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Influence of selenium on enzymes of nitrogen metabolism and the phenolic compounds in garlic under oxidative stress

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The search for safe means of influencing individual metabolic processes for the action of heavy metals is of considerable interest in plant growing. The influence of selenium on the activity of nitrogen metabolism enzymes and phenolic compounds in garlic under conditions of stress caused by cadmium chloride has been investigated. The activity of AsAT and AlAT was determined by the Reitman and Frankel method, the activity of NAD-GDH by the NADH oxidation rate, the PC content by reaction with the Folin-Chocalteo reagent, the content of TBA-action products, by reaction with thiobarbituric acid. As a result of the article, the founded that in conditions of stress there is an increase in the activity of AsAT and AlAT with a conjoined increased in the activity of NAD-GDH, which can be considerated as exhibition of an adaptive reaction directed at the synthesis of pyruvate, oxaloacetate and glutamate, associated with the activation of sugars in the formed garlic bulbs, as well as an increase in the content of phenolic compounds and TBA-active products, which indicates the development of oxidative stress. Applicate of natrium selenate in the investigated concentration only results in a slight increase in AlAT activity and a tendency to increase AsAT activity in garlic bulbs, which proves the specific effect of the test drug and may be explained by its form and duration of exposure, but more significant effect was observed on the activity of NAD-GDH, in particular, in the direction of lowering the enzyme. The reduction of the content of phenolic compounds with a simultaneous decrease in the content of TBA-active products by treatment with the drug is shown, which proves the antioxidant effect of the investigated preparation under conditions of oxidative stress development. Applicate of natrium selenate in the stress concentration tested shows a partial decrease in the toxic effects of stress, which is confirmed by the data of the content of phenolic compounds and TBA-active products in garlic bulbs under experimental conditions. Perspective direction of this work is to find out the expediency of using selenium for stresses of different genesis, experiments on this are already being carried out.

Keywords: *Garlic sativum* L.; natrium selenite; alaninaminotransferase; aspartataminotransferase; glutamate gehydrogenase; phenolic compounds; malondialdehyde

Introduction

Introduction of agricultural production of ecologically-safe to increase the adaptive capacity of agricultural plants become actually in recent years. One of the advanced future research directions in this aspect can be the production technology using selenium (Se). The latter is an essential trace element, which can play in favour the adaptation of plants to various natural and climatic changes. Selenium is a component of the triplepted glutathione (GSH), selenium-containing amino acids and enzymes (Garousi, 2017).

So, garlic is sensitive to environmental changes by the culture that is selenium accumulators (White et al., 2016). Increase of non-specific resistance of plants that is, general adaptive mechanisms for the actions of stressors, can promote the activation of metabolic processes of the plant organism and the ability to adapt to probable stressful influences (Gupta & Gupta, 2017; Choundhury et al., 2017).

Cadmium (Cd) is a heavy metal, which, even at concentrations, is able to reduce the content of SH-groups of proteins and non-protein compounds in plant tissues, including components of antioxidant protection, which is accompanied by the induction of oxidative stress, one of the indicators of development of the latter is the content of malondialdehyde (MDA) (Asgler et al., 2014). Stress is the protector role of Se in plants under stress conditions caused by Cd (Han-wen, S. et al., 2010; Wu et al., 2016).

Nitrogen metabolism exhibits high sensitivity to stress factors. The key enzymes of nitric metabolism are aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2). Aspartate aminotransferase (AspAT) catalyzes the reversible transfer of an amino group from glutamate to oxaloacetate to form aspartate and α-ketoglutarate. Alanine aminotransferase (AlaAT) catalyses the reversible reaction of conversion of alanine and 2-oxoglutarate into pyruvate and glutamate. It is known that amino acids are regulatory and signal molecules under stress conditions. Thus, an increase in the content of alanine and glutamate in plants under stressful conditions was detected (Kumar et al., 2014). Syntheses of alanine and aspartate can be one of the metabolic transformations of selenium-containing amino acids as a result of transamination (Torre et al., 2014).

Glytamatedehydrogenase NAD+-depended (GDH) (EC 1.4.1.2) catalyzes the interconversion of α-ketoglutaric acid and glutamate, this enzyme plays an important role in the nitrogen metabolism. In the work (Shao et al., 2015) reduced the activity of NAD-GDH in wheat under salt stress conditions.

Besides, in plants there can be non-enzymatic deamination of amino acids with the participation of chlorogenic acid in the polyphenol-polyphenol oxidase system (Taranto et al., 2017). Phenolic compounds (FS) in plants are the most common class of biologically active compounds of secondary nature, which have antioxidant properties will be explained by the presence in their composition of OH- groups, it is proved to increase their content in plants under stressful conditions (Manquián-Cerda et al., 2016).

The aim of this article is to investigated the effect of sodium selenate on the activity of the enzymes of nitrogen metabolism and the content of phenolic compounds in garlic bulbs under oxidative stress conditions.

Materials and methods

The study carried out of the bulbs of winter garlic (*Allium sativum* L.) of cv Dushes. The garlic growned in the vegetarian builging of the Kharkiv National Agrarian University named after V.V. Dokuchaeva, plants are treated in the phase of the fifth leaf when used according to the following scheme: 1 – water (control); 2. cadmium chloride (CdCl2 2,5 H2O) in concentration 1,6 мМ (stress); 3. sodium selenate (Na_2SeO_4) (Merck, Hungary) in concentration 80 μM (Haw-Wen Sun et al., 2010); 4 – $CdCl_2$ 2,5 H_2O, and a day later – Na_2SeO_3. The cadmium chloride was introduced under root 0,4 L/m^2, and Na_2SeO_4 – at the same time under the root and over the leaves. The garlic bulbs were analyzed day after the appropriate treatments. After the experiment, the soil from the experimental sites was replaced. In each variant there were 20 garlic plants, each variant was executed in triple repetition.

The weight of material plants is homogenized in a medium cooled to 4 ± 1 °C, that contained 50 mM of Tris-HCl buffer, pH 7.5, 0.5 M of sucrose, 1 mM of EDTA, 5 mM of dithiotreitol, 1 mM $MgCl_2$. The homogenate is filtered through four layers of gauze, squeezed and centrifuged on a centrifuge MSE ROTOR LOG SHEET SUPERSPEED 65 at 3000 g during 15 min. The supernatant fluid is centrifuged at 16000 g for 20 min. The supernatant is used to determine the activity AlAT. The precipitate is resuspended in 0.01 M Na-K-phosphate buffer, pH 7.4, to remove part of soluble ballast proteins and precipitate is again centrifuged at 8500 g for 20 min. The supernatant is used to determine the activity AsAT and NAD-GDH. The extraction procedure was carried out at 4-6 °C.

Activity of both enzymes (AlaT and AsAT) is defined in the enzyme preparations according to the Reitman and Frankel method (Reitman & Frenkel, 1966). The medium for incubation contained 1 ml of the substrate, 1.8 mM α-oxoglutaric acid and 0.2 M aspartic acid in phosphate buffer pH 8.5, incubated at 30 °C for 10 min. The enzyme homogenate is added and the mixture is incubated for 60 min, and then added 1 ml 20% solution of 2.4-dinitrophenylhydrasine in 1 M HCl. After 20 min, the reaction is stopped by the addition of 10 ml of 0.4 M NaOH, and absorbance Extinction is defined on a spectrophotometer SF-26 at wavelength 505 nm. Activity of AsAT as expressed in μmol pyruvate/(h×mg of protein). The analogical procedure is defined of an activity AlAT, to the 0.05 M buffer Tris-HCl, pH 7.25, enzyme preparation added and and the substrate has been added to the 1.8 mM of α-oxoglutaric acid and 0.1 M of alanine in phosphate buffer, pH 7.25. Activity of AlAT is expressed as μM oxaloacetate / (h×mg of protein).

The activity of NAD+-depended glytamatedehydrogenase (EC 1.4.1.2) is defined in the enzyme preparations according to (Singh & Srivastava, 1983) determined by the rate of oxidation of the NAD in the reaction mixture containing of enzyme homogenate, 50 мМ of tris-HCl buffer, pH 8.3, 10 mM of α-ketoglutarate, 0.025 mM NAD 20 mM $(NH_4)_2HPO_4$. Extinction is defined on a spectrophotometer SF-26 at wavelength of 340 nm. Activity of NAD-GDH is expressed as μmol NAD/(min mg of protein).

The of phenolic compounds is determined by the Folin-Ciocalteu reagent according to Singleton and Rossi method (Singleton et al., 1999) in modifications. The weight of material plants is extracted in ethanol 80%, when extract boiled in a water bath using a glass funnel as a return fridge. After that, the filtrate is added of 3.0 ml of the 20% TCA for deposition of proteins and centrifugation at 4000 g for 15 min. Then, 3 ml of ethanol-ether mixture added to the precipitate to wash the protein. followed by centrifugation under specified conditions. The aliquot of the extract is added to 3.0 ml of Folin-Ciocalteu (1:6 v/v diluted with distilate water) reagent, after 3 min addided of sodium carbonate 7.5% 3 ml, and incubated at 20 °C in the dark. Extinction is defined on a colorimeter at wavelength of 750 nm. The content of phenolic compounds is calculated according to the calibration curve prepared for the chlorogenic acid as a standard (8.3-100 μg/ml) and expressed as μg chlorogenic acids per mg of fresh tissue (f.t.).

The content of TBA-active products is determined by reaction with 3 2-thiobarbituric acid accoding to (Hodges et al., 1977) in modifications. The weight of material plants is extracted in 10 ml 0.1 M K_2HPO_4 (pH 7.8). Samples are centrifuged at 3000 g for 15 min. To precipitate homogenatics in volume 2.0 ml added 3.0 ml 0.8% solution of thiobarbituric acid in 20% solution of

trichloroacetic acid and the samples are centrifuged at 3000 g for 15 min. Then the filtrate is kept in a water bath at 95 °C for 45 min, after this, the test tubes are cooled rapidly in the ice. To samples of cooled samples, 4.0 ml of butanol added the samples are centrifuged again at 3000 g for 15 min. The content of MDA is calculated by difference of the absorbance and 532 and 600 nm using the extinction coefficients of 15.600 $M^{-1}cm^{-1}$ and expressed as nM malindialdehyde (MDA) per g of fresh tissue (f.t.). We used reagents of firms Sigma (USA), Merck (Germany), Reanal (Hungary).

The reliability of the difference were evaluated using ANOVA using a non-parametric method using the U Wilcoxon-Mann-Whitney criterion, Statistica 8.0 software package in excel format. The reliability of the differences between the groups were considered reliable at $p<0.05$.

Results and discussion

As shown in Table 1, one day after treatment with a stressor, the activity of AsAT and AlAT increases in garlic bulbs by an average of 1.5 times relative to control. The treatment of Na_2SeO_4 plants increases the activity of AlAT by an average of 16%, while the AsAT activity indicator only shows a tendency to increase it relative to the control variants. Treatment of Na_2SeO_4 garlic plants on the background of stress leads to a decrease in the activity of AlAT by an average of 13% compared to indicators under stress and an average increase of more than 2 times relative to control values.

If one comes from the notion that AsAT and AlAT play a key role in the metabolism of ala, asp and glu, then an increase in the activity of aminotransferases in garlic bulbs under stress caused by cadmium (Table 1), can be considered as manifestation of an adaptive reaction directed at the synthesis of pyruvate and activation of glucose syntheses in actively growing bulbs.

Table 1. Influence of selenium on an activity of aspartate aminotransferase, alanine aminotransferase and glutamategehydrogenase in garlic bulbs under oxidative stresses, Mean ± sem, n=6.

Variants	AsAT, μmol pyruvate/h mg^{-1} of protein	AlAT, μmol oxaloacetate/h mg^{-1} of protein	GDH nmole NAD/h mg^{-1} of protein
Control	2.63 ± 0.21	1.51 ± 0.08	26.84 ± 1.96
Stress	6.11 ± 0.51*	4.44 ± 0.25*	71.32 ± 5.36*
Na_2SeO_4	2.91 ± 0.16	1.83 ± 0.13*	15.40 ± 0.09*
Stress+Na_2SeO_4	6.32 ± 0.43*	3.90 ± 0.19	46.04 ± 2.85*#

Note: * – differences are significant at $p<0,05$ relative to control; # – differences are significant at $p<0,05$ relative to condition of stresses.

In the work (Mwamba et al., 2016), an increase in the total content of amino acids in the context of increasing the content of asparagine and glycine in *Brassica napus* L. plants under conditions of cadmium stress has been proved.

Treatment of Se plants (Table 1) in the concentration used contributes to a slight increase in the activity of AlAT in relation to control variants. This may be due to the specificity of the action of this compound on individual amino acids of plants, in the interest of which there are data of work (Šindelářová et al., 2015), here an increase in the content of ala and glu in *Brassica oleracea* was observed under conditions of Na_2SeO_4 treatment. Under the combined treatment of garlic plants with Stressor and Se, there is a significant decrease in the activity of AlAT – on average almost 2 times, and AsAT – 33% relative the indicated parameters on variants with influence of a stressor. Absence of influence on the activity of AsAT under the conditions of Na_2SeO_4 plants in the concentration used after a day of exposure, probably, may be explained by the conversion of inorganic form Se into organic, in particular, selenium-containing amino acids and enzymes, which requires more exposure time. So, as coenzymes for the activity of AsAT and AlAT is pyridoxal phosphate, which participates in the synthesis of selenocysteine (Se-Cys) (Garousi, 2017).

The activity of aminotransferases can be controlled by the level of synthesis of substrates and corresponding amides and, as a results, of ammonium nitrogen content in plants. It is known that the acceptors of the latter are, on the one hand, ketoacids, in particular, pyruvic acid, oxalic acid, on the other – its biosynthesis is related to the content of glutamic acid and its glutamine amide. A key link in nitrogen exchange is the conversion of α-ketoglutaric acid and glutamate with the participation of glutamate hydrogenase (GDH), this enzyme is involved in the assimilation of ammonium nitrogen.

As shown in Table 1, a day after treatment with garlic bulbs by the stressor, the activity of GDH increases by an average of almost 3 times relative to the indicators in the control variants. Treatment with Na_2SeO_4 contributes to a decrease in the activity of GDH by an average of 43% relative to the activity of this enzyme in the control.

The treatment of the Na_2SeO_4 garlic of stress leads to a lower reduction in GDH activity, namely an average of 31% in relation to stress indicators and an average increase of 44% relative to control parameters.

Find GDH activity is reduced in salt stress conditions in wheat, but our data demonstrate an increase in the activity of this enzyme due to stress caused by cadmium. There are data (Dresler et al., 2014) to increase the content of gluten meal in the processing of cadmium in *Zea mays* as well as (Takeda & Fukui, 2015), a decrease in the activity of NADH-GDH in Arabidopsis thaliana seeds by the action of Na_2SeO_4.

In addition, non-enzymatic deamination of amino acids in plants is carried out in the polyphenol-polyphenol oxidase system with the participation of chlorogenic acid. The ammonia source for amino acid synthesis may be a deamination reaction of amino acids, in particular aspartate and alanine, which are catalyzed by aspartate ammonia lyase and alanine ammonia,

respectively, which can participate in the synthesis of polyphenols such as coffee, ferulous and chlorogenic acid. Chlorogenic acid is one of the components of the polyphenol-polyphenol oxidase system, in which there is active deamination of amino acids, therefore the content of phenolic compounds (PC) under the conditions of the experiment is investigated.

As can be seen in Table 2, the content of FS in formed onion Garlic sativum in stress conditions increases by an average of 1.52 times relative to the control values. Plant treatment Na_2SeO_4 reduces the FS content by an average of 27% relative to control values. Treatment of Na_2SeO_4 against the background of stress reduces the stress caused by cadmium, as evidenced by a decrease in the content of the PC on average by 46% relative to indicators of stress, as well as the trend towards to control values.

Table 2. Influence selenium on the content of phenolic compounds in garlic bulbs under oxidative stresses, Mean ± sem, n=6.

Variants	**Phenolic compounds, µg chlorogenic acids/g f.t.**
Control	206.4 ± 13.6
Stress	367.2 ± 27.4*
Na_2SeO_4	261.6 ± 10.2*
Stress+Na_2SeO_4	187.3 ± 8.6*#

Note: * – differences are significant at p<0,05 relative to control; # – differences are significant at p<0,05 relative to stresses.

One of the causes of phenolic exchange induction under stress conditions may be an increase in the activity of the key enzyme of this link of metabolism – phenylanalin-ammonia-lyase in stressful conditions.

The experiment revealed an increase in the content of phenolic compounds under conditions of stress caused by cadmium (Table 2). The literature contains data on changes in the content of individual classes of the PS by the action of cadmium. So, accoding (Kosyk et al., 2017) activation of the synthesis of anthocyanins – compounds of flavonoid origin has been discovered for the action of Cd ions and an increase in the antocyanic zone that may be interesting to ascertain the adaptive role of antocyans, and is explained by the antioxidant properties of the latter, as well as the ability of the flavonoids to control the level of hydrogen peroxide that increases in the specified conditions (Manquian-Cerda, 2016). Also, the manifestation of the regulatory properties of compounds of phenolic nature, as oxidic stress can lead to activation of oxidative polymerization of the PS.

A decrease in the content of the FS in the action of Se was found (Table 2). In work (Bo et al., 2017) reduction of the content of PC in garlic bulbs during the action of Se that is consistent with our data, and is associated with the induction of phenylanaline-ammonia-lyase in the action of Se. Besides, Se can directly accelerate the synthesis of chlorogenic acid in plants (Dong et al., 2013), and in our experiments, when calculating the content of the PC as a standard, it is precisely chlorogenic acid used.

The H_2 acceptor in the reactions of deamination of amino acids in plants are quinones, which in the polyphenol-polyphenol oxidase system generate active forms of oxygen (AFO). The activity of the key enzymes of nitric metabolism and the content of the PC may depend on the content of the AFO in plant tissues.

Table 3. Influence selenium on the content of TBA-action products and total SH-groups in garlic bulbs under oxidative stresses, Mean±sem, n=5-7.

Variants	**TBA-action products. nM MDA/g f.t.**
Control	62.18 ± 3.5
Stress	147.73 ± 8.0*
Na_2SeO_4	38.30 ± 2.7*
Stress+Na_2SeO_4	84.6 ± 7.1*#

Note: * – differences are significant at p<0,05 relative to control; # – differences are significant at p<0,05 relative to stresses.

As shown in Table 3, the content of TBA-active products increases on condition of processing by the stressor on average 2.3 times relative to the control indicators that indicates the development of oxidative stress in the conditions of experiment. Under the conditions of processing of wheat plants Na_2SeO_4 there is a decrease in the content of TBA active products on average 1.8 times relative to the control values. In article (Grosh & Biswas, 2017) it has been proved that the effect of Na_2SeO_4 at a concentration of 40 µM leads to an increase in the activity of enzymes of antioxidant defense, in particular, ascorbate oxidase, glutathione reductase and glutathione peroxidase in Triticum aevistinum in conditions of stress caused by the influence of arsenic. Treatment of garlic Na_2SeO_4 against the background of stress leads to a decrease in the content of TBA active products on average 1.7 times in relation to stress indicators, as well as the probable tendency to achieve control parameters, indicating a partial neutralization of the Se toxic effect of cadmium in the investigated concentration. Proved adaptogenic and stress-protective properties Se, its ability to correct the negative effects on plants of stress factors, processing Se increases the content of glutathione and changes in the activity of oxidative-reducing enzymes.

Conclusion

As a result of the study, it was found that in conditions of stress there is an increase in the activity of AsAT and AlAT with a conjoined increase in the activity of NAD-GDH, which can be considerated as exhibition of an adaptive reaction directed at the synthesis of pyruvate, oxaloacetate and glutamate, associated with the activation of sugars in the formed garlic bulbs, as well as an increase in the content of phenolic compounds and TBA-active products, which indicates the development of oxidative stress. Applicate of natrium selenate in the investigated concentration only results in a slight increase in AlAT activity and a tendency to increase AsAT activity in garlic bulbs, which proves the specific effect of the test drug and may be explained by its form and duration of exposure, but more significant effect was observed on the activity of NAD-GDH, in particular, in the direction of lowering the enzyme. The reduction of the content of phenolic compounds with a simultaneous decrease in the content of TBA-active products by treatment with the drug is shown, which proves the antioxidant effect of the investigated preparation under conditions of oxidative stress development. Applicate of natrium selenate in the stress concentration tested shows a partial decrease in the toxic effects of stress, which is confirmed by the data of the content of phenolic compounds and TBA-active products in garlic bulbs under experimental conditions.

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