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PROTECTIVE EFFECT OF HUMIC ACIDS AGAINST HEAVY METAL STRESS IN TRITICALE

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Abstract

The effects of copper and cadmium and the preparation Biomin (natural substance extracted from coal with a.i. humic acids) on the biometric parameters, some stress markers and enzymatic activities in leaves and roots of triticale plants were investigated. The functional carbon distribution in Biomin was analyzed by ^{13}C NMR. It was found that all treatments retarded the growth and fresh weight of the plants. Heavy metals enhanced the content of stress markers proline and malondialdehyde and the activities of guaiacol peroxidase, superoxide dismutase and glutathione-S-transferase. The opposite trend was observed after Biomin application. Catalase activity was not affected considerably by the treatments. The free thiol-containing compounds were increased only in the roots of cadmium-treated plants. Comparative analysis of the measured parameters suggested that Biomin possessed protective effect against heavy metal toxicity.

Key words: defense enzymes, heavy metal stress, humic acids, NMR, stress markers, triticale

Abbreviations: Bm – Biomin, CAT – catalase, CPMAS – cross-polarization magic angle spinning technique, GPOX – guaiacol peroxidase, GST – glutathione-S-transferase, HA – humic acids, HM – heavy metal, HS – humic substances, MDA – malondialdehyde, NMR – nuclear magnetic resonance, ROS – reactive oxygen species, SOD – superoxide dismutase, TOSS – total suppression of the spinning sideband

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Introduction. The excess of Cu and Cd in soil or nutrient solution is cytotoxic and causes retardation of plant growth, leaf chlorosis, induces oxidative stress via overproduction of reactive oxygen species, disturbs the normal physiological processes and even leads to plant death. To minimize the detrimental effects of HM and their accumulation, plants have evolved various detoxification mechanisms [1]. The endogenous plant defence systems could be strengthened by application of various chemicals (for example polyamines) that prevent HMs to damage plants [2]. Humic substances, including humic acids, are natural organic polyelectrolytes contained in the humus, which stabilize the soil organic matter [3]. Several authors [3–6] have reported the ability of HS to increase the growth of different plant species grown under diverse conditions. However, the exact mechanism responsible for this effect of HS is poorly understood. Some authors suggest that HS promote plant growth by improving the soil bioavailability of certain nutrients, mainly iron and zinc [3, 4], others propose that HS can directly influence the plant metabolism both by activating the root plasma membrane-ATPase activity and increasing the nitrate uptake rates in roots [7]. This could act as a signal for root-to-shoot distribution of certain plant growth regulators (polyamines) and phytohormones (cytokinins and abscisic acid). To our knowledge, limited information is available about the possibilities of HS to protect plants grown under unfavourable conditions [8, 9] – the authors report that HA could protect pea plants from the toxic action of high concentrations of Cu and Cd. The goal of our investigation was to examine the potential of natural substance extracted from coal (a.i. HA), namely Biomin, to protect triticale plants against copper and cadmium toxicity.

Materials and methods. ***Plant material, treatment and measurements.*** The hybrid seeds of triticale (× *Triticosecale* Wittm.) were produced by cross-breeding of wheat (*Triticum*) and rye (*Secale*) in the Institute of Plant Physiology and Genetics – Bulgarian Academy of Sciences. Plants were grown under controlled conditions with 16/8 h light/dark regime, 120 µmol m^{-2} s^{-1} photon flux density; 26/22 °C day/night temperature; 60% air humidity. The triticale seedlings were grown on half-strength Hoagland–Arnon nutrition medium. The Cu ($CuSO_4$ 15 mg/l), Cd ($CdSO_4$ 5 mg/l) and Bm (500 mg/l) were added to the nutrition medium when the plants were five-day-old and were re-supplied with each solution change. Samples for analysis were taken 13 days after the beginning of the treatment. Growth parameters (fresh weight and plant length) and biochemical analyses according to the appropriate methods were measured using shoots and roots of triticale plants. Free proline content was evaluated according to Bates et al. [10]. MDA content was determined according to Kramer et al. [11]. GPOX activity was determined by Dias and Costa [12]. CAT activity was measured by Aebi [13]. SOD activity was measured according to Beauchamp and Fridovich [14] with slight modification. GST activity was determined according to Gronwald et al. [15]. The content of free thiol groups was measured

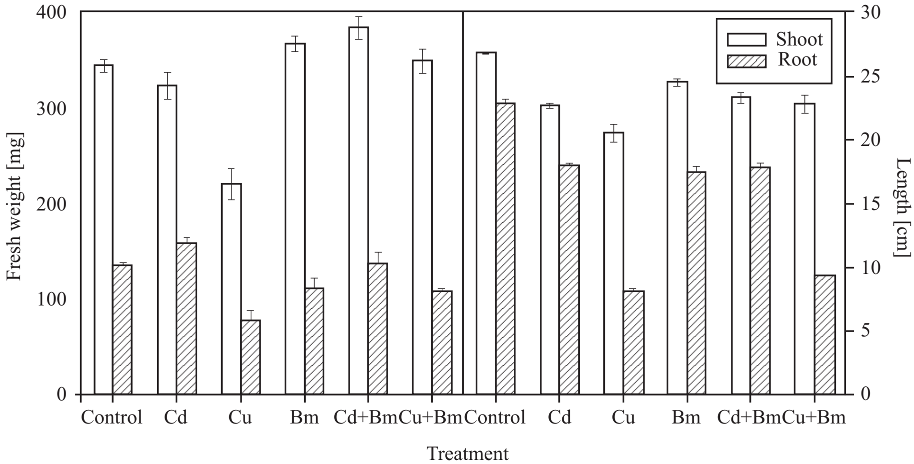

Fig. 1. Shoot and root fresh weight and length of triticale plants treated with Cu, Cd and Bm. Data are mean values ± SE

according to Ellman [16] with modification by Edreva and Hadjiyska [17]. Functional C distribution in Bm was analyzed using ^{13}C NMR technique. Spectra were obtained on an Avance 600 II+ Bruker spectrometer at 150.92 MHz using CPMAS–TOSS. Spinning speed of 6 kHz has been used with 3 µs 90° pulse width for carbon, 50 ms acquisition time, 2 ms contact time, 2 k scans and 5.0 s recycle delay.

Replication and statistics. All experiments were repeated three times with three to five replications. The results reported in the figures are means of the values with standard error (SE).

Results and discussion. Plant roots are the first point of contact with toxic HM concentrations in the nutrition medium. It was obvious that HM significantly decreased the length of both roots and shoots of triticale seedlings, which was accompanied by a reduction in fresh weight of Cu-treated plants (Fig. 1). Treatment with Bm also caused a slight decline of root and shoot length when applied alone. Only the shoot fresh weight of the plants treated with Bm was a little increased. The combination of Bm with each of the HMs overcame to some extent the negative effects of Cu and Cd on these parameters. It was expected that Bm application would improve substantially the fresh weight and

Table 1

Functional carbon distribution from ^{13}C CPMAS TOSS NMR spectra

HS	Alkyl C	O-Alkyl C	Aromatic C	Carboxylic C	Carbonylic C
Bm	49.3	13.4	31.4	4.6	1.3
HA (according to [6])	27.4	12.6	59.3	12.2	1.3

plant length but our data were in contrast with results reported by other authors [3–6] who showed that the root application of HS (particularly HA) caused a significant increase in shoot growth. However, it should be noted that the other studies were performed with purified HA [6]. Moreover, the ^{13}C NMR analyses of Bm (Table 1) revealed substantial structural differences to HA, described by the same method in the literature [6]. In Biomin formulation, the quantity of aliphatic carbons was almost double at the expense mainly of the aromatic carbons (approximately 2 times lower than the corresponding structures in HA) [6]. Additionally, the carboxylic content was 3 times less than in HA.

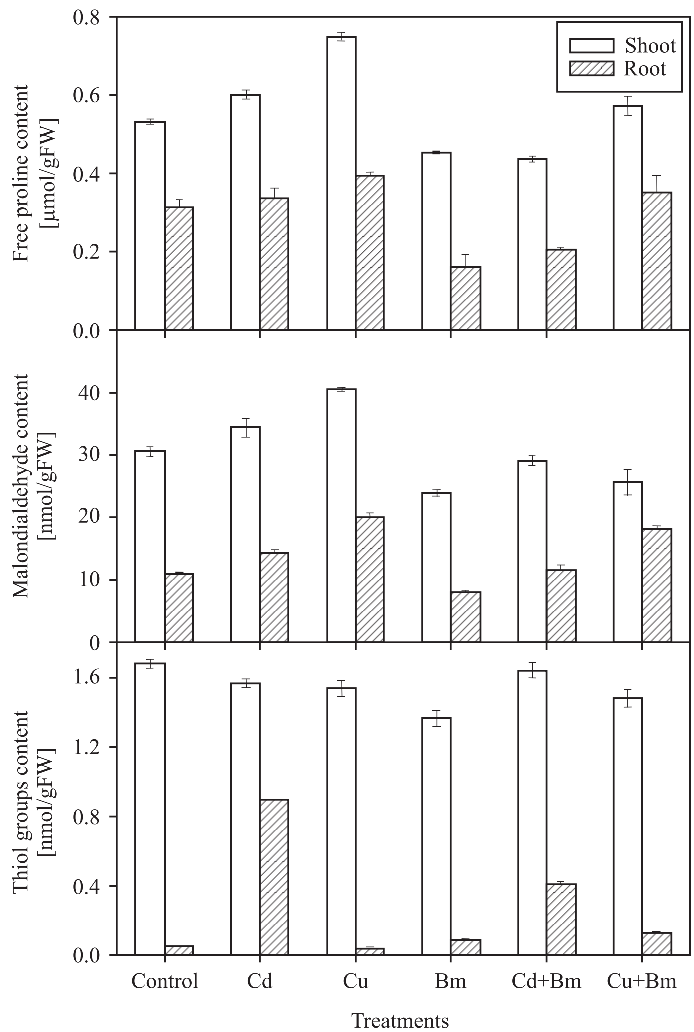

Fig. 2. Content of MDA, free proline and total free thiols in shoots and roots of triticale plants treated with Cu, Cd and Bm. Data are mean values ± SE

HM applications caused an increase in MDA and proline content (Fig. 2) measured in both organs. Bm treatment alone had an opposite effect on these stress markers. In plants treated with combination of HMs and Bm, this tendency of decreased amounts of stress biomarkers remained unchanged. Similarly to other investigations [2, 18, 19], MDA levels were increased more significantly by Cu than Cd application, most probably because Cu ions are a component of the Fenton-reaction, which produces the highly-toxic hydroxyl radicals. Likewise, free proline content was also increased in a comparable manner by Cu and Cd. Both parameters are usually used to demonstrate oxidative injuries in plants provoked by various stress factors [20]. Typically, increased quantity of MDA is associated with negative effects of ROS on biomembrane integrity, which result in peroxidation and fragmentation of unsaturated fatty acids [11]. The changes of MDA and proline demonstrate that oxidative stress occurred in variants treated with HMs only. The decreased MDA and proline amounts in plants treated with combination Bm + HM as compared to HM-treated plants might be evidence that the oxidative stress was not largely spread out and that Bm in some way could possibly protect triticale plants against HMs.

To cope with the negative effects of Cu and Cd, various enzymatic and nonenzymatic antioxidants are also mobilized to quench ROS. Accumulation of thiol-containing compounds is a universal stress response under HM stress [1]. The most obvious alteration of free thiol-containing compounds was provoked by treatment with Cd and it was root-specific (Fig. 2). Probably Cd provoked an increase in the glutathione content as the most abundant low-molecular thiol in plants. Furthermore, glutathione serves as a precursor for the biosynthesis of phytochelatins, which are HM-binding peptides predominantly synthesized inductively by exposure to Cd, but also by other HMs such as Hg, Cu, Zn, Pb and Ni [1]. Additional investigations concerning the phytochelatins and phytochelatin synthase could disclose the mechanisms of plant tissue-specific responses to HM toxicity.

In our study we also measured the enzymatic activities of CAT, GPOX, GST and SOD (Fig. 3). It was established that Cu and Cd enhanced the activities of GPOX, GST and SOD in both organs, while CAT activity was decreased after Cu treatment. Bm applied alone had no effect on CAT and GPOX, and slightly decreased SOD and GST activities. The simultaneous application of Bm and HMs altered the activity of the enzymes in an opposite manner as compared to the respective activities in the HM-treated plants. CAT is the key enzyme which eliminates H_2O_2. Increased CAT activity in HM-treated plants is an indication that plants maintain a high level of CAT activity in order to diminish the harmful accumulation of H_2O_2 and to cope with the oxidative stress induced by HMs [18]. However, we found that this enzyme was not influenced significantly as compared to the other three enzymes. On the other hand, the activity of GPOX was considerably increased by the HMs, probably because this enzyme is involved in the prevention of oxidative damage in plasma membranes acting as major hy-

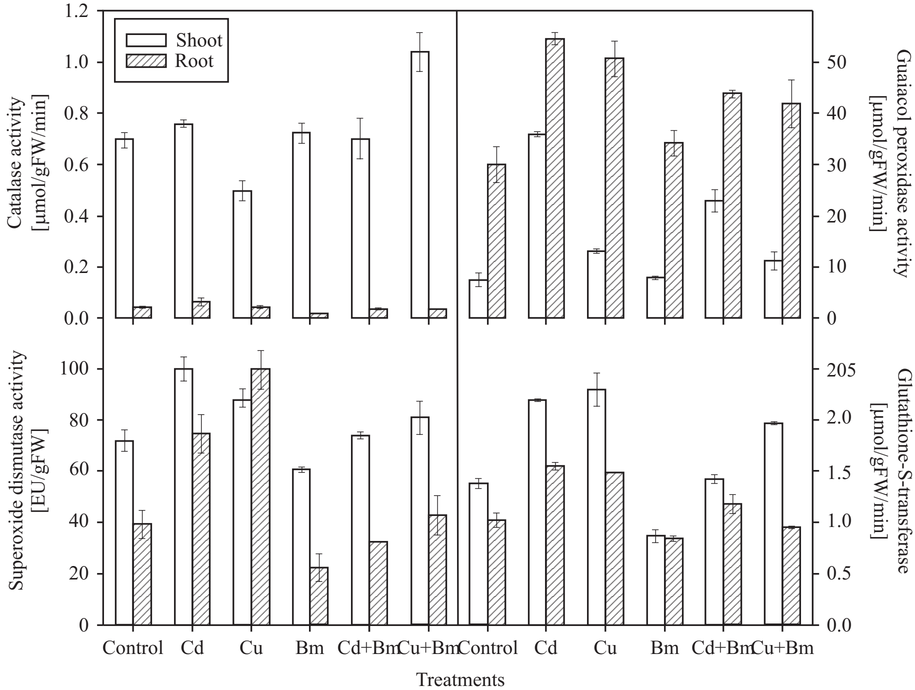

Fig. 3. Activities of CAT, GPOX, SOD and GST in shoots and roots of triticale plants treated with Cu, Cd and Bm. Data are mean values ± SE

drogen peroxide scavenger and is also proposed as "heavy-metal-stress related enzyme" [18, 21]. Similarly to other studies [2, 18, 19] SOD and GST activities were also increased by both HMs. SOD is a major enzyme that converts the highly toxic superoxide radical to hydrogen peroxide [18]. GST also plays an important protective function by catalyzing the glutathione-dependent isomerization and reduction of toxic organic hydroperoxides to the less-harmful monohydroxy alcohols [19]. In our experiment, these antioxidant enzymes were effectively activated in order to scavenge the damaging ROS generated by HMs. After the simultaneous application of Bm and HMs, the enzyme activities were less increased as compared to the HM-treated variants. It seems that Bm prevented the HM-induced oxidative stress to be fully evolved. Additionally, in our previous investigations on Cd and Cu root uptake in triticale (unpublished data) we have found that the presence of Bm in the medium causes a 3 and 1.5 times decrease of Cd and Cu uptake respectively. Most probably this effect could be due to the possibility for Bm to form chelating complexes with HMs in the nutrition medium or in the plant.

Crop plants exposed to stress need effective mechanisms of adaptation to survive, to continue to grow and to yield. Some substances with plant growth-regulating activity can improve this adaptation [2]. Here we found that Bm could protect to some extent triticale plants from HM stress. However, the exact mechanism by which Bm counteracts the HM toxicity is not known and additional investigations have to be made to elucidate if Bm acts at a physiological level or it is a chelating agent. Further research in this area will also be of benefit for exploiting the possibilities for growing triticale in regions contaminated with heavy metals as well as the application of environmentally friendly formulations like Biomin in the modern agricultural practices.

REFERENCES

[1] Yadav S. South Afr. J. Bot., **76**, 2010, No 2, 167–179.

[2] Groppa M., M. Tomaro, M. Benavides. BioMetals, **20**, 2007, No 2, 185–195.

[3] Chen Y., C. Clapp, H. Magen. Soil Sci. Plant Nutr., **50**, 2004, No 7, 1089–95.

[4] Chen Y., M. De Nobili, T. Aviad. In: Soil organic matter in sustainable agriculture (eds F. Magdoff, R. Weil), New York, CRC Press, 2004, 103–129.

[5] Chen Y., T. Aviad. In: Humic Substances in Soils and Crop Science: Selected Readings (eds P. MacCarthy, C. Clapp, R. Malcom, P. Bloom), Madison, Soil Science Society of America, 1990, 161–186.

[6] Mora V., E. Bacaicoa, A.-M. Zamarreno, E. Aguirre, M. Garnica, M. Fuentes, J.-M. Garcia-Mina. J. Plant Physiol., **167**, 2010, No 8, 633–642.

[7] Nardi S., D. Pizzeghello, A. Muscolo, A. Vianello. Soil Biol. Biochem., **34**, 2002, No 11, 1527–1536.

[8] Kamenova-Jouhimenko S., Y. Markovska, V. Georgieva. Biol. Plant., **40**, 1997, No 3, 405–410.

[9] Kamenova-Jouhimenko S., V. Georgieva, N. Georgieva, M. Boychinova, G. Popov. Plant Sci., **37**, 2000, No 6, 654–657 (in Bulgarian).

[10] Bates L. S., R. P. Waldren, I. D. Tearee. Plant Soil, **39**, 1973, No 1, 205–207.

[11] Kramer G., H. Norman, D. Krizek, R. Mirecki. Phytochemistry, **30**, 1991, No 7, 2101–2108.

[12] Dias A., M. Costa. J. Exp. Bot., **34**, 1983, No 5, 537–543.

[13] Aebi H. Methods Enzymol., **105**, 1984, 121–126.

[14] Beauchamp C., I. Fridovich. Anal. Biochem., **44**, 1971, No 1, 276–287.

[15] Gronwald J., P. Fuerst, C. Eberlein, M. Egli. Pestic. Biochem. Physiol., **29**, 1987, No 1, 66–76.

[16] Ellman G. Arch. Biochem. Biophys., **82**, 1959, No 1, 70–75.

[17] Edreva A., E. Hadjiyska. Physiol. of plants, **9**, 1984, No 3, 73–82 (in Bulgarian).

[18] Medouni C., A. Ben, G. Houlné., M.-E. Chabouté, F. Jemal. Plant Growth Regul., **57**, 2009, No 1, 89–99.

[19] Skórzyńska-Polit E., M. Drazkiewicz, Z. Krupa. Acta Physiol. Plant., **32**, 2010, No 1, 169–175.

[20] Todorova D., I. Moskova, I. Sergiev, V. Alexieva, S. Mapelli. In: Abiotic Stress and Plant Responses (eds N. Khan, S. Singh), New Delhi, I. K. International Publishing House, 2008, 205–215.

[21] Van Assche F., H. Clijsters. Plant Cell Environ., **13**, 1990, No 3, 195–206.

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