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Dihydroquercetin increases the adaptive potential of wild soybean against copper sulfate and cadmium sulfate toxicity

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Abstract: In this study, the mechanism of the biochemical adaptation of wild soybean to the experimentally modeled effects of cadmium sulfate and copper sulfate in approximately double permissible concentration was investigated. The extracted concentrations of cadmium and copper in the experimental soil were determined by inversion voltammetry – 1.46 and 48.25 mg/kg, respectively. Growing soybeans in soil with the addition of copper and cadmium sulfates led to an increase in the concentration of malonic dialdehyde in soybean seeds relative to control by 62% and 38%, respectively, which confirmed the strengthening of oxidative processes. There was also an increase in the specific activity of peroxidase by 198% under the action of copper sulfate and 122% under the action of cadmium sulfate. Copper in the studied concentration was more toxic than cadmium. Acid phosphatase showed stable specific activity under the action of the studied metals. PAGE revealed multiple forms that were absent from the control: under the action of copper sulfate–AP7, AP12; cadmium sulfate–AP12. Dihydroquercetin treatment of soybean seeds before sowing in soil contaminated with copper and cadmium sulfates led to a decrease in the level of malonic dialdehyde by 20% and 11%, respectively, and a decrease in the specific activity of peroxidase by an average of 12%. There was a decrease in specific activity and the appearance of new multiple forms of acid phosphatase: under the action of copper sulfate by 18%, AP13; cadmium sulfate – 25%, AP2 and AP13. Thus, we suggest that flavonoids may take part in the adaptation of plants to the effects of copper and cadmium.

Keywords: Glycine soja Sieb. & Zucc., acid phosphatase, biochemical adaptation, flavonoids, heavy metals, oxidative stress, peroxidase

1. Introduction

Soybean (*Glycine max* L. Merrill) is the most important agricultural legume crop with a unique and outstanding nutritional composition and versatile use (FAO, 2017; Li et al., 2019). Global food security needs a refocusing of soybean breeding towards achieving yield increase with production technology suitable to reduce the effects of biotic and abiotic stresses (Considine et al., 2017). Wild soybean (*Glycine soja* Sieb. & Zucc.) is the ancestor of domesticated soybean and has a higher level of genetic diversity and adaptive potential to the harsh environmental conditions making it suitable research material and inclusion in soybean breeding programs as a climate-resilient specie (Nawaz et al., 2018; Li et al., 2019).

Recently, soil contamination of agricultural land by heavy metals has become a serious problem (Miransari, 2016; Zhang et al., 2018). Cadmium (Cd) is a toxic element for plants. Cd can inhibit plant growth, development, and productivity by disrupting amino acid biosynthesis, inhibiting enzyme activity, interfering with mineral nutrition, and metabolic imbalances (Liu et al., 2015a; Yang et al., 2018). Copper (Cu) is essential as a plant microelement but in excess amounts it becomes highly toxic and inhibits photosynthesis (Dey et al., 2014), nutrient uptake, plant growth (Adrees et al., 2015), and ultimately causes cell death (Printz et al., 2016). In addition, excessive amounts of heavy metals induce oxidative stress, causing an increased formation of reactive oxygen species (ROS)

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(Zagoskina and Nazarenko, 2016). ROS can be formed by inhibiting, for example, Cd ions, the complex III of the mitochondrial electron transport chain producing unstable semiquinones, which transfer an electron to molecular oxygen with the formation of superoxide anion leading to the formation of other ROS. Transition metals are also able to interact with H_2O_2 by the Fenton reaction, with the formation of hydroxyl radicals (Chumakov et al., 2016).

The antioxidant defense system of the plant body includes enzymatic (peroxidase, catalase, superoxide dismutase, etc.) and nonenzymatic systems (carotenoids, vitamins, polyphenols, etc.) (Xu et al., 2018). Appropriate protective mechanisms to nullify the toxic effects of Cu and Cd sulfates are prerequisite of growing soybean in soils contaminated with these heavy metal sulfates. Understanding the metabolic profiles of soybean through the study of polymorphism of protein marker systems is an ideal genetic basis for solving practical problems of soybean breeding with improved heavy metal stress tolerance and agronomic traits (Xu et al., 2017). In this regard, several studies have noted the responsiveness of the soybean antioxidant system to changes in environmental conditions (Ivachenko et al., 2016; Xu et al., 2017; Yang et al., 2017).

Many physiological processes, including the activity of hydrolytic enzymes, are capable of being activated or inhibited by heavy metals. Heavy metals are able to accumulate in high concentrations in lysosomes. The assumption of the participation of lysosomes in the metabolism of heavy metals was put forward on the basis of the well–known ability of a number of metals (Cd^{2+}, Cu^{2+}, Pb^{2+}, Fe^{2+}, and Ni^{2+}), especially with their prolonged or excessive entry into the body, to concentrate in the vesicular structures of the cytoplasm, which give a positive reaction to acid phosphatase (APase) or other lysosomal enzymes (Yaqoob et al., 2017).

APase (EC 3.1.3.2) belongs to the class of hydrolases and plays an important role in the regulation of metabolic processes by hydrolysis of various intracellular phosphorylated biomolecules, and also participates in maintaining the energy pool in biological systems (Ferreira et al., 1998; Srivastava and Anand, 2014; Liu et al., 2015 b). APase acts as a multifunctional enzyme as it participates in plant growth and development, regulation of peroxidase activity (Kong et al., 2018), and salt tolerance responses of plants (Li et al., 2008).

Since soil contamination by heavy metals is a widespread issue, a search for materials and methods that can lead to a decrease in plant stress caused by their action is needed (Zafar et al., 2019). One of the approaches to solve this problem may be the treatment of plants of various kinds of antioxidants. The well-known antioxidants are flavonoids, which are found in wood, bark, leaves or roots of plants. Flavonoids are able to serve as traps for free radicals, as well as to chelate metal ions involved in peroxidation (Li et al., 2016). Twelve forms of flavonoids are found in soybeans. They exist in 4 different types: aglycones, β-glucosides, acetyl glucosides, malonyl glucosides (Tsukamoto et al., 2018). Soybean isoflavones have been found to have antioxidant properties and play an important role in various biological processes in plants (Grosser et al., 2015; Li et al., 2016; Tuskamoto et al., 2018). Dahur larch (Larix Dahurica), which is a rich source of flavonoids, namely dihydroquercetin (DHQ), is widespread in the Amur taiga. DHQ – analog of quercetin, hydrogenated to positions 2 and 3, belongs to the group of flavanones. DHQ is highly soluble in polar solvents and water–alcohol solutions, especially when heated. In solutions of DHQ may be present in the trans and cis forms. Moreover, the trans form of flavanone is more chemically active. DHQ has a high P vitamin activity, exhibits antioxidant activity, is resistant to autooxidation, and is a low toxic substance (Gogotov et al., 2014). The mechanism of antioxidant action is to neutralize the action of active oxygen and nitrogen molecules by preventing lipid peroxidation and the formation of chelate complexes with metals. The inhibitory effect of DHQ on the development of apoptosis was also noted (Koroteev et al., 2014). It was previously established that DHQ plays an important role in the formation of the protective mechanism of soybean seeds under conditions of oxidative stress (Kuznetsova et al., 2015) such as protection against pathogens (Fusarium sp.) (Skadhauge et al., 1997), salinity, and heat (Martinez et al., 2016).

The purpose of this study was to examine the activity of APase of wild soybean seeds under conditions of oxidative stress caused by cadmium sulfate and copper (II) sulfate, and to assess the ability of DHQ to reduce the level of oxidative stress of soybean.

2. Material and methods

2.1. Plant material

Wild soybean (*G. soja*) seeds were grown in a greenhouse at a temperature of 18–28 °C. Before planting, the seeds were surface sterilized in ethanol, washed with distilled water, and then planted in darkened vessels filled with soil. The study was conducted in brown forest loam soil of a natural field site (50°17'N. L., 127°24'E. L.) with naturally low concentrations of Cu and Cd. The soil was collected from the upper arable horizon (20 cm), humus content 4.0%, pHKCl = 5.9. Total concentrations of Cd and Cu in the soil were < 0.1 and 5.52 ± 1.66 mg/kg, respectively, and the extractable concentrations were < 0.1 and 1.12 ± 0.34 mg/kg, respectively (Chernyshuk et al., 2018).

Intoxication with salts of heavy metals in the form of copper sulfate and cadmium sulfate was carried out by adding the corresponding salts to the soil to a concentration twice as high allowed in the soil, which is 4 mg/kg for Cd and 264 mg/kg for Cu (Hygienic regulations 2.1.7.2511–09). Seeds grown in the soil without adding the salts of heavy metals served as controls. Before sowing, seeds were treated with 2 mm DHQ solution. All plants were divided into 6 experimental groups; control (seeds without presowing treatment), DHQ+ (presowing treatment with DHQ), Cu+ (Copper (II) sulfate treatment in the soil), DHQ + Cu (presowing treatment of seeds with DHQ and Cu added to the soil), Cd+ (Cadmium sulfate added to the soil), and DHQ + Cd (presowing treatment of seeds with DHQ and Cadmium sulfate added to the soil). The experiment was laid out in a completely randomized design and each treatment was repeated thrice.

2.2. Physicochemical analysis of soil

Soil acidity (pH) was determined by a potentiometric method in accordance with the state standard (State Standard 26483). 30 g of the sample was extracted for 1 h with 75 mL of 1 N KCl, filtered and measured using an Ecotest–2000 liquid analyzer (Econix, Russia).

The extracted concentrations of Cu and Cd available for plants were determined in the soil by inversion voltammetry using a TA analyzer (Tomianalit, Russia). 50 mL of ammonium acetate buffer solution (pH = 4.8) was added to the air-dried sample (5.00 ± 0.01 g). Extraction was carried out for 24 h with constant stirring. The sample was then filtered into a measuring flask and the volume was brought to the mark with double–distilled water. 2 mL of the resulting extract was evaporated at a temperature of 160–180 °C to a dry residue. Before analysis, the ash was dissolved in 0.2 mL of concentrated formic acid and 1.8 mL of double–distilled water (Methodical Instructions 31–11/05). The composition of soil used for growing the seeds before and after Cd and Cu supplementation is given in the Table.

2.3. Biochemical analysis of plant samples

Soybean protein extract was obtained by homogenizing soybean seeds (500 mg) in 0.15 M NaCl at 4 °C for 15 min. The obtained extract was then centrifuged at 3000 rpm for 15 min. Protein was determined by the biuret method (Gornall et al., 1949). The content of malonic dialdehyde (MDA) was used as an indicator of the intensity of oxidation. The content of MDA was determined by the method based on the reaction between MDA and thiobarbituric acid (DIA • M, Russia). The sample (250 mg) was homogenized in 4 mL of 20% trichloroacetic acid. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. To 1 mL of the supernatant, 4 mL of 0.5% thiobarbituric acid were add in 20% trichloroacetic acid. The reaction mixture was incubated for 30 min at 95 °C followed by rapid cooling in an ice bath, and centrifuged for 12 min at 10,000 g. The MDA concentration was determined spectrophotometrically by measuring the optical density (OD) at a wavelength of 532 nm and the nonspecific clouding of the solution at a wavelength of 600 nm using a spectrophotometer (Celekli et al., 2013).

Table. The composition of intact and experimental soil.

Indicator	Soil (without supplementation)	Soil (with supplementation)
Cd (mg/kg)	< 0.1	1.46 ± 0.44
Cu (mg/kg)	1.12 ± 0.34	48.25 ± 14.48
pH	5.90 ± 0.20	5.62 ± 0.20

Peroxidase (POase) activity was determined by the Boyarkin method with the modification of Mokronosov with benzidine (Vekton, Russia) using a CFC–2 photoelectric colorimeter (Russia). 4 mL of 200 mM acetate buffer (pH 5.4) was added to 3 mL of benzidine hydrochloric acid solution, the arrow of the galvanometer was set to 0, 0.5 mL of 0.3% hydrogen peroxide was added, and the time was noted using a stopwatch till the OD of 0.5.

APase activity was determined with *p*-nitrophenyl phosphate (disodium salt) (PanReac AppliChem, Germany) as a substrate. The reaction mixture consisting of 0.1 mL of soybean protein extract, 3 mM *p*-nitrophenyl phosphate in 1.4 mL of 100 mM acetate buffer was incubated for 20 min at 37 °C. The reaction was stopped by adding 2 mL of cooled 0.1 M NaOH. The OD of the solution was measured at 415 nm relative to the control, in which the protein extract was added after the alkali solution (Saeed et al., 2014). The analysis was performed on a spectrophotometer (PromEcolab, Russia). The specific activity of enzymes was expressed in units per mg of protein.

Multiple forms of enzymes were detected by electrophoresis on polyacrylamide gel column (PAGE). Fractionation of soluble proteins was carried out in 7.5% polyacrylamide gel at 4 °C according to Davis in the modification for soybean proteins (Davis, 1964). Bromophenol blue dye (Reanal, Hungary), 0.1 mL of soybean protein extract was applied to the column and electrophoresis was carried out on a PEFA–1 device (Russia) in a tris-glycine buffer (pH 8.3) at a temperature of 2–6 °C and a voltage of 200–500 V. For 15 min, a current of 2.5 mA was passed through the column followed by a current of 5 mA for 1.0–1.5 h.

Multiple forms of POases were detected with a benzidine reagent in acetate buffer with a pH of 4.8. The gel was placed in a benzidine reagent and then transferred

to a 0.1% aqueous hydrogen peroxide solution, after a few seconds the forms appeared as blue bands.

APase activity was detected in the gel after electrophoresis with 11 mM a-naphthylphosphate (DIA • M, Russia). The gel was incubated in 100 mM acetate buffer for 20 min at 37 °C. Places of localization of the forms of the enzyme, in the form of bright pink stripes, were established after staining the gels with 2 mM dye of durable blue B (DIA • M, Russia) (Ivachenko et al., 2008).

The localization multiple forms of POases and APases were studied by the relative electrophoretic mobility (Rf). Each form of soybean enzymes was analyzed according to previously designated Rfs: for POases – P1-P18, for APases – AP1–AP13 (Ivachenko et al., 2016).

2.4. Statistical processing of experimental data

The experimental data were processed using the STATISTICA 10 software, the graphical representation of the data was done in Microsoft Excel (2010). Results were expressed as mean (n = 6) ± standard deviation, the differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Responsiveness of the antioxidant system of soybeans to the effects of cadmium sulfate and copper sulfate

In response to the effects of a stressor, plants include a specific set of biochemical and physiological reactions to overcome impairments to its vital activity, which ensures nonspecific or urgent adaptation (Yaqoob et al., 2017). Toxic effects of heavy metals cause the formation of ROS in the plant cell (a state of oxidative stress) and, consequently, the intensification of lipid peroxidation processes which leads to the accumulation of a product such as MDA (Kolesnichenko and Kolesnichenko, 2012; Celekli et al., 2013). Kulikova et al. (2011) noted an increase in the MDA content during soybean incubation on a medium with excess Cu content; the excess Cu inhibited both biomass accumulation and linear plant growth. An increase in MDA content and the specific activity of antioxidant enzymes in soybean has been reported under stress caused by Cd exposure (Merve and Burcu, 2012). In this study, we found that Cu and Cd sulfates caused an increase in the concentration of MDA in soybean seeds by 62 and 38%, respectively, which indicates a state of oxidative stress (Figure 1A). A significant increase in the specific activity of POase was also observed, which may be associated with the establishment of redox homeostasis, which is necessary for the proper function of plant cells (Figure 1B).

Cd also caused oxidative stress in soybean plants, but it was less toxic than Cu at the studied concentration. Our results demonstrated that a presowing treatment of seeds of wild soybean with DHQ led to a decrease in MDA concentration in all experimental groups, which indicate the possible involvement of this flavonoid in reducing oxidative stress (Figure 1A). A decrease in the specific activity of POase in groups 4 (DHQ + Cu) and 6 (DHQ + Cd) relative to groups 3 (Cu+) and 5 (Cd+) was also observed, indicating that DHQ effectively complements the enzymes of the antioxidant system (Figure 1). The literature has previously noted the ability of various compounds to concentrate MDA and regulate the antioxidant activity of enzymes under stress, thereby reducing oxidative damage in soybean plants (Zengin, 2014).

The electrophoretic zymogram of the wild soybean POases, the P6 form was detected in all the samples under study (Figure 2). In the soybean seeds treated with DHQ, a highly mobile form P1 was found which was absent in the control. It is known that an increase in

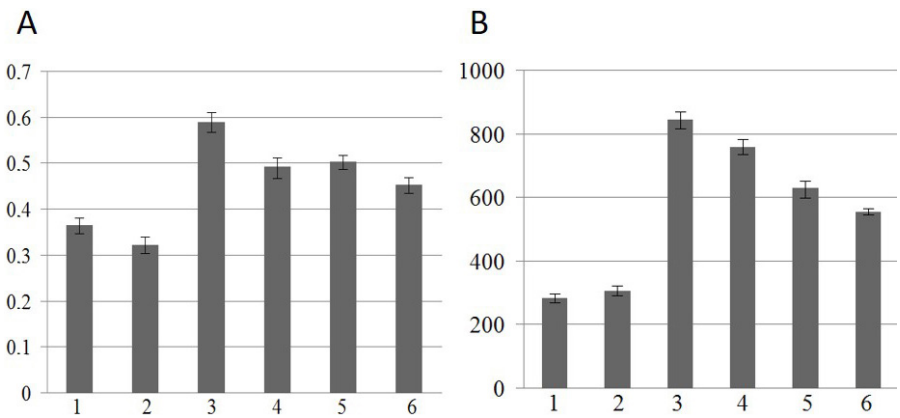

Figure 1. Concentration of MDA (μmol/g wet weight) (A) and the specific activity of peroxidase (units/mg protein) (B) in soybean seeds grown under experimental conditions: 1 - control; 2 - DHQ+ (2 mM); 3 - Cu+ (48.25 mg/kg); 4 - Cu ((48.25 mg/kg) + DHQ (2 mM)); 5 - Cd+ (1.46 mg/kg); 6 - Cd ((1.46 mg/kg) + DHQ (2 mM)).

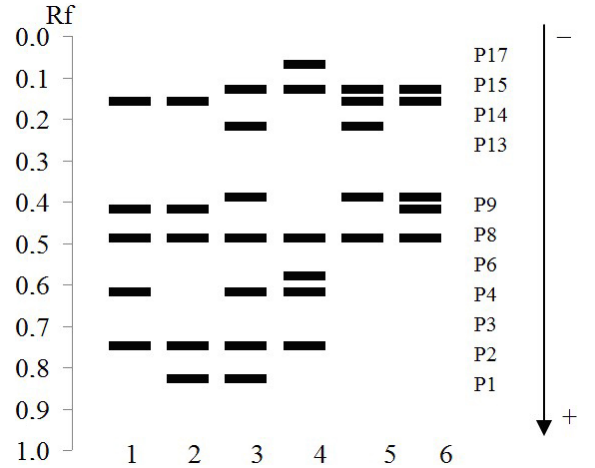

Figure 2. Electrophoretic zymogram of peroxidase in soybean seeds grown under experimental conditions: 1 - control; 2 - DHQ (2 mM); 3 - Cu (48.25 mg/kg); 4 - Cu ((48.25 mg/kg) + DHQ (2 mM)); 5 - Cd (1.46 mg/kg); 6 - Cd ((1.46 mg/kg) + DHQ (2 mM)). Arrow - electrophoresis direction (from the cathode to the anode). The numbering of the identified forms of the enzyme is indicated on the right.

POase activity is associated with the increased formation of ROS and activation of mechanisms that protect the plant from oxidative damage caused by stress. The adaptive restructuring of the POase enzymatic system is accompanied by the synthesis of multiple forms with new properties (Semenova, 2012). When wild soybean seeds were grown in soil with high Cu content, we observed an increase in the concentration of MDA, specific activity, and the number of multiple forms of POases (4) relative to the control. When intoxication with cadmium sulfate, the number of multiple forms POase in soybean seeds did not change relative to the control, but their electrophoretic mobility changed, new medium and low mobility forms appear (P9, P13, and P15), which were observed under the influence of copper sulfate. These data probably indicate a high adaptive potential of soybean and its resistance to the effects of the studied metals.

Kuznetsova et al. (2015) noted that the addition of DHQ to heavy metal salts led to a decrease in the specific activity of POases and the appearance of new forms of the enzyme in soybean seedlings. In our study, in the experimental group 4 (DHQ + Cu), 2 new forms of P4 and P17, functioning under these conditions, were observed. In this group, a decrease in the specific activity of POase relative to group 3 (Cu) was also noted, which is apparently due to the cessation of the functioning of forms P9 and P13. In experimental group 6 (DHQ + Cd), no new multiple forms of POase were detected but a decrease in the specific activity of the enzyme relative to group 5 (Cd) was observed, which is probably due to the cessation of the functioning of form P13, as in the experiment with copper sulfate (Figure 2) .

3.2. Effect of copper sulfate and cadmium sulfate on APase activity

Under stress, depolarization of cell membranes occurs; its permeability increases and H^+-ATP–ases are inhibited, which leads to acidification of the cytoplasm. Reducing the pH of the cell environment contributes to the activation of enzymes of the class of hydrolases, most of which have an optimum pH in an acidic environment. As a result, hydrolysis processes are enhanced (Zhang et al., 2017). Due to metal toxicity, a change in the specific activity and composition of the multiple forms of a number of enzymes, that are not directly related to intoxication, occurs. Largely, this applies to enzymes with broad metabolic functions–nonsubstrate-specific hydrolases, which include APase (Yaqoob et al., 2017).

Seed treatment with DHQ resulted in a 25% increase in the specific activity of APase relative to the control (Figure 3A), which corresponds to the appearance of one additional form of the AP7 enzyme (Figure 3B).

Earlier, Shapoval et al. (2015) found that the treatment of plants with DHQ contributes to an increase in the resistance of soybean plants to unfavorable environmental factors, diseases, and increases plant growth and productivity. APase is involved in the mobilization of phosphate, which in turn is necessary in the processes of plant growth, and therefore, it is possible to observe an increase in the specific activity of the enzyme in these conditions.

We observed that with the influence of copper sulfate and cadmium sulfate on wild soybean, the specific activity of APase was stable relative to the control (Figure 3A). Tabaldi et al. (2007) noted that Cd did not affect the activity of APase in cucumber seedlings (*Cucumis sativus* L.). It should be noted that in the experimental groups 4 (DHQ + Cu) and 6 (DHQ + Cd) there was a decrease in the specific activity of APase relative to groups 3 (Cu) and 5 (Cd), which may indicate the role of DHQ in regulating the activity of APase (Figure 3A).

Analysis of the electrophoretic spectra of APases of wild soybean seeds showed that stable experimental forms of the enzyme (AP4 and AP9) were found in all experimental groups. In general, the number of multiple forms of the enzyme increased relative to the control in all samples (Figure 3B).

Under the influence of copper sulfate, we detected AP7 and AP12 forms while only the AP12 form was observed under the cadmium sulfate, which were absent in the control. In group 6 (DHQ + Cd), wound rendered multiple forms of AP2 and AP13 were found, while the low mobile form of AP13 was also found in group 4 (DHQ + Cu).

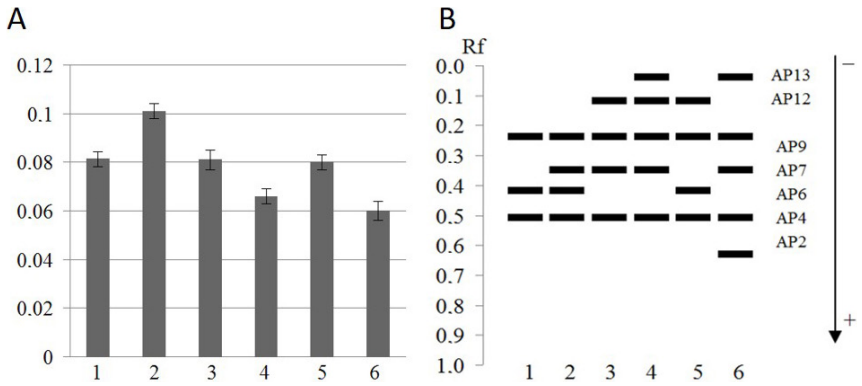

Figure 3. Specific activity (units/mg protein) (A) and the electrophoretic zymogram (B) of acid phosphatase in soybean seeds grown under experimental conditions: 1 - control; 2 - DHQ (2 mM); 3 - Cu (48.25 mg/kg); 4 - Cu ((48.25 mg/kg) + DHQ (2 mM)); 5 - Cd (1.46 mg/kg); 6 - Cd ((1.46 mg/kg) + DHQ (2 mM)). Arrow - electrophoresis direction (from the cathode to the anode). The numbering of the identified forms of the enzyme is indicated on the right.

Experimental groups 4 (DHQ + Cu) and 6 (DHQ + Cd) were characterized by the largest number of multiple forms of APase (5) and a decrease in the specific activity relative to the control. An increase in the number of multiple forms of the enzyme probably indicates the resistance of the species, which is characterized by a decrease in the activity of hydrolytic reactions and an increase in the processes of synthesis, including proteins.

4. Conclusions

Our results demonstrated that the cultivation of wild soybean in soil with the addition of copper sulfate and cadmium sulfate at a concentration of 48.25 and 1.46 mg/kg, respectively, led to an increase in the MDA content and an increase in the specific activity of POase, which in turn confirmed the increased oxidation processes in soybean. Cu in the studied concentration was more toxic than Cd. Additional treatment of soybean seeds with DHQ before sowing into soil contaminated with cadmium sulfate and copper sulfate increased the adaptive potential of soybean, reducing the level of MDA, the specific activity of POase, and causing the appearance of new multiple forms of POase.

Growing wild soybean on the soil supplemented copper sulfate and cadmium sulfate showed that wild soybean exhibited stable specific activity of APase and high variability of multiple forms. It was shown that the additional treatment of soybean seeds with DHQ before sowing in contaminated soil caused a decrease in specific activity and an increase in the number of multiple forms of APase relative to the control. The increase in the number of multiple forms of APase was probably indicative of the resistance of the specie, which was characterized by a decrease in the activity of hydrolytic reactions and an increase in the processes of synthesis, including proteins.

Thus, it is stated that DHQ may be involved in the adaptation of plants to the effects of Cu and Cd. Our results are important for soybean breeders for resistant variety development to the adverse effects of the studied heavy metals.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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