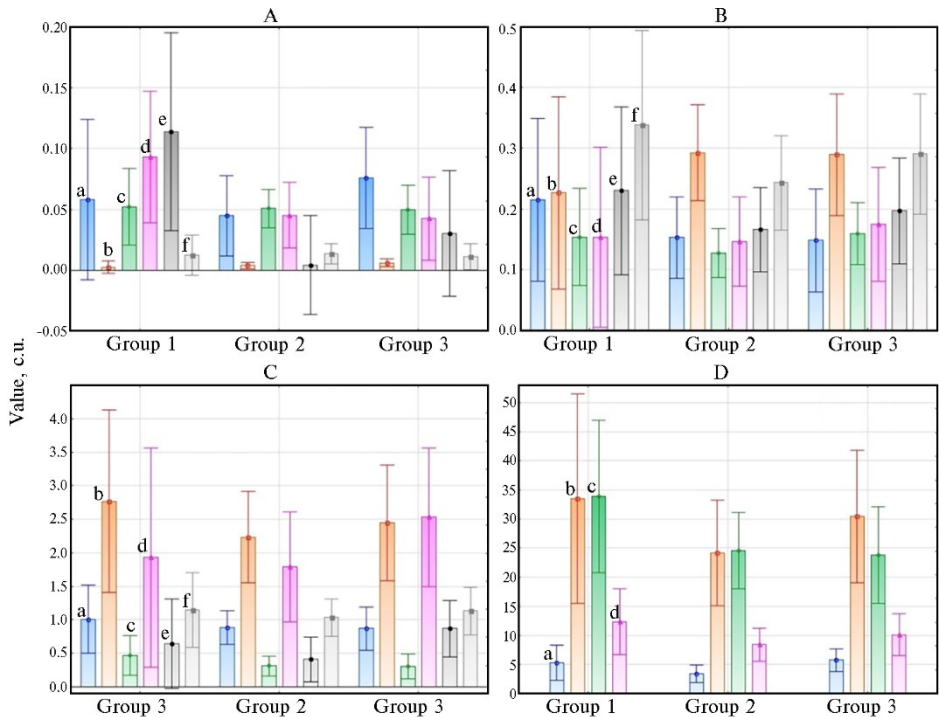
VIR Catalogue No. (k)	Group	n	Root length, cm; min-max, $M \pm SEM$			Root weight, g (treatment)
			control	treatment	$\Delta$	
57573	1	24	2.2-8.3 5.9±0.4	2.0-5.7 3.6±0.2	2.3	0.109
63523	1	27	2.4-7.0 5.3±0.5	1.2-4.4 2.8±0.3	2.5	0.133
29466	2	30	7.2-10.2 8.8±0.2	3.4-6.8 5.1±0.1	3.7	0.131
32715	2	30	6.7-10.5 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

Note. *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 susceptibility, group 3 — samples with high susceptibility to  $Al^{3+}$ . 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 treatment 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  $\text{Al}^{3+}$  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 accessions 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  $\text{Al}^{3+}$  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  $\text{Al}^{3+}$ , only arabic acid lactone (RI = 1657) was characteristic. It can be assumed that lactones of sugar acids are involved in plant protection from  $\text{Al}^{3+}$ , 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 concentrations [6, 8, 21-24].

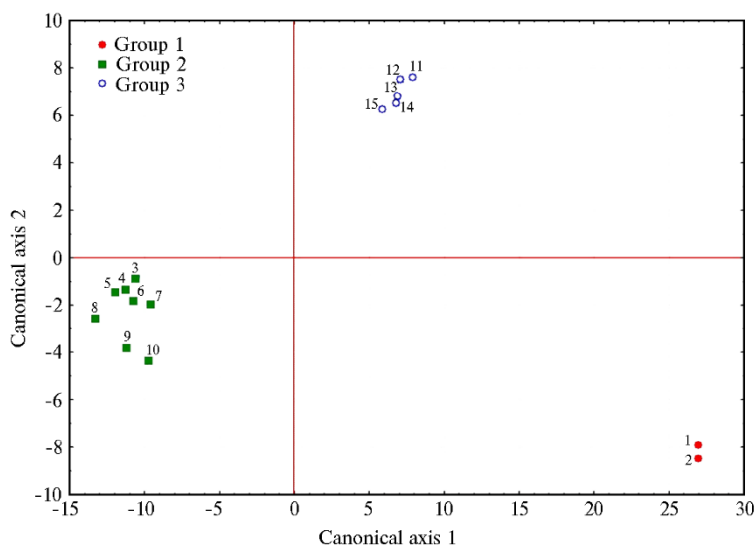
Dispersion analysis of all metabolites, except for unidentified ones, showed that in seedlings with more pronounced resistance to  $\text{Al}^{3+}$  (group 1), the MPs of roots significantly differs from the MPs of other groups in values for succinic, caprylic, stearic, oleic, linoleic acids, methyl 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 seedlings 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 ( $0.1 > p > 0.05$ ), it can be noted that MPs of accessions 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  $\text{Al}^{3+}$ . 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* accessions with medium susceptibility to  $\text{Al}^{3+}$  may be due to this defense mechanism. Oligosugars can be part of root mucilage which acts as a diffusion barrier, limiting the entry of  $\text{Al}^{3+}$  into plant root cells [6]. In addition, accumulation of oligosugars occurs 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 nitroalkenes that the precursors of which these compounds 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 accessions 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 susceptibility 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 parameters 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  $\text{Al}^{3+}$  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 parameters 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 susceptible ones, although the predominant secretion of this acid is a feature of the protective response of wheat to contact with  $\text{Al}^{3+}$  [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  $\text{Al}^{3+}$  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  $\text{Al}^{3+}$  [13, 15, 45]. According to published data [4, 15, 46], amino acids and phenolic compounds, like organic acids, are capable of chelating  $\text{Al}^{3+}$  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 susceptible forms. The enhanced accumulation of phosphoric acid and methyl phosphate in the roots of seedlings that we observed in accessions susceptible to  $\text{Al}^{3+}$ , 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  $\text{Al}^{3+}$ , as reported by M.W. Oh et al. [37]. The decrease in the amount of nucleosides we noted in forms susceptible to  $\text{Al}^{3+}$  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  $\text{Al}^{3+}$  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 considered 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<sup>3+</sup> [6, 48-50]. In the MPs of the Al<sup>3+</sup>-resistant accessions 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<sup>3+</sup>-resistant *T. aestivum* accessions 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 concentrations 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 Al<sup>3+</sup>, 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<sup>3+</sup>. 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<sup>3+</sup>-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<sup>3+</sup> 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*

forms 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 breeding resistant triticale varieties.

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