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Biochemical responses of triticale plants treated with UV-B irradiation and nutrient solution enriched with humic acids

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Abstract: A natural substance extracted from coal with humic acids as its active ingredients, namely Biomin, was added to nutrient medium and applied to triticale roots 3 days prior to UV-B irradiation treatment. UV-B treatment increased malondialdehyde and anthocyanin contents and the activities of peroxidase and superoxide dismutase, while it decreased chlorophyll content, fresh weight, and shoot length. Catalase and total phenolics content did not change in UV-B–treated shoots. The pretreatment with Biomin showed favorable effects on growth, decreased the oxidative damage provoked by UV-B irradiation, increased the content of UV-B–absorbing compounds, and positively influenced enzymatic activities. The application of Biomin on triticale plants was beneficial for counteracting the UV-B–induced oxidative stress by increasing the content of nonenzymatic antioxidants and antioxidant enzyme activities involved in detoxification of reactive oxygen species.

Key words: Defense enzymes, humic acids, Biomin, triticale, UV-B stress

1. Introduction

As a result of stratospheric ozone depletion, the amount of solar ultraviolet-B (UV-B) radiation reaching Earth has been increasing. Negative effects of increased UV-B irradiation on plant ecosystems are well known. UV-B modifies plant morphology, reduces growth, alters biosynthesis of secondary metabolites, induces oxidative stress via overproduction of reactive oxygen species (ROS), disturbs the normal physiological processes, and even leads to plant death (Frohnmeyer and Staiger, 2003; Bassman, 2004; Edreva, 2005). To minimize the detrimental effects of UV-B radiation, plants have evolved various detoxification mechanisms, such as enhancement of the antioxidant system (Brosché and Strid, 2003), induction of photolyases, and accumulation of UV-absorbing compounds (Frohnmeyer and Staiger, 2003; Fedina et al., 2007).

When the strength of the stressor pressure does not exceed the endogenous defense capacity, plants are able to overcome negative stress effects. The effectiveness of the antioxidant defense systems could be enhanced by application of compounds possessing different chemical natures or physiological modes of action. Applied in low doses, these substances could activate cell metabolism, improve plant physiological processes, and increase plant resistance to various unfavorable stress factors (Park et al., 2006; Todorova et al., 2008; Habibi, 2012; Kabiri et al., 2012; Pandey et al., 2012; Todorova et al., 2012).

Humic substances (HSs), including humic acids (HAs), are natural organic polyelectrolytes in the soil humus that stabilize the soil organic matter (Chen et al., 2004a). Several authors (Chen and Aviad, 1990; Chen et al., 2004a, 2004b; Mora et al., 2010) have reported the ability of HSs to increase the growth of different plant species grown under adverse conditions. However, the exact mechanism responsible for this effect of HS is poorly understood. Some authors suggested that HSs promote plant growth by improving the bioavailability of certain nutrients, mainly iron and zinc (Chen et al., 2004a, 2004b). Others proposed that HSs can directly influence the plant metabolism by both activating the root plasma membrane ATPase activity and increasing the nitrate uptake rates in roots (Nardi et al., 2002). This could act as a signal for root-to-shoot distribution of certain plant growth regulators (polyamines) and phytohormones (cytokinins and abscisic acid) (Mora et al., 2010). To our knowledge, limited information is available about the possibilities of HSs to protect plants grown under unfavorable conditions

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(Kamenova-Jouhimenko et al., 1997, 2003; Sergiev et al., 2013; Zhang et al., 2013). The authors reported that HAs could protect pea and triticale plants from the toxic action of high concentrations of Cu and Cd as well as *Malus robusta* seedlings from drought stress. In the light of the positive action of HS on plants, the goal of the present investigation was to examine the effect of a natural substance HA extracted from coal, namely Biomin, on triticale plants and its potential beneficial effect in preventing damage caused by UV-B irradiation.

2. Materials and methods

2.1. Plant material and treatments

The triticale seeds (× *Triticosecale* Wittm.) were produced by cross-breeding wheat (*Triticum*) and rye (*Secale*) in the Institute of Plant Physiology and Genetics of the Bulgarian Academy of Sciences. Plants were grown as a water culture on half-strength Hoagland-Arnon nutrient medium in a growth chamber with a 16/8-h light/dark regime, 120 mmol m^{-2} s^{-1} photon flux density, 26/22 °C day/night temperature, and 60% air humidity. Seven-day-old triticale seedlings were treated with UV-B (Philips TL 20W/12 RSSLV/25, λ_{max} 312 nm) for 4 days at 7.7 kJ m^{-2} day^{-1} in the middle of the light period. A natural substance extracted from coal with HA as the active ingredient, namely Biomin, was added to the nutrient medium at a concentration of 500 mg L^{-1} 3 days prior to UV-B irradiation and it was resupplied with nutrient solution changes. Plant material was collected from 12-day-old seedlings 1 day after the end of the UV-B treatment.

2.2. Biometrical and biochemical analyses

Growth parameter (fresh weight and plant length) measurements and biochemical analyses were performed according to the appropriate methods. The measurements were carried out on shoots and roots of triticale plants. The frozen and lyophilized plant material was used to determine concentration of pigments. The amount of photosynthetic leaf pigments was extracted with 90% acetone and determined according to Arnon (1949). Anthocyanins were extracted with acetone and 2 N HCl (90:10) and measured spectrophotometrically according to Lindoo and Caldwell (1978).

Fresh material was homogenized with 0.1 % (w/v) trichloroacetic acid for soluble phenol and malondialdehyde (MDA) determinations. The amount of total soluble phenols was measured by the method of Simonovska et al. (2003); caffeic acid was used as the reference standard. MDA content was estimated as a parameter reflecting biomembrane integrity deterioration. It was determined as the thiobarbituric acid product according to Kramer et al. (1991) by using a extinction coefficient of 155 mM^{-1} cm^{-1}.

For the assay of antioxidant enzymes, fresh plant material was homogenized in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (w/v). The homogenates were centrifuged at 12,000 × *g* for 15 min. The enzyme activities were determined according to previously described methods as follows: catalase (CAT; EC 1.11.1.6), Aebi (1984); guaiacol peroxidase (POX; EC 1.11.1.7), Dias and Kosta (1983); superoxide dismutase (SOD; EC 1.15.1.1), Beauchamp and Fridovich (1971).

CAT activity was measured by following the decomposition of hydrogen peroxide and was determined by monitoring its decrease in absorbance at 240 nm (ε = 36.8 mM^{-1} cm^{-1}) for 30 s.

POX activity was measured using guaiacol as a substrate and the increase in absorbance at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) due to the guaiacol oxidation was recorded for 3 min.

Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

2.3. Statistics

All experiments were repeated 3 times with 3 replicates each. The data reported are mean values ± standard errors (SEs). The significances of differences were examined by one-way ANOVA. Treatment means were compared with their Fisher's least significance difference (LSD), $\alpha \leq 0.05$.

3. Results

The Biomin application positively influenced fresh weight of triticale shoots and roots (18% and 19%, respectively, as compared to the respective control) but did not have a significant effect on plant length (Figure 1). It was observed that UV-B treatment reduced the length (by 17% in comparison to the control) and the fresh weight (by 25%) of triticale shoots (Figure 1). In the Biomin-pretreated plants, the negative effects of UV-B radiation on the measured growth parameters of shoots were reduced.

The UV-B irradiation drastically affected levels of chlorophyll a and b, which decreased by 42% and 37%, respectively (Figure 2A). Biomin applied alone did not influence the amount of chlorophyll considerably. The UV-B treatment caused a substantial increase of anthocyanin content in shoots (129% as compared to the control; Figure 2B), while in roots a decrease of 31% was observed. Applied alone, Biomin did not considerably influence anthocyanin concentrations measured in both organs, but in combination with UV-B there was an enhancement of 156% in shoots.

Total soluble phenolic content (Figure 3A) was slightly affected in roots only: UV-B led to a decrease of 12%, while

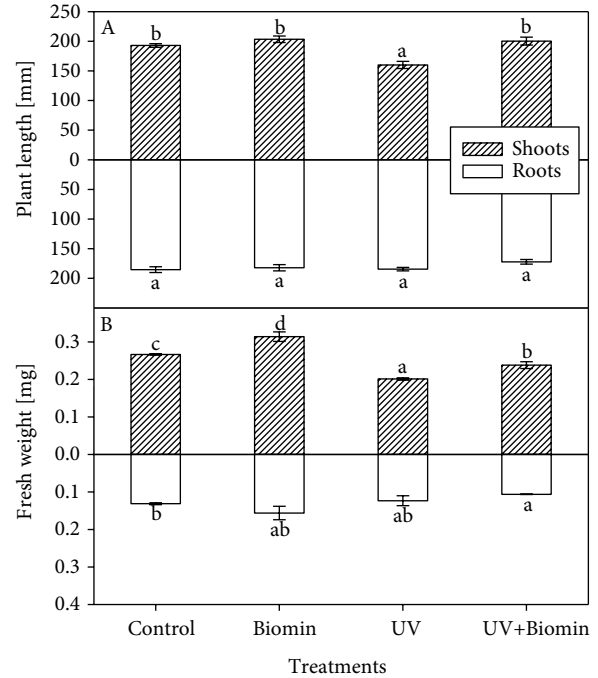

Figure 1. Length (A) and fresh weight (B) of triticale plants subjected to treatment with 500 mg/L Biomin, UV-B (7.7 kJ m^{-2} day^{-1}), or both combined (UV-B+Biomin). Data are mean values ± SEs. The means of parameters denoted with different letters are significantly different ($\alpha \leq 0.05$) for either roots or shoots.

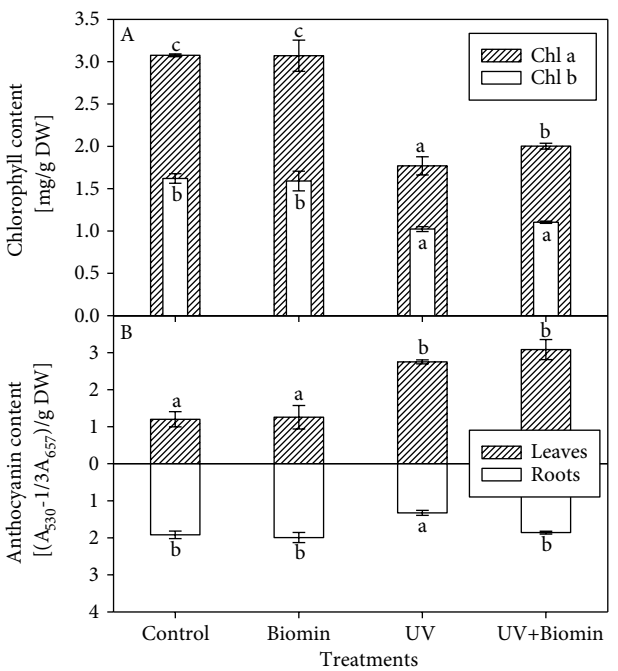

Figure 2. Content of chlorophyll (A) and anthocyanins (B) in triticale plants subjected to treatment with 500 mg/L Biomin, UV-B (7.7 kJ m^{-2} day^{-1}), or both combined (UV-B+Biomin). Data are mean values ± SEs. The means of parameters denoted with different letters are significantly different ($\alpha \leq 0.05$).

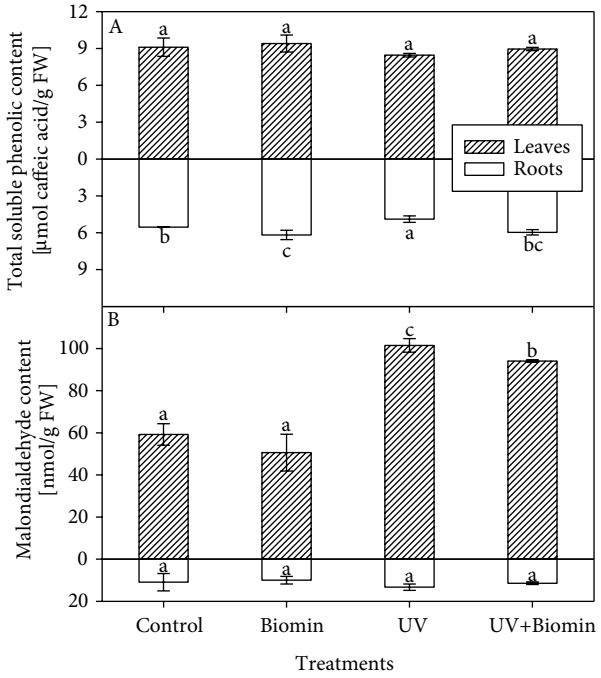

Figure 3. Total soluble phenolics (A) and malondialdehyde (B) in triticale plants subjected to treatment with 500 mg/L Biomin, UV-B (7.7 kJ m^{-2} day^{-1}), or both combined (UV-B+Biomin). Data are mean values ± SEs. The means of parameters denoted with different letters are significantly different ($\alpha \leq 0.05$) for either roots or leaves.

HA caused an increase (11% as compared to control). In plants subjected to the combined treatment, the value of this parameter was comparable to the control level.

MDA content was not significantly influenced in roots by any treatments (Figure 3B). On the contrary, in shoots UV-B caused a considerable rise of MDA, with 71% augmentation as compared to the control. The combined treatment, Biomin + UV-B, led to lower MDA content (by 12%) as compared to treatment by UV-B alone.

SOD activity inhibition was observed in roots of plants treated with Biomin and with Biomin + UV-B (18% and 28%, respectively), while in shoots the enzymatic activity was enhanced (21% and 18% for UV-B and Biomin + UV-B) (Figure 4A). In addition, the SOD activity in roots treated with Biomin + UV-B was 23% lower as compared to plants treated by UV-B alone.

The application of Biomin did not change significantly POX activity (Figure 4B) in either studied part of triticale plants. The UV-B treatment increased POX activity in roots (33%) and a huge enhancement of up to 12-fold as compared to the control was observed in shoots. In the combined treatment, the presence of Biomin reduced to some extent the POX activity, which was amplified in shoots by UV-B irradiation (Figure 4B).

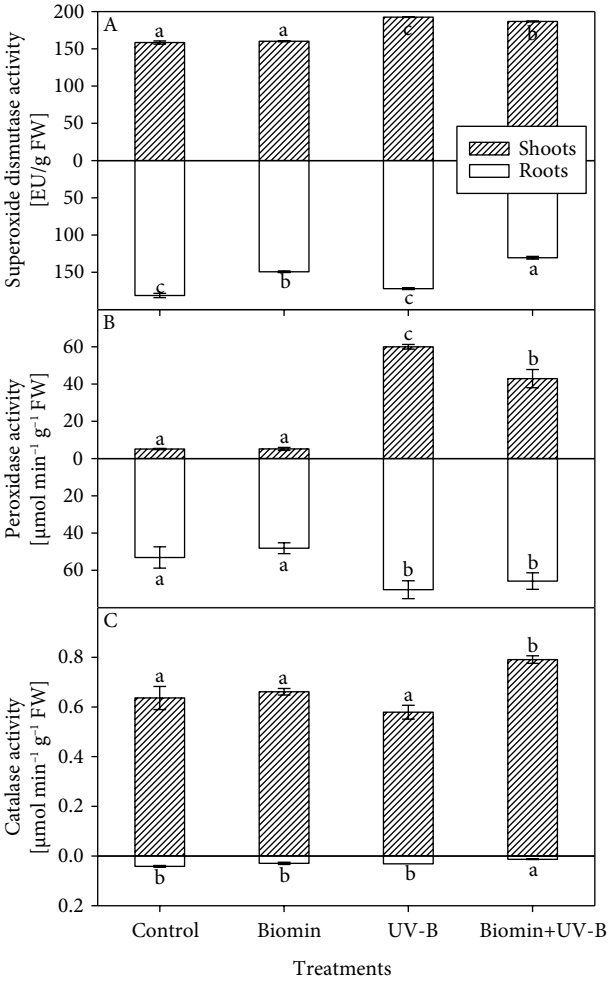

Figure 4. Activities of superoxide dismutase (A), guaiacol peroxidase (B), and catalase (C) in triticale plants subjected to treatment with 500 mg/L Biomin, UV-B (7.7 kJ m^{-2} day^{-1}), or both combined (UV-B+Biomin). The means of parameters denoted with different letters are significantly different ($\alpha \leq 0.05$) for either roots or shoots.

The CAT activity (Figure 4C) was not significantly influenced by the application of Biomin or UV-B in either shoots or roots of triticale. However, the enzymatic activity, which was increased in shoots (24%), was equally reduced in roots by the combined treatment (Figure 4C).

4. Discussion

The application of Biomin did not show significant effects on the length of triticale plants; only an increase in root and shoot fresh weights occurred (Figure 1). Similar increases of plant growth caused by the application of HA were reported (Nardi et al., 2002; Mora et al., 2010). On the other hand, UV-B irradiation negatively affected the experimental plants. Fresh weight and length of shoots (Figure 1) and photosynthetic pigments (Figure 2A) were significantly decreased by the UV-B treatment. This was expected since similar effects were established previously in pea, wheat, bean, and Indian mustard plants (Alexieva et al., 2001; Singh et al., 2011; Li et al., 2012; Pandey et al., 2012). Additionally, when Biomin was added to the nutrient medium, the harmful effects of UV-B decreased.

In general, the observed changes were more pronounced in shoots than in roots. The deviations in the parameters measured in roots could be explained by the direct influence of HA on them rather than by the UV-B treatment of the aboveground plant parts. However, the lower fresh weight of roots in plants subjected to combined treatment was likely an indicator of interaction between UV-B irradiation and Biomin application. The higher fresh weight, length, and chlorophyll content in the shoots exposed to the combined treatment as compared with UV-B stress only could be interpreted as an indicator of better plant fitness due to HA treatment.

Accumulation of anthocyanins and other UV-absorbing compounds after UV irradiation has been reported (Alexieva et al., 2001; Steyn et al., 2002; Guo et al., 2008). These compounds may act in the leaf as screens by absorbing UV before it reaches UV-sensitive targets such as chloroplasts, other organelles, and macromolecules. An increase of anthocyanins was also observed in UV-B–treated shoots (Figure 2B). However, the increase of total soluble phenolics by UV-B was not detected (Figure 3A). Anthocyanins have a lower UV absorbance than colorless flavonoids and simpler phenolics (Landry et al., 1995; Steyn et al., 2002) and contribute only a little to UV-B absorbance (Woodall and Steward, 1998). The increase of only anthocyanins and no alteration of phenolics in our model system seemed to be insufficient to mitigate the UV-induced damage as evidenced by chlorophyll and biomass loss. This is in agreement with the results stated by Hatier and Gould (2009) that anthocyanins can enhance the antioxidant capacity but cannot substitute the major antioxidant pool in plants. The combined treatment (Biomin + UV-B) did not have a negative effect on anthocyanin content in roots, despite it being additionally enhanced in the shoots. The preliminary application of HA showed some beneficial effects on plants subsequently subjected to UV-B stress. The enhanced synthesis of UV-B–screening compounds in plants treated with UV-B + Biomin resulted in a better ability to cope with UV-B stress, evidenced by lesser damage of chlorophyll and also lesser reduction of fresh weight and length as compared to UV-B–irradiated plants.

Abiotic stresses, including UV irradiation, lead to general disturbance of plant metabolism, which causes an increase of ROS production. Typically, the increased quantity of MDA is associated with the negative effects

of ROS on biomembrane integrity, which result in peroxidation and fragmentation of unsaturated fatty acids (Kramer et al., 1991). Our data showed that ROS caused oxidative stress and membrane damage since MDA (estimated as an oxidative stress marker) was considerably enhanced in UV-B–treated shoots. However, preliminary application of HA led to a decline of MDA content, suggesting that UV-B–induced damage was partly alleviated.

Under normal growth conditions, the formation and the removal of ROS are in a delicate balance. This equilibrium is impaired when stress conditions occur (Gill and Tuteja, 2010). Furthermore, plant resistance to stress factors has been associated with their antioxidant capacity, and increased levels of antioxidant constituents may prevent stress damage. Some of the most important enzymatic antioxidants triggered in response to ROS generation are SOD, CAT, and POX. SOD is a metal-containing enzyme that acts as the first line of defense against ROS, catalyzing the dismutation of superoxide to H_2O_2. Subsequently, CAT and POX detoxify H_2O_2 (Foyer and Noctor, 2005; Gill and Tuteja, 2010). CAT is a tetrameric heme-containing enzyme that directly scavenges hydrogen peroxide (Gill and Tuteja, 2010). The Biomin application caused some changes in the antioxidative capacity of plant: significant reduction of SOD in roots (Figure 4A) and a lower reduction of MDA and POX (Figures 3B and 4B). Few data were reported about HAs and ROS in plants. Bowden et al. (2010), comparing corn and soybean, concluded that HAs had effects specific for plant species.

In UV-B–treated pea shoots, the superoxide anion radical was demonstrated to be the dominant ROS, while singlet oxygen was minor (Hideg et al., 2002). Therefore, the increased SOD activity in irradiated triticale shoots (Figure 4A) indicated that this enzyme system was switched on to detoxify the superoxide anion induced by UV-B irradiation. Peroxidases use a wide range of substrates (i.e. phenolics) to scavenge hydrogen peroxide and were noted to be important enzymes in UV-B stress reactions and tolerance (Jansen, 2002). Besides hydrogen peroxide detoxification, under UV-B radiation POX serves various physiological functions in plants, including lignin biosynthesis and cell wall linkage (Jansen, 2002; Marjamaa et al., 2009). The extremely increased POX activity in UV-B–irradiated triticale (Figure 4B) suggested that peroxidase was the main enzyme in detoxifying H_2O_2, since CAT activity was not changed by UV-B treatment (Figure 4C). Additionally, since the POX activity in UV-irradiated seedlings was tremendously enhanced, it is possible that lignification processes were stimulated, also. Similar data were obtained by other authors, who showed that silicon can increase plant defense systems of soybean against UV-B stress by reduction of SOD and POX activity in silicon-treated UV-B–stressed seedlings as compared to only UV-B–treated seedlings (Shen et al., 2010). On the other hand, while the combined treatment reduced SOD and POX activities in triticale, it increased the CAT in shoots as compared to irradiated plants (Figure 4C). These results indicate that a compensation mechanism for attenuating ROS (and H_2O_2 in particular) was probably activated by application of HS prior to plant irradiation.

The data from the combined treatment showed that Biomin can protect triticale plants against UV-B irradiation, as Biomin treatment positively influenced the growth of irradiated plants, lessened chlorophyll loss and membrane damages, and increased the content of UV-B–absorbing compounds and the activities of antioxidant enzymes. Our data show that the application of Biomin might render beneficial and protective effects on triticale seedlings exposed to UV-B stress through a coordinated action of nonenzymatic antioxidants and ROS detoxification enzymes.

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