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EFFECTS OF BRASSINOSTEROID ON THE INDUCTION OF PHYSIOLOGICAL CHANGES IN *HELIANTHUS ANNUUS* L. UNDER COPPER STRESS

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Abstract

FILOVÁ ANGELIKA, SYTAR OKSANA, KRIVOSUDSKÁ ELEONÓRA: *Effects of brassinosteroid on the induction of physiological changes in Helianthus annuus L. under copper stress.* Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2013, LXI, No. 3, pp. 623–629

The purpose of our experiments was to consider sensitivity of the chosen 6 sunflower cultivars (*Helianthus annuus* L. cv. Belinda, cv. Codiwer, cv. ESPrim, cv. MAS 95, cv. MAS 97 and cv. Spirov) to copper ions with the possibility of elimination by means of brassinosteroids (BRs) on the ground of physiological characteristics (content of dry weight, amount of chlorophylls, proline content, Relative Water Content – RWC as well as malondialdehyde - MDA) and to show possible resistance mechanisms of this plant to copper ions. Lipid peroxidation (LP) is a biochemical marker for the free radical mediated injury. Character of changes in lipid peroxidation depends on intensity of influence stressor and from plant sensitivity. In leaves of experimental plants cultivars MAS 97 and SPIROV after Cu treatment by concentration 5 mM the level of malondialdehyde (MDA) content has been increased on 11% and 30% respectively. The higher MDA content has been observed in leaves of cultivar ESPrim. In the other experimental variants under Cu treatment content of MDA was on control level which evidence about more less sensitivity these cultivars to Cu treatment. The combination of Cu^{+} BRs has been shown MDA content in leaves of all experimental plants on control level which can evidence about protection effect of BRs under Cu treatment on the leaves of experimental cultivars of sunflower plants.

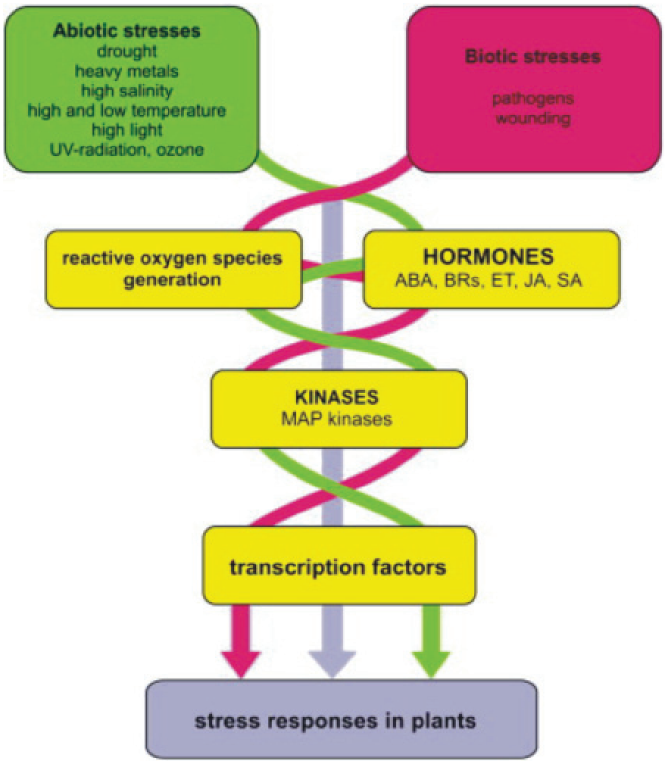
brassinosteroids, copper, *Helianthus annuus* L., MDA, proline, chlorophylls

Plants are during their life exposed to the effect of many stressful factors of biotic and abiotic disposition (e.g. heavy metals), which can not only slow down their life functions, but also harm particular organs and in isolated instances it can lead to the plant death. The stress factor influence on plants can cause oxidative stress, which is typical of rapid temporary production of big amount of active forms of oxygen (AFO), and break a balance between the production and reduction of AFO. Malondialdehyde (MDA) is a product of oxidative harm of lipids and it represents the best explored marker of the oxidative stress (Behnamnia *et al.*, 2009).

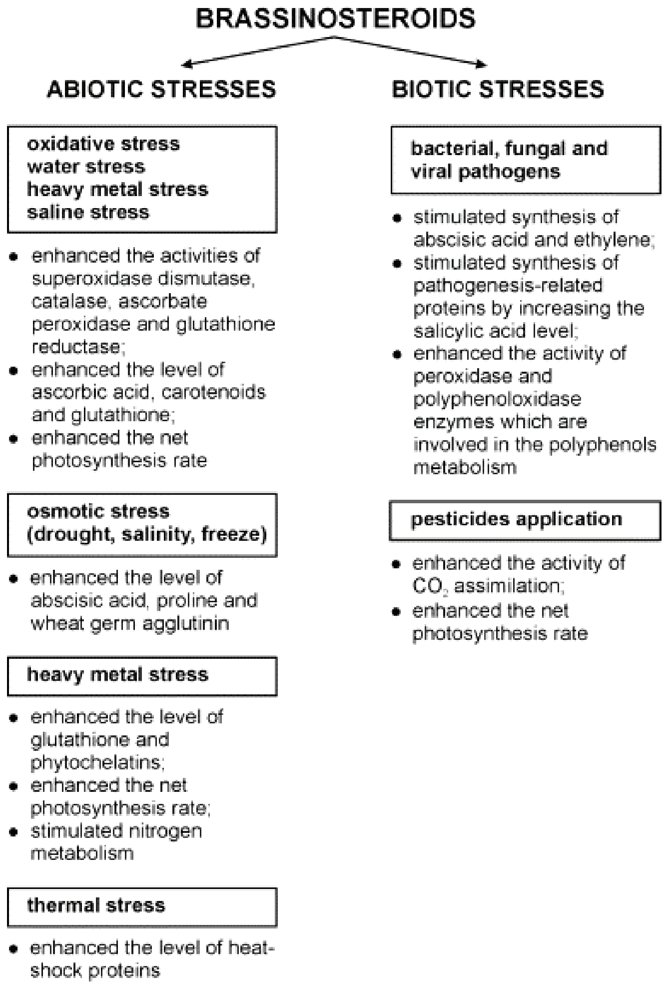
Copper is an activator of the enzymes which participates on a metabolism N and synthesis of proteins. In a case of copper lack the content of free nitrogenous substances in the cells is increasing at the expense of protein production (Heidari, 2010). Therefore it is necessary, especially by an intensive fertilization, to ensure that a plant has got enough copper (Lošák *et al.*, 2011; Škarpa, Lošák, 2008). The biggest supply of copper is in the chloroplasts in which it works as a stabilizer of the chlorophyll. The lack of copper causes a disruption of the photosynthesis process and it leads to a limitation of the growth intensity. In spite of the physiological importance of copper, on the other hand excessive content of this element in the soil becomes the stressful factor for the plants or microorganisms (Hayat *et al.*, 2000; Divi, Krishna, 2009). The effect of copper toxicity on the plants is truly provable. However many aspects of the toxic effect of copper on the plants are clarified, the results of several physiological and biochemical analysis are controversial. Changes in the processes of photosynthesis and water regime influenced by the copper effect in the plants from a family *Asteraceae* are markedly different (Ali *et al.*, 2007). Big variability in seed germination noticed for instance in the various hybrids of sunflower. Big variety in a reaction of plants on the heavy metals is constantly revealing new questions which solution can play very important role from the point of view of preserving pure environment and man's health. A sunflower is thanks to the big biomass an ideal plant for decontamination of soil (phytoremediation), whereas it accumulates notable content of heavy metals in the roots (Fariduddin *et al.*, 2009).

Brassinosteroids (BRs) are steroidal phytohormones with a wide scale of effects. By exposing plants to the drought stress as well as to the heavy metals their survival is improved and also resistance and yield are increased. The plant response is regulated directly (by the synthesis of metabolites) or indirectly (by induction of antioxidizing compounds and enzymes), often in the interaction with other phytohormones. Experiments studying an impact of brassinosteroids on the reaction of plants stressed e.g. by water deficit and toxicity of heavy metals are different in various parameters. Therefore their proper interpretation is difficult. Thanks for better understanding of their influential mechanisms, it is possible to discover new possibilities for their usage in agricultural biotechnology. Meanwhile, a research of other aspects like reception, transport and stability of Brs, as well as development of Brs analogues with high activity should continue. Only with the knowledge of unique mechanisms of plant resistance to stress and with the predictable effects in the field conditions, it will be possible to use a whole potential of BRs in the practise. In the majority of studies demonstrating anti-stress effects of BRs it is used an exogenous treatment. Nowadays the exogenous application of BRs is common field method in Japan, China, Russia and Belarus (Bajguz, Hayat, 2009; Divi, Krishna, 2009).

We tested the hypothesis that application of brassinolide 24-epibrassinolide (EBL) will alleviate the toxic effect of copper applied through shotgun approach on sunflower growth and metabolism and will ultimately result in improved yield at harvest and give the farmers a better option to grow their crops under stress condition. The purpose of our experiments was to consider sensitivity of the chosen sunflower cultivars to copper ions with the possibility of elimination by means of brassinosteroids on the ground of physiological characteristics (content of dry matter, amount of



1: *Generalized model of the plant stress signalling networks. ABA, abscisic acid; BRs, brassinosteroids; ET, ethylene; JA, jasmonic acid; SA, salicylic acid. (Bajguz, Hayat, 2009)*



2: *Effects of BRs on plants exposed or subject to different stresses (Bajguz, Hayat, 2009)*

assimilatory pigment, proline content, relative water content – RWC as well as MDA) and to show possible resistance mechanisms of this plant to copper ions.

MATERIAL AND METHODS

There were used 6 hybrids of sunflower (*Helianthus annuus* L.): Belinda (FRA), Codiwer (FRA), ESPrim (FRA), MAS 95 (SVK), MAS 97 (HUN) a Spirov (BUL) which were provided by the company FINAGRO, spol. s. r. o. Bratislava. Sterilized seed were budding for 24 hours in the distilled water and consequently were sprouting on Petri dish (Ø 0.15 m) with wetted filter paper in darkness. After 3 and 4 days of sprouting were approximately equally sprouted seeds sown into 15 l plastic bowls with a mixture of peat and perlite (in a ratio of 4:1), poured with distilled water which corresponds to maximum soil sorption capacity (~1000 ml). Plants were left to grow to the beginning phase of butonization – stars (32 day). Afterwards they were poured with distilled water (control variant plants) or with the solution enriched with copper with concentration 80 µM $CuSO_4.5H_2O$ and the last chosen combination was Cu + Brassinolide 24-epibrassinolide (EBL) (80 µM $CuSO_4.5H_2O$ + 100 µM EBL). Waterings in the next phases of the experiment did not contain metal or brassinosteroids. According to the needs distilled water was applied which quantity corresponded to the half of dose of maximum sorption substrate capacity (500 ml). After 10 days sprouts were separated from the roots, roots were cleaned off soil and thoroughly washed with water. Consequently, we measured their length, fresh and dry weight. The experiment was realized in three repetitions. The gained data was analysed by mathematical-statistical methods of programme MS Excel. Meaning of the differences by comparing the sets were determined by the Student test.

Hormone preparation

Brassinolide 24-epibrassinolide (EBL) was purchased from Sigma-Aldrich, USA. Stock solution (10^{-4}M) was prepared by dissolving the hormone in 5 ml of ethanol in a 100 ml volumetric flask. Five millilitres of 0.5% surfactant "Tween-20" was added to it, and the final volume was made up to the mark by using double distilled water (DDW). The 10^{-8}M concentration of EBL was prepared by dilution of the stock solution.

Analysis of lipid peroxidation

Malondialdehyde (MDA) was measured spectrophotometrically by the reaction with Thiobarbituric acid. Plant material (roots 500 mg and leaves 200 mg) was homogenized in 3 ml 0.1 M Trichloroacetic acid (TCA). The mixture was warmed up to temperature 95 °C for 30 min and afterwards was centrifugated by 10,000 × g per 5 min. Quantity of MDA was determined according to Heath and Packer (Heath, Packer, 1968) by wave length 532 nm and conversion using molar extinction coefficient 155 $m.M^{-1}.cm^{-1}$.

Chlorophyll and photosynthesis measurements

The chlorophyll content in fresh leaf samples was measured by using a SPAD chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan).

Determination of proline content

The proline content in fresh leaf and root samples was determined by the method of Bates *et al.* (1973). Samples were extracted in sulfosalicylic acid. To the extract, an equal volume of glacial acetic acid and ninhydrin solutions was added. The sample was heated at 100 °C, to which 5 ml of toluene was added. The absorbance of the aspired layer was read at 528 nm on a spectrophotometer JENWAY 6405 UV/VIS.

Relative water content (RWC) was calculated by placing the values in the following formula:

$$RWC = \frac{(\text{fresh mass} - \text{dry mass})}{(\text{turgor mass} - \text{dry mass})} \times 100\,\%.$$

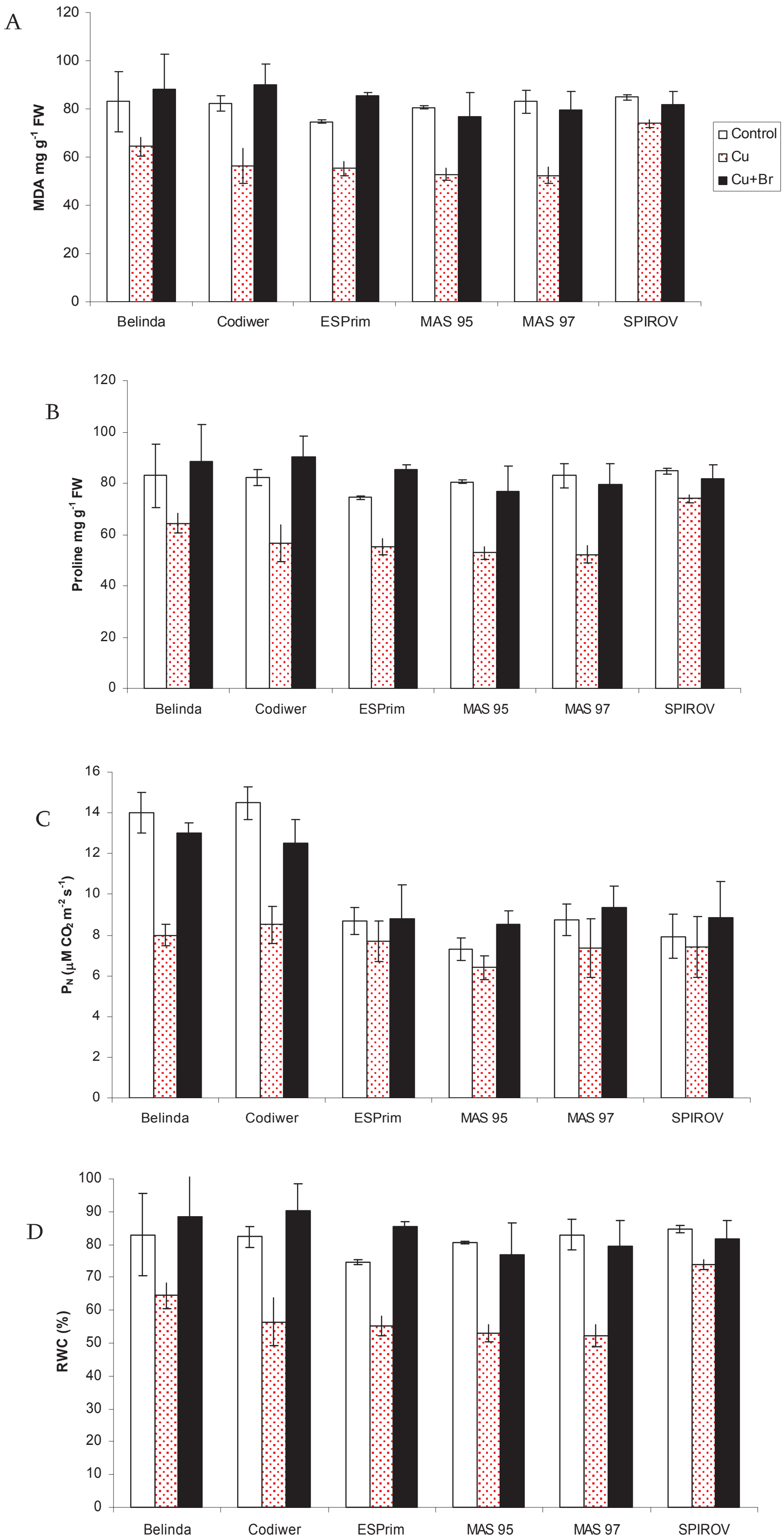
RESULTS AND DISCUSSION

Tested cultivars of sunflower showed according to the tested parameters, such as length and weight of sprouts and fresh weight of roots, relatively high rate of tolerance. In spite of the fact that no significant visual symptoms of toxic effect of metal were markedly noticeable, we detected decrease in the content of dry weight of roots (less than 25–39% in comparison with the control of two tested cultivars treated by Cu cv. MAS 97 and cv. SPIROV). Cv. Belinda, cv. Codiwer a cv. MAS 95 tends to be the most resistant or tolerant to Cu toxicity from the point of view of evaluation of morphological parameters of particular cultivars.

BRs treatment completely compensated reduction of biomass caused by Cu toxicity. In the comparison with the untreated plants (control and Cu stress) there was noticed an improvement of root growing in the treated plants. Yu *et al.* (2004) and Zhang *et al.* (2008) observed that BRs application improved assimilation of carbon and nitrogen by the stabilization of membrane structures in the stressful conditions and also improved general growth and plant photosynthesis. Photosynthetic apparatus cv. MAS 97, cv. SPIROV and cv. ESPrim reacted the most sensitive to the dose of copper what expresses in the content reduction especially of chlorophyll a (by 41%), chlorophyll b (by 22%) and carotenoids (by 29%). Negative impact on the photosynthetic apparatus efficiency is one of the typical signs of the effect of various kinds of abiotic stress such as drought, high temperature, and also heavy metals. Brassinosteroids induced an improvement in photosynthesis efficiency which can be caused by stomatal or non-stomatal factors or by their combination. Influence of BRs on a conductivity of stomas was noticed by Hayat *et al.* (2000, 2010) and Fariduddin *et al.* (2009). Non-stomatal efficiency limitations of photosynthesis can be related with the photosynthetic pigments, concentration and activity of enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) and use of assimilative products. Yuan *et al.* (2010) consider as an acceptable explanation that exogenous application of BRs is increasing the capacity of carbon oxide assimilation in Calvin cycle using increased initial activity of RuBisCo. In the following experiments the BRs treatment reduced the decreasing of chlorophyll content and assimilation speed, increased efficiency of photosystem II and activity of enzymes, such as RuBisCo, nitroreductase and glutamine synthesis (Li *et al.*, 2004). Carotenoids are pigments protecting chlorophylls from the photo-oxidative damage (Yu *et al.*, 2010). Their content is decreasing with the increasing level of the oxidative stress caused by the Cu toxicity in all cultivars of sunflower and is significantly higher in the plants treated by BRs, in the comparison with the controlled ones.

According to the measurement of MDA level it is likely that BRs help the effective catching of ROS by increased activity of the antioxidative enzymes system. Production of MDA and other aldehydes in the plants exposed to the Cu toxic stress is a dependable indicator of the production of free radicals in the plant tissue and today it serves as the indicator of lipid peroxidation and meanwhile of stress damage, such as heavy metals at the cell level (Heidari, 2010). The cultivars resistant to the copper toxicity are able to catch the free radicals and less MDA is produced with the decreasing content of hydrogen peroxide in a comparison with the sensitive cultivars (Divi, Krishna, 2009). On the basis of gained data we can claim MAS 97, SPIROV and ESPrim belong to the sensitive cultivars of sunflower as they produced increased amount of MDA (more than 11–30% in a comparison with the control). On the second hand, among tolerant to Cu toxicity sunflower cultivars belong Belinda, Codiwer and MAS 95. Stated cultivars produced lower part of MDA in a comparison with the controlled variant.

With the Cu + BRs combination we noticed decreased MDA content in the leaves of all common Sunflower cultivars, what indicates



3: *Effect of EBL on A: MDA content, B: proline content, C: net photosynthetic rate and D: relative water content in sunflower plants subjected to Cu stress* possible elimination of copper toxicity in the sunflower plants using the exogenous application of brassinosteroids.

Plants raised from the seeds given presowing seed soaking treatment in 80 μM$CuSO_4.5H_2O$ showed significant reduction in all measured growth parameters (i.e., length, fresh and dry mass of root and shoot, and leaf area) irrespective of varietal difference. However, treatment with EBL to the foliage favored growth and neutralized the negative effects generated by Cu more effectively in all cultivars. Significant reduction in photosynthetic parameters occurred from Cu application, net photosynthetic rate (PN), and relative water capacity (RWC) of six cultivars, given presowing seed soaking treatment in $CuSO_4$, as compared to their respective controls.

All varieties showed significantly different responses to the spray treatments. Enzyme activity increased in response to both metal and hormone treatment in six cultivars, but Codiwer, showed higher enzymatic activity than did Belinda, ESPrim, MAS 95 and MAS 97 in response to the treatments. The foliar spray of either with 24-epiBL significantly enhanced the growth, photosynthesis, antioxidant enzymes and proline content in copper stressed sunflower plants.

The exogenous application of plant hormones has been found to counter toxic effects of various abiotic stresses. Brassinosteroids (BRs) are a new type of phytohormones that elicit a wide range of physiological processes in plants (Bajguz, Hayat, 2009; Behnamnia, 2009; Hayat, 2010). At present, 70 analogs have been identified, and among these, three natural brassinosteroids (brassinolide, 24-epibrassinolide (EBL), and 28-homobrassinolide (HBL)) are known to have an economic impact on plant metabolism, growth and productivity, and experience high stability under field conditions (Ali, 2007).

CONCLUSIONS

Many aspects of the copper toxic efficiency on the plants are clarified, however results of several physiological and biochemical analysis are controversial. At the same time, high variability of plant reaction to the heavy metal ions depending up the genotypes complicates unambiguity of the conclusions. Deeper biochemical and molecular-biological analysis can contribute to revealing of other possible mechanisms of sunflower resistance to copper ions or other heavy metals. Wide scale of BRs effects on the plants stressed by the heavy metals instigates to search for mechanisms and connections which disproves old concepts and motivates development of new methods.

SUMMARY

The purpose of our experiments was to consider sensitivity of the chosen 6 sunflower cultivars (*Helianthus annuus* L. Cv. Belinda, cv. Codiwer, cv. ESPrim, cv. MAS 95, cv. MAS 97 and cv. Spirov) to copper ions with the possibility of elimination by means of brassinosteroids on the ground of physiological characteristics (content of dry matter, amount of assimilatory pigment, P_N, proline content, RWC as well as MDA) and to show possible resistance mechanisms of this plant to copper ions. After 3–4 days of sprouting were approximately equally sprouted seeds sown into 15 l plastic bowls with a mixture of peat and perlite, poured with distilled water which corresponds to maximum soil sorption capacity (~1000 ml). Plants were left to grow to the beginning phase of butonization – stars (32 day). Afterwards they were poured with distilled water (control variant plants) or with the solution enriched with copper with concentration 80 μM $CuSO_4.5H_2O$ and the last chosen combination was Cu +EBL (80 μM $CuSO_4.5H_2O$ + 100 μM EBL). Waterings in the next phases of the experiment did not contain metal or brassinosteroids. According to the needs distilled water was applied which quantity corresponded to the half of dose of maximum sorption substrate capacity (500 ml). Tested cultivars of sunflower showed according to the tested parameters, such as length and weight of sprouts and fresh weight of roots, relatively high rate of tolerance. In spite of the fact that no significant visual symptoms of toxic effect of metal were markedly noticeable, we detected decrease in the content of dry basis in the roots (less than 25–39% in comparison with the control of two tested cultivars treated by Cu cv. MAS 97 and cv. SPIROV). Cv. Belinda, cv. Codiwer a cv. MAS 95 tends to be the most resistant or tolerant to Cu toxicity from the point of view of evaluation of morphological parameters of particular cultivars. All varieties showed significantly different responses to the spray treatments. Enzyme activity increased in response to both metal and hormone treatment in six cultivars, but Codiwer, showed higher enzymatic activity than did Belinda, ESPrim, MAS 95 and MAS 97 in response to the treatments. The foliar spray of either with 24-epiBL significantly enhanced the growth, photosynthesis, antioxidant enzymes and proline content in copper stressed sunflower plants.

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