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SOMACLONAL VARIABILITY AND BARLEY BREEDING ON RESISTANCE TO ALUMINUM

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S u m m a r y

In barley callus culture on acid selective media with aluminum the authors selected the resistant lines, from which the regenerated plants were obtained. During a growing on acid sod-podzol soil the seed progeny of regenerated lines was compared with initial varieties on biochemical parameters, on determinants of productivity and yield. It was revealed, that hereditable in regenerated progeny the determinants of somaclonal variability can be used for creation of high productive and resistant to toxic action of aluminum forms of plants.

Keywords: barley, callus, regenerates lines, cell selection, aluminium stress, chlorophyll, damage of membranes, crop.

More than 50% agriculture lands in the world have acid soil (1), and very often negative effects of this factor on plants are enhanced by the presence of mobile ions of aluminum. According to a modern concept, cytotoxic action of aluminum is associated with oxidative stress in plant cells (2, 3). This assumption is supported by the report on activation of genes for peroxidase and glutathione-S-transferase in plants exposed to toxic concentrations of this element (4). Defense reactions are performed by the antioxidant system including both enzymes (superoxide dismutase, glutathione peroxidase, catalase) and low molecular weight compounds (e.g., carotenoids) that interact with free radicals, reduce reactive oxygen species (ROS) and products of oxidative metabolism (5). Anthocyanins - flavonoid plant pigments – have similar properties (6).

Under a long-term stress, antioxidant systems of plants can't longer stand against increasing amounts of ROS, which leads to serious violations of cellular metabolism - photooxidation of chlorophyll (2, 7), lipid peroxidation (8), leakage of ions, and protein degradation (9). Other mechanisms for countering aluminum-related acid stress, such as accumulation of antioxidants (10) or release of organic acids (11), are less efficient, therefore, creation of crop varieties resistant to this factor is an important task of selection. Realization of resistance mechanism on the cellular level (12, 13) is a prerequisite for using cultures of isolated cells and tissues to solve this task.

Cell selection allows relatively rapid and low-cost identification of resistant samples and significantly increased diversity of somaclonal changes arising in the cultured tissue during mutations, gene expression, somatic crossing-over, etc. (14).

The purpose of this work - a comparative study of cultivated barley varieties and seed progenies of lines-regenerants obtained on the aluminum-containing acidic medium, based upon the analysis of biochemical parameters reflecting the arise of oxidative stress, productivity properties and yield.

Technique. The calli were derived from the explants - immature embryos of barley (*Hordeum vulgare* L.) cultivars 999-93, Novichok, Kumir, 637-98, 530-98, Dobry, 889-93.

The embryos were isolated from caryopses on the 10^{th}-14^{th} day after flowering and placed on a modified MS-medium (15) containing sucrose (25 g/l), 2,4-dichlorophenoxyacetic acid (2,4-D, 2 mg/l) and agar (7 g/l) (pH 5.6-5.8). The explants were cultured in the light (4 klx) at 25 °C and photoperiod 16 h. The resulting calli were transplanted onto MS medium with reduced content of 2,4-D (1 mg/l) for cell proliferation. Duration of the first sub-culturing was 15-20 days, the second - 21 days. The morphogenic calli were transferred on MS medium containing kinetin (1 mg/l), β-indoleacetic acid and gibberellic acid (0,5 and 0,1 mg/l, resp.). During the periods of cell proliferation and morphogenesis, the media were added with aluminum sulfate (Al^{3+} concentration of 40 mg/l, pH 3.8-4.0), a dense consistency was formed using agar-gelatin mixture (6 g/l;12 g/l). At the final stage, regenerated plants were transplanted in vessels filled with a mixture of soil, sand and peat (volume ratio 1:1:1) and grown in a climatic chamber ("Nema Netzschkau", Germany) to obtain seed progenies.

Field experiments were conducted in 2008 in nurseries of preliminary and competitive variety trials (Science and Research Development Institute of North-Eastern Agriculture). Soil - sod-podzol containing 2,27% humus, P_2O_5 and K_2O (resp., 223 and 190 mg/kg), mobile aluminum – 4,72 mg/100 g, pH_{salt} 4.1. A common agrotechnics was performed. The plants-regenerants (generations R_3-R_5) derived from the initial varieties Novichok, 999-93 and 637-98 were grown. Seeding rate - 5 million/ha (standard quality seeds), test-site area - 10 m^2, 4-fold. Meteorological conditions of the year 2008 were favorable, close to a multiyear average. Field germination, timing of phenological phases and conservation of plants to a harvest were recorded. The yield structure was measured around 20 plants by plant height, total and productive tillering, ear length, number and weight of grains per ear, weight of 1000 grains.

Biochemical analysis was performed on joint samples of leaves collected from 20 plants from each test-site during different phenophases (tillering, heading and milky-wax seeds). LPO intensity was determined by a color reaction of thiobarbituric acid with the product of LPO malondialdehyde (MDA) (16). Exosmos of electrolytes into distilled water was evaluated on the conductometer INOLAB ("WTW", Germany) by the change in electrical conductivity of the extract during a 3-hour period, calculated as a percentage relative to the value of complete release of electrolytes from leaf cells after boil. The contents of photosynthetic pigments were determined on the spectrophotometer Specol ("Analytik Jena", Germany) in acetone extracts (17) at wavelengths (λ) of 662, 644 (chlorophylls) and 440,5 nm (carotenoids). Anthocyanins were extracted with 1% hydrochloric acid solution, contents of pigments in samples were measured at λ=510 and 657 nm as described (18).

The obtained data were calculated using standard statistical methods (19) in Microsoft Excel.

Results. The share of calli died on the selective aluminum-containing nutrient media ranged from 11,6 to 70,0% depending on sensitivity of the original genotype (Table 1).

1. Survival rates of calli derived from different barley cultivars cultured in vitro on acid selective medium containing aluminum

Cultivar	Planted, pcs.	Survived, pcs.		Died, %
		total	morphogenic	
999-93	40	26	5	34,7
Novichok	32	28	7	11,6
Kumir	24	8	0	66,7
637-98	54	18	6	67,2
530-98	111	92	2	17,4
Dobry	60	18	1	70,0
889-93	32	13	1	59,4

The greatest number of morphogenic callus lines resistant to the selective factor were obtained from the cultivars Novichok, 999-93 and 637-98. Some dense areas of the calli developed the leaf-like green structures (initials) and after 2-3 weeks there were formed regenerated plants which then produced a seed progeny.

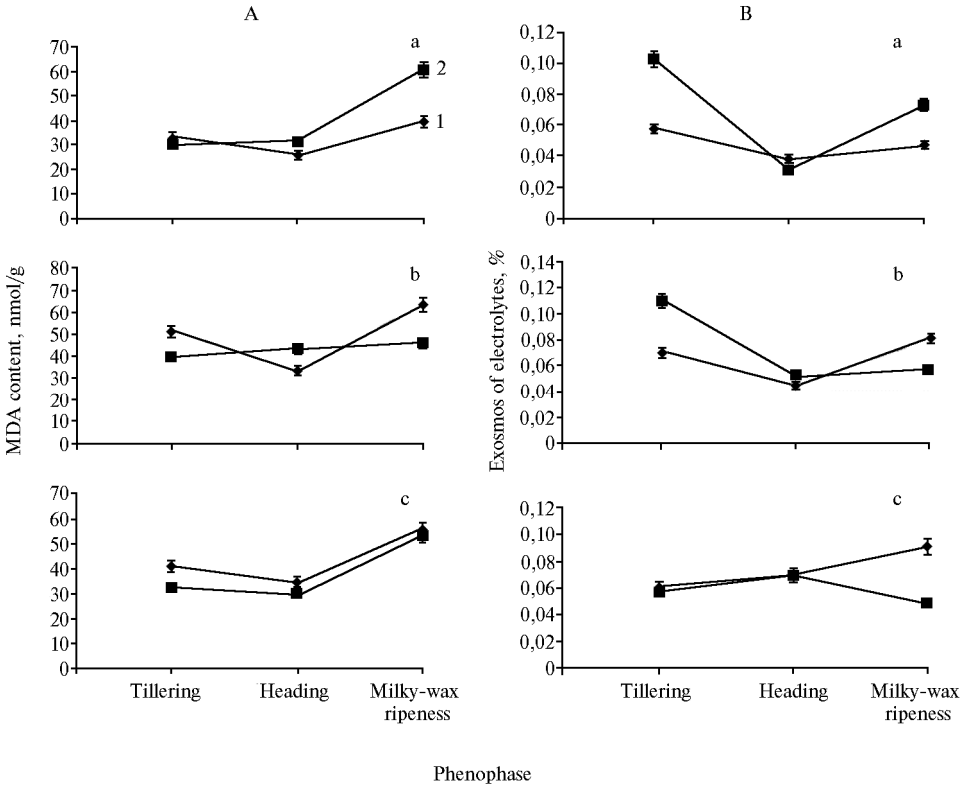

Fig. 1. Malonaldehyde (MDA) contents (A) **and exosmos of electrolytes** (B) **in leaves of barley grown on acid soil containing aluminum - initial cultivars** (1) **and derived lines-regenerants (R)** (2) **in different phenophases:** a, b, c – respectively, cultivars 999-93, Novichok and 637-98 (nurseries of preliminary and competitive variety trials, Science and Research Development Institute of North-Eastern Agriculture, Kirov, 2008).

When growing the seed progenies of lines-regenerants (R) on sod-podzolic acid soil, the line R 637-98 stably manifested LPO intensity lower than that of the initial cultivar through all growing season (Fig. 1, A, b). In leaves of R Novichok, MDA content exceeded that of the initial form only during the period of heading, in R 999-93 - in the interphase between heading and milky-wax seeds (Fig. 1 A, a).

Oxidative processes caused by the presence of aluminum in soil result in damage to cell membranes, some of which lose the barrier function along with reduce in contents of chlorophylls and carotenoids (10).

Exosmos of electrolytes in the line R 637-98 was close to that in the source cultivar in early ontogeny, and much lower - during milky-wax ripeness, which probably reflects a lower level of experienced stress (Fig. 1, B, b). R 999-93 manifested the highest value of released electrolytes during almost all vegetation season except the period of heading (Fig. 1B, a), which indicates the strongest suppression of the barrier function of membranes compared to the donor cultivar. In R Novichok, exosmos during the tillering period exceeded that of the parental form by more than 1,5 times and decreased in further development to the value inferior (0,058%) to the initial cultivar (0,080%).

The contents of photosynthetic pigments in leaves of lines-regenerants in early developmental periods were significantly higher than in corresponding initial varieties. The content of chlorophylls A,B in R Novichok during tillering was 8-10% higher than that in the original cultivar, and a similar trend was established in lines R 637-98 and R 999-93 in the phase of heading (Table 2). During the ripening of seeds, the advantage of this parameter reached 19% in R 637-98 and 39% - in R Novichok.

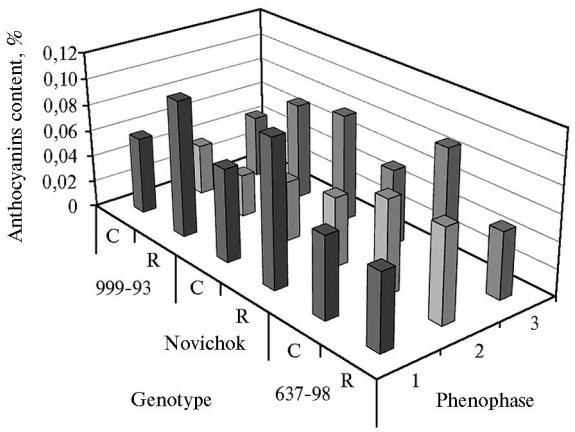

Fig. 2. Anthocyanins content in leaves of barley grown on acid soil containing aluminum - initial cultivars (C) **and derived lines-regenerants** (R) (2) **in different phenophases:** 1 – tillering, 2 – heading, 3 – milky-wax ripeness (nurseries of preliminary and competitive variety trials, Science and Research Development Institute of North-Eastern Agriculture, Kirov, 2008).

Significantly lower contents of photosynthetic pigments (including carotenoids) were observed in the line-regenerant R 999-93 (by 16-20% lower than in the donor cultivar) during tillering and milky-wax ripeness, i.e. in the periods of maximum accumulation of MDA in plant tissues and intense release of electrolytes. The relationship of these processes can be explained by the fact that the oxidative degradation of chlorophyll involves ROS which provoke LPO of membrane phospholipids resulting in MDA. Along with it, in the lines R 999-93 and R Novichok, the maximum accumulation of carotenoids and anthocyanins in leaves occurred simultaneously with periods of increased (compared to the initial cultivars) LPO intensity and exosmos of electrolytes. Thus, R Novichok and R 999-93 during tillering developed the contents of carotenoids exceeding those of the source cultivars by, respectively, 14% and 18% (Table 2). R Novichok differed from the initial variety by higher content of anthocyanins during tillering, R 999-93 - during tillering and seed ripening (Fig. 2). The line R 637-98 exhibited the absence of significant deviations in these parameters.

2. Contents of pigments (*M*±*m*) **in leaves of barley grown on acid soil containing aluminum – initial cultivars and derived lines-regenerants in different phenophases** (nurseries of preliminary and competitive variety trials, Science and Research Development Institute of North-Eastern Agriculture, Kirov, 2008).

Genotype	Content, mg/g			Ratio chlorophylls / carotenoids	Sum of chlorophylls A and B, mg/g
	chlorophyll A	chlorophyll B	carotenoids		
			Phenophase: tillering		
999-93	4,39±0,47	1,36±0,07	1,83±0,21	3,14	5,75
	3,52±0,44	1,07±0,13	1,54±0,74	2,98	4,59
Novichok	4,16±0,87	1,26±0,27	1,72±0,35	3,15	5,42
	4,59±0,27	1,37±0,26	1,97±0,36	3,03	5,96
637-98	4,65±0,23	1,44±0,12	1,98±0,11	3,08	6,09
	4,43±0,23	1,33±0,11	1,96±0,09	2,94	5,76
			Phenophase: heading		
999-93	5,15±0,81	1,62±0,27	1,74±0,27	3,89	6,77
	5,17±0,69	1,74±0,33	2,05±0,47	3,37	6,91
Novichok	4,32±0,16	1,35±0,11	1,68±0,12	3,38	5,67
	4,34±0,54	1,42±0,21	1,69±0,16	3,41	5,76
637-98	5,49±0,35	1,69±0,41	2,16±0,43	3,32	7,18
	5,95±0,47	1,82±0,16	2,22±0,10	3,50	7,77
			Phenophase: milky-wax ripeness		
999-93	5,50±0,42	1,90±0,07	2,15±0,16	3,43	7,41
	4,59±0,63	1,49±0,20	1,76±0,16	3,44	6,09
Novichok	2,98±0,36	1,01±0,12	1,35±0,07	3,43	4,00
	4,96±0,54	1,62±0,22	1,78±0,24	3,44	6,59
637-98	4,37±0,46	1,61±0,25	1,67±0,16	3,57	5,98
	5,51±0,28	1,86±0,09	1,99±0,24	3,71	7,37

Note. Above the line – value of the initial cultivar, below the line – value of the line-regenerant derived from this cultivar

3. Grain yield and productivity traits of barley grown on acid soil containing aluminum – initial cultivars and derived lines-regenerants (nurseries of preliminary and competitive variety trials, Science and Research Development Institute of North-Eastern Agriculture, Kirov, 2008).

Cultivar / line	Grain yield, g/m^2	Plant height, cm	Tillering, pcs.		Length of a major ear, cm	Number, pcs.		Weight, g	
			total	productive		spikelets in a major ear	grains in a major ear	grain in a major ear	1000 grains
999-93	320	61,3	3,4	3,0	5,9	19,3	18,6	0,8	40,0
R 999-93	370*	72,4*	3,8	3,3	6,1	20,9*	20,3*	0,9	46,8*
Novichok	300	70,4	2,3	2,0	6,5	22,6	21,4	1,0	43,2
R Novichok	210	66,2*	1,9*	1,7*	6,1	21,9	20,6	0,9	41,2
637-98	350	52,3	3,2	2,8	5,6	18,8	18,4	0,8	43,6
R 637-98	410*	53,2	3,8*	3,4*	6,1*	18,8	18,2	0,8	44,1

* Differences from the initial cultivar are reliable at P ≥ 0,95.

Thus, the minimum oxidative stress was found in plants R 637-98 having lower LPO intensity during the growing season (Fig. 1, A), low exosmos of electrolytes (Fig. 1 B), especially during seed maturation along with stable contents of chlorophylls (Table 2.) and anthocyanins (Fig. 2). The obtained results show a better redox balance in R 637-98, which, in turn, resulted in formation of higher yields than in the initial cultivar (increase in average length of the main ear, total and productive tillering) (Table 3).

The line R Novichock showed 30% lower productivity than the original cultivar. During the first half of growing season, this line showed more pronounced symptoms of the oxidative stress related to membrane disorders (POL, release of electrolytes) than cv Novichok (Fig. 1). The content of chlorophylls was similar in both forms (Table 2). The lower yield of R Novichok was most likely associated with morphometric changes of plants regenerated from a callus culture along with biochemical modifications. This line significantly differed from the original cultivar by plant height, total and productive tillering (Table. 3).

The line R 999-93 exceed the donor cultivar by grain yield. At the same time, this line showed the higher degree of oxidative stress (damage to membranes, destruction of chlorophylls in early ontogeny and in its final stage), which resulted in lower grain

weight per plant (1,4 ± 0,5 g vs. 1,9 ± 0,5 g in the initial variety) despite the increase in average number of spikelets and grains per main ear, weight of 1000 grains and plant height. R 999-93 produced better grain yield owing to good survival of plants under Al-stress, especially in early ontogeny. Germination (97,8%) and conservation of plants to harvest (336 pcs./m^2) in R 999-93 exceeded those of the initial variety (91,2% and 264 pcs./m^2), which resulted in greater density of tillers on a field and, therefore, better grain yield per unit area eventhough the decrease in productivity of individual plants.

Changes in biochemical parameters and productivity traits of seed progenies of regenerants indicate the genetic nature of these deviations. These changes can be provided by the somaclonal variation in a callus culture exposed the selective factor - toxic concentrations of aluminum ions. Previously, it has been confirmed the somaclonal variation of plant resistance to soil salinity (20) and osmotic stress (21).

Thus, seed generations R_3-R_5 of barley lines-regenerants obtained through the cell selection against the aluminum-containing acidic substrate were found to be distinct from the initial variety by the degree of cellular oxidative stress and productivity. In the authors' opinion, these modifications can be the result of somaclonal variation. Such induced variability was not always adaptable, as it can be seen in the derived regenerant lines that manifested both improved and worsened individual traits relative to the initial varieties. However, findings of this research confirm the feasibility of using cell technology in breeding programs aimed at increased resistance of barley to aluminum contamination of acidic soils.

REFERENCES

1. Barceló J. and Poschenrieder C., Fast Root Growth Responses Root Exudates and Internal Detoxification as Clues to the Mechanisms of Aluminum Toxicity and Resistance: a Review, *Environ. Exp. Bot.,* 2002, vol. 48, pp. 75-92.
2. Zhang H., Li Y.H., Hu L.Y., Wang S.H., Zhang F.Q. and Hu K.D., Effects of Exogenous Nitric Oxide Donor on Antioxidant Metabolism in Wheat Leaves under Aluminum Stress, *Fiziol. rast.,* 2008, vol. 55, no. 4, pp. 523-528.
3. Ezaki B., Gardner R.C., Ezaki Y. and Matsumoto H., Expression of Aluminum-Induced Genes in Transgenic Arabidopsis Plants Can Ameliorate Aluminum Stress and/or Oxidative Stress, *Plant Physiol.,* 2000, vol. 122, pp. 657-665.
4. Richards K.D., Schott E.J., Sharma Y.K., Davis K.R. and Gardner R.C., Aluminum Induces Oxidative Stress Genes in *Arabidopsis thaliana*, *Plant Physiol.,* 1998, vol. 116, pp. 409-418.
5. Kapitanov A.B. and Pimenov A.M., Carotenoids – Antioxidant Modulators of Cellular Metabolism, *Usp. sovr. biol.,* 1996, vol. 116, no. 2, pp. 179-190.
6. Zaprometov M.N., *Fenol'nye soedineniya: rasprostranenie, metabolism i funktsiya v rasteniyakh* (Phenolic Compounds: Distribution, Metabolism and Functions in Plants), Moscow, 1993.
7. Ohki K., Photosynthesis, Chlorophyll and Transpiration Responses in Aluminum Stressed Wheat and Sorghum, *Crop Sci.,* 1986, vol. 26, pp. 572-575.
8. Blokhina O., Virolainen E. and Fagerstedt K.V., Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review, *Annal. Bot.*, 2003, vol. 91, pp. 179-194.
9. Heath R.L., The Biochemistry of Ozone Attack on the Plasma Membrane of Plant Cells, *Rec. Adv. Phytochem.*, 1987, vol. 21, pp. 29-54.
10. Ezaki B., Tsugita S. and Matsumoto H., Expression of a Moderately Anionic Peroxidase is Induced by Aluminum Treatment in Tobacco Cells: Possible Involvement of Peroxidase Isozymes in Aluminum Ion Stress, *Physiol. Plant.,* 1996, vol. 96, pp. 21-28.
11. Klimashevsky Je.L., *Geneticheskii aspekt mineral'nogo pitaniya rastenii* (Genetic Aspect of Mineral Nutrition of Plants), Moscow, 1991.
12. Foy C.D., Chaney R.L. and Parberry D.G., Aluminium Toxicity for Plants, *Annu. Rev. Plant Physiol.*, 1978, vol. 29, pp. 511-566.
13. Taylor G.J., The Physiology of Aluminum Tolerance in Higher Plants, *Commun. Soil Sci. Plant Anal.*, 1998, vol. 15, pp. 1179-1194.
14. Larkin P.J. and Scowcroft W.R., Somaclonal Variation — a Novel Source of Variability from Cell Culture for Plant Improvement, *Theor. Appl. Genet.,* 1981, vol. 60, pp. 197-214.
15. Murachige T. and Skoog F., A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue, *Physiol. Plant.*, 1962, vol. 15, pp. 473-497.
16. Lukatkin A.S., *Kholodovoe povrezhdenie teplolubivykh rastenii i okislitel'nyi stress* (Cold Damage to Heat-Loving Plants and Oxidative Stress), Saransk, 2002.
17. Shlyk A.A., *Opredelenie khlorofillov i karotinoidov v jekstraktakh zelenykh list'ev. Biokhimicheskie metody v fiziologii rastenii* (Determination of Chlorophylls and Carotenoids in Green Leaf Extracts. Biochemical Methods in Plant Physiology), Moscow, 1971.
18. Murav'eva D.A., Bubenchikova V.N. and Belikov V.V., Spectrophotometrical Identification of Sum Anthocyanins in Blue Cornflower Inflorescences, *Farmatsiya,* 1987, vol. 36, pp. 28-29.
19. Lakin G.F., *Biometriya* (Biometry), Moscow, 1990.
20. Barakat M.N. and Adbel-Latif T.H., In Vitro Selection of Wheat Callus Tolerant to High Levels of Salt and Plant Regeneration, *Euphytica,* 1996, vol. 91, no. 2, pp. 127-140.
21. Tuchin S.V. and D'yachuk P.A., Creation of Drought-Resistant Wheat Forms by a Single-Stage Selection of Callus Cultures, *S.-kh. biol.,* 1994, no. 5, pp. 21-23.