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An Effect of Various Selenium Forms and Doses on Antioxidant Pathways at Clover (*Trifolium pratense* L.)

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The aim of this study was to determine the effect of different doses and forms of foliar applied selenium on antioxidant indicators and selenium content in the forage of red clover (*Trifolium pratense* L.). This was done in the pot experiment, where the plants were placed in chambers under controlled-environment. In the experiment, different forms of selenium (sodium selenite, and/or sodium selenate), in concentrations corresponding to 2, 4 and 20 $mg.m^{-2}$ was used. Samples of aboveground tissues were taken at 14th, 28th and 42nd day after foliar application of selenium. The obtained samples were analysed using chromatographic and spectrophotometric techniques. After successful foliar application of selenium, the significant ($P < 0.05$) increase of the selenium content in aboveground mass of red clover was found. The maximum values for both forms were achieved in experimental groups treated with the highest concentration of selenium (20 $mg.m^{-2}$). In comparison with selenate, selenite showed approximately 50% less efficient accumulation of selenium in the aboveground mass. From the determined antioxidants indicators, a positive effect in particular on the level of reduced glutathione was observed ($P < 0.05$) in both experimental groups (selenate, and/or selenite). The group treated with selenate had also higher content of phytochelatin 3 ($P < 0.05$). Despite the use of high selenium concentration (20 $mg.m^{-2}$) its toxic effect on plants was not observed.

Keywords: Selenium; Forage; Grass; Multi-Instrumental Analysis; Antioxidant

1. INTRODUCTION

Selenium is an essential element significantly influencing the state of health of plants, animals and humans. Se is incorporated in selenoproteins during their translation, in the form of the amino acid selenocysteine (Sec), a cysteine analogue in which the sulphur-containing thiol is substituted by a selenol group [1]. The selenium containing enzyme - glutathione peroxidase belongs to the best-known selenoproteins, but selenium is also part of other enzymes and antioxidants [2,3]. Adequate intake of selenium in the diet is essential for the protection of biological membranes from oxidative destruction and prevents damage to the body from heavy metals [4]. As a result of the involvement of selenium compounds in numerous biological functions, its deficit impairs overall health status of the animals, increases the susceptibility of juveniles to infectious diseases, causes reproductive disorders or may be directly cause of an illness [5-7]. At the same time, however, it is important to note that the difference between the accepted maximum daily dose and toxic one is very small in case of this micronutrient, and its excess can cause serious poisoning, which can lead to death [8,9]. For food supplementation of animals, plants are the primary source, where one may support the concentration of selenium in an organism. On the other hand, an amount of selenium deposited in animal products depends on the supply of this element, wherein the organic form is more suitable than the inorganic [10,11].

Selenium concentration in plant biomass is derived from its content in the soil and may vary considerably depending on the region [12-14]. Selenium occurs in inorganic form as selenide, selenate and selenite in soil [15,16]. Adequate supply of selenium in plants positively influences the growth and resistance to biotic and abiotic stresses [17].

The main aim of this paper was to reveal the effect of two forms of selenium (selenite vs. selenate) on growth and antioxidant pathways in red clover (*Trifolium pratense* L.). Electrochemical and chromatographic methods and assays were used to reach the main aim.

2. EXPERIMENTAL PART

2.1. Chemicals and material

Reduced (GSH) and oxidized (GSSG) glutathione, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, USA). Phytochelatins (PCs) $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ were synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity above 90 %. HPLC-grade methanol (>99.9%; *v*/*v*) was from Merck (Dortmund, Germany). Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless noted otherwise. Stock standard solutions of the thiols (1 $mg.mL^{-1}$) were prepared with ACS water (Sigma-Aldrich, USA) and stored in dark at -20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany).

2.2. Plant experiment

Clover (*Trifolium pratense* L.) was used in our experiment. The selenium was applied in two forms (IV or VI) as sodium selenite and/or sodium selenite on the leaves in the concentrations of 2, 4 or 20 $mg.m^{-2}$. The experiment was performed in vessels, which were placed in a climate chamber. During the whole experiment, plants kept in the climate chamber were grown at 24 °C (day temperature), 20 °C (night temperature) and 65% humidity (for all 24 h) with a day length of 12 h (light intensity 380 $\mu mol \cdot m^{-1} \cdot s^{-1}$). The above-mentioned selenium doses were applied on leaves 25 days after sieving. The control samples were not affected by selenium during the whole experiment. The leaves from all plants (treated and control) were sampled at 14^{th}, 28^{th} and 42^{nd} day after application of the target substances. The experimental groups were divided into (*A*) selenate and/or (*B*) selenite. The sampled leaves were immediately weighed and frozen to -20 °C prior to detection of total content of selenium and antioxidant activity.

2.3. Preparation of samples for chromatographic and spectrometric measurements

Leave samples (100 mg) were homogenized by mortar and liquid nitrogen for three minutes. Then, 0.5 ml of phosphate buffer (0.2 M, pH 7) was applied to the mortar and homogenization continued for another five minutes. The mixture was processed by manual homogenizer ULTRA-TURRAX T8 (IKA, Germany) at 25,000 rpm for 3 minutes [18] and then by ultrasound needle for 2 minutes. The homogenate was then transferred to a new test-tube. The mixture was further homogenised by shaking on a Vortex–2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (16 400 *g*) for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Prior to analysis, the supernatant was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore, Billerica, Mass., USA) and used for chromatographic and spectrometric measurements.

2.4. Determination of GSH, GSSG, phytohcelatin 3 (PC3) and phytochelatin 4 (PC4)

Chromatographic analysis was determined using high performance liquid chromatography with electrochemical detection (HPLC-ED). HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 $ml.min^{-1}$ (Model 582 ESA Inc., Chelmsford, MA), and a chromatographic column Zorbax eclipse AAA C18 (150 × 4.6; 3.5 nm particles, Agilent Technologies, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes one flow cell (Model 6210, ESA, USA). The cell consists of four working carbon porous electrodes, each one with auxiliary and dry Pd/H_2 reference electrodes. Both, the detector and the reaction coil/column were thermostated. The sample (20 μL) was injected using autosampler (Model 542 HPLC, ESA, USA). Samples were kept in the carousel at 8 °C during the analysis. The column was thermostated at 35 °C. Mobile phase consisted of 80 mM TFA (A) and 100% methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 7 min. (3% B), 7

→ 15 min. (15% B), 15 → 28 min. (30% B), and 28 → 33 min (98% B). Flow rate of the mobile phase was 1 $ml.min^{-1}$ and working electrode potential was set to 900 mV

2.5. Spectrophotometric measurements of total protein content and oxidative stress

Spectrophotometric measurements of total protein content and oxidative stress (ABTS, DPPH) were carried using an automated chemical analyser BS-400 (Mindray, Shenzhen, China). Reagents and samples were placed on cooled sample holder (4 ± 1 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37.0 ± 0.1 °C. Mixture was consequently stirred. The washing steps of pipetting needle with distilled water (18 mΩ) were done in the midst of the pipetting. For detection itself, the following range of wave lengths can be used - 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm. The instrument was operated using the BS-400 software (Mindray).

2.6. Determination of total proteins by the pyrogallol red

The pyrogallol red protein assay (Skalab) is based upon formation of a blue protein-dye complex in the presence of molybdate under acidic conditions (pH=2.5). Briefly, 150 µl volume of reagent mixture (1:1) R1+R2 (50 mM succinic acid, 3.47 mM sodium benzoate, 0.06 mM sodium molybdate, 1.05 mM sodium oxalate and 0.07 mM pyrogallol red) was pipetted into a plastic cuvette with subsequent addition of 8 µl of sample. Absorbance was measured at λ = 605 nm after 10 minutes of incubation. Resulting value was calculated from the absorbance value of the pure reagent mixture and from the absorbance value after 10 minutes of incubation with the sample.

2.7. Determination of antioxidant activity by the ABTS method

The ABTS radical method is one of the most used assays for determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-electron oxidation of the synthetic chromophore 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): ABTS• – e- ABTS•+. This reaction was monitored spectrophotometrically by the change of the absorption value. Briefly, 150 µL volume of reagent (7mM ABTS• (2,2´-azinobis 3-ethylbenzothiazoline-6-sulfonic acid and 4.95 mM potassium peroxodisulphate) was incubated with 3 µL of sample. Absorbance was measured at λ = 660 nm for 12 minutes. For calculating of the antioxidant activity, values determined before decrease of the absorbance (2^{nd} minute of measurement – A2) and the last measurement value (12^{th} minute of measurement – A12) were used. Resulting value was calculated in accordance with following formula: Differential absorbance A = A12 – A2.

2.8. Determination of antioxidant activity by the ferric reducing antioxidant power (FRAP) method

The FRAP method is based on the reduction of complexes of 2,4,6-tripyridyl-s-triazine (TPTZ) with ferric chloride hexahydrate (FeCl3•6H2O), which are almost colourless, and eventually slightly

brownish. This chemical forms blue ferrous complexes after its reduction. The method has its limitations, especially in measurements under non-physiological pH values (3.6). In addition, this method is not able to detect slowly reactive polyphenolic compounds and thiols. Briefly to the conditions, reagent preparation – Solution 1: 10 mM solution of TPTZ in 40 mM of hydrochloric acid; Solution 2: 20 mM solution of ferric chloride hexahydrate in ACS water; and Solution 3: 20 mM acetate buffer, pH 3.6. These three solutions are mixed in a 1:1:10 ratios. Reagent can be used for seven days if stored at 4 °C in the dark. Briefly, to the procedure, 150 μL volume of reagent was pipetted into a plastic cuvette with subsequent addition of 3 μL sample (gallic acid). Absorbance was measured at λ = 605 nm for 12 minutes. For calculating of the antioxidant activity, values before decrease of absorbance (2^{nd} minute of measurement – A2) and last (12^{th} minute of measurement – A12) were used. Resulting value was calculated in accordance with following formula: Differential absorbance A = A12 – A2.

2.9. Determination of antioxidant activity by the free radicals method

This method is based on ability of chlorophyllin (the sodium-copper salt of chlorophyl) to accept and donate electrons with a stable change of maximum absorption [19]. This effect requires an alkaline environment and the addition of a catalyst. Briefly, to the procedure, the reagent (150 μL) was injected into a plastic cuvette with the subsequent addition of 6 μL sample. The absorbance was measured at 450 nm in the second and last (12^{th}) minute of the assay. The difference between two absorbances was considered to be the output value.

2.10. Determination of antioxidant activity by the DPPH test

The DPPH test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors. The DPPH• radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolourized after reduction with antioxidant (AH) or a radical (R•) in accordance with the following scheme: DPPH• + AH → DPPH-H + A•, DPPH• + R• → DPPH-R. Briefly to the procedure, 150 μl of R1 reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl - DPPH) was pipetted into plastic cuvette. Subsequently, volume of 15 μL of sample was added. DPPH has strong absorption in UV-VIS spectrum, absorbance was measured for 12 min at λ = 505 nm. To assess the production of free radicals absorbance difference of the reagent without sample and reagent with sample after ten-minute incubation was taken. Then, the absorbance difference was recalculated per gram of proteins determined in the sample. After the subtraction of the blank value, the highest absorbance difference was taken as 100% of amount of free radicals. The higher was the amount of free radicals, the higher was oxidative stress.

2.11. Preparation of samples for electrochemical determination selenium

A sample (10 mg) was weighed out to glass vials and 500 μL of mineralization mixture (hydrogen peroxide (150 μL, 30%, *v*/*v*) and nitric acid (350 μL, 65%, *w*/*w*)) was added. Prepared

samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria). Rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under the following conditions: power 100 W, ramp 10 min, hold 99 min, cooling 10 min, maximum temperature 80 °C. A blank digestion was simultaneously carried out in the same way.

2.12. Determination of selenium

Determination of selenium by differential pulse voltammetry were performed with 797 VA Computrace instrument connected to 889 IC Sample Centre (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4mm^2 was used as the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was auxiliary. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%). 0.128 M ammonium sulphate, 0.123 mM copper sulphate and sulphuric acid (to adjust pH to 2.2) as a supporting electrolyte were used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as it follows: initial potential of -0.4 V, end potential -0.9 V, deoxygenating with argon 120 s, accumulation time 200 s, deposition potential -0.6 V, time interval 0.05 s, potential step 6 mV, pulse amplitude 30 mV, volume of injected sample: 20 μL, and volume of measurement cell 2 mL (20 μL of sample + 1980 mL electrolyte).

2.13. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences between content of selenium, thiols and antioxidant activity were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one-way ANOVA test (particularly Scheffe test), which was applied for means comparison.

3. RESULTS AND DISCUSION

3.1. Phytochelatins

PC3 level was significantly reduced ($P<0.05$) in tissues obtained from plants treated with 4 mg.m^{-2} Se in the form selenite (B4 group) after 28th and/or 42nd days of the experiment. In comparison with the control group, the content PC 3 in this group was lower by 22 % and/or 21 %, respectively (Fig. 1A). After successful foliar application of sodium selenate at concentration of 4 and 20 mg.m^{-2} concentration of PC3 was found to be significantly increased ($P<0.05$) in all evaluated sampling periods. In the case of experimental A2 group (2 mg.m^{-2}), this increase was determined at the 28th and 42nd day after a foliar application (Fig. 1B). After application of selenite, PC4 level was reduced ($P<0.05$) compared with the control group at 14th of sampling (experimental group B4, Fig.

1C). In comparison with the control group, there was observed decrease ($P<0.05$) of the content of PC4 in plants treated with 20 mg.m^{-2} Se (A20 group) at 28th day. The content of PC4 was lower by 45 % (Fig. 1D).

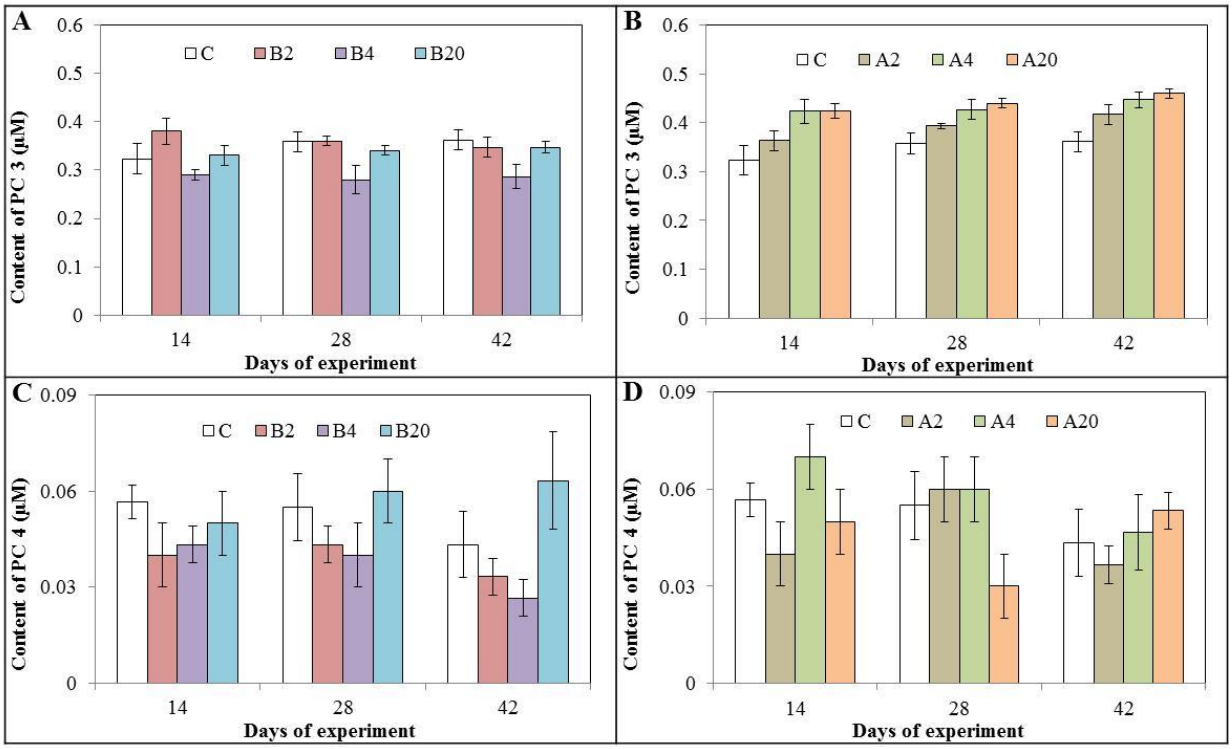

Figure 1. The influence of different doses of selenium on PC 3 level: selenite (A); selenate (B) and on PC 4 level: selenite (C), selenate (D) C – control, A2 – selenate 2 mg.m^{-2} Se, A4 – selenate 4 mg.m^{-2} Se, A20 – selenate 20 mg.m^{-2} Se, B2 – selenite 2 mg.m^{-2} Se, B4 – selenite 4 mg.m^{-2} Se, B20 – selenite 20 mg.m^{-2} Se

3.2. Selenium content

The selenium content in samples was determined using cathodic stripping voltammetry (CSV) in the presence of copper. Among other electroanalytical techniques, anodic stripping voltammetry at gold electrodes [20,21] and adsorptive stripping voltammetry [22], is this technique the most sensitive. Applied determination of Se(IV) is based on the cathodic stripping of of Cu2Se at mercury electrode. In CSV at a hanging mercury drop electrode, acidic media gave a much better sensitivity for the deposition of selenium than did neutral or basic electrolytes. In acidic solutions, Se(IV) is reduced to HgSe at a mercury electrode:

$$H_2SeO_3 + Hg + 4H^+ + 4e^- \longrightarrow HgSe + 3\,H_2O$$

Codeposition of Se(IV) and Cu(II) has been proposed for increasing the sensitivity of CSV. Copper(I) selenide is most probably formed as a result of the reaction of HgSe with Cu(Hg) at a potential of -0.5 V at a mercury drop electrode [23]. The deposition of selenium in the presence of Cu(II) can be represented by [21]:

$H_2SeO_3 + 2Cu^{2+} + 4H^+ + 8e^- \rightarrow Cu_2Se + 3H_2O$

The accumulated Cu_2Se is reduced in a single stripping reaction represented with characteristic peak at a potential of –0.7 V:

$Cu_2Se + 2H^+ + 2e^- \rightarrow H_2Se + 2\ Cu(Hg)$

Se(IV) can also be accumulated as HgSe but the Cu_2Se stripping peak yields a lower detection limit (about 20 pM of Se(IV) [23]), a longer linear calibration curve and is less subject to interference from peak splitting and from metal ions present in the sample.

The selenium content was significantly ($P<0.05$) increased in all periods of sampling in the experimental group treated with the highest concentration of selenite (B20). The increase ($P <0.05$) in the content of selenium was also observed for B2 group after 14 and 42 days after the performed foliar application and after 42 days in B4 group (Fig. 2A). Higher ($P<0.05$) effectiveness of selenium accumulation was observed in the groups treated with selenate. Similar to groups treated with selenite, the highest values were found in experimental group treated with the highest concentration of selenate. In comparison with the control group, eight-fold increase selenium content in aboveground mass was determined. The significant increase ($P<0.05$) of selenium content was also found at A4 group in all sampling periods and A2 group at 28th and 42nd day of collection (Fig. 2B).

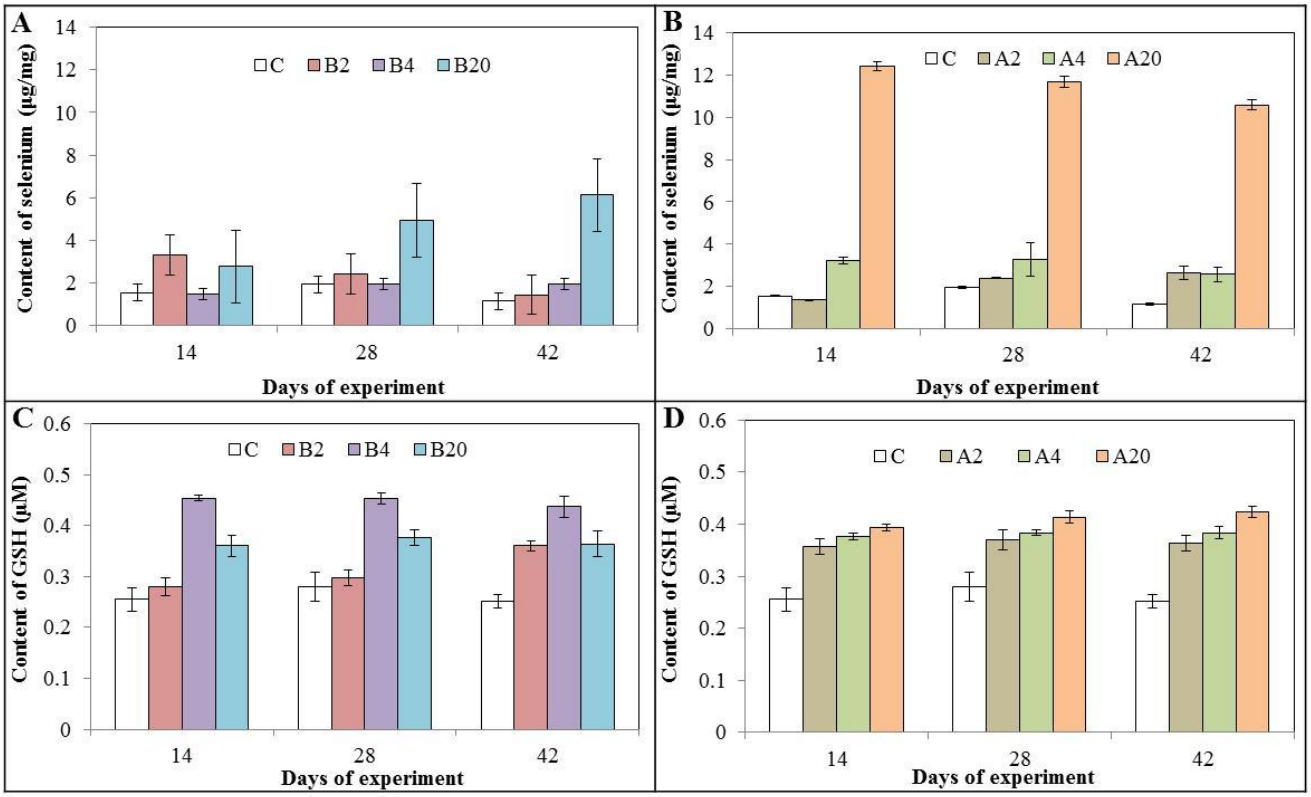

Figure 2. The influence of different doses and form of selenium on selenium content in the biomass of red clover: selenite (A); selenate (B) and on GSH level: selenite (C), selenate (D) C – control, A2 – selenate 2 mg.m^{-2} Se, A4 – selenate 4 mg.m^{-2} Se, A20 – selenate 20 mg.m^{-2} Se, B2 – selenite 2 mg.m^{-2} Se, B4 – selenite 4 mg.m^{-2} Se, B20 – selenite 20 mg.m^{-2} Se

3.3. Reduced glutathione and mass

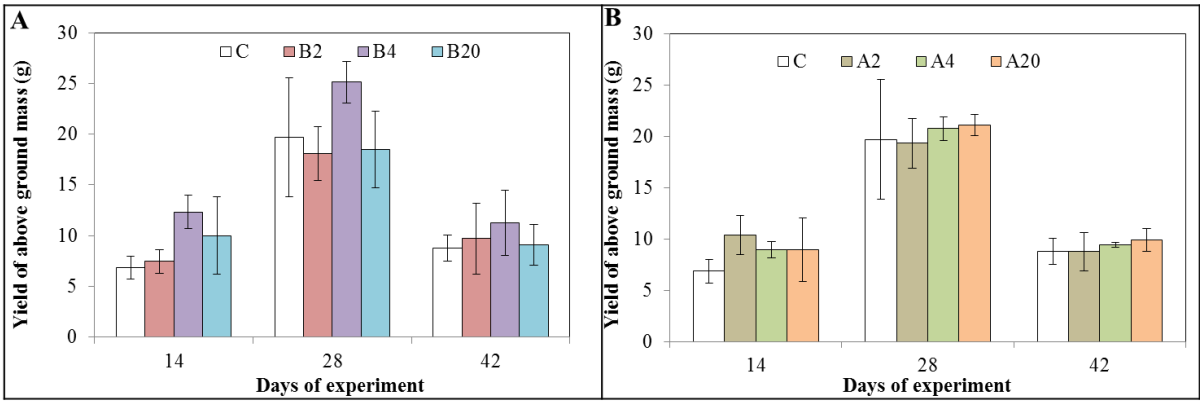

Figure 3. The influence of different doses and forms of selenium on yield of above ground mass of red clover: selenite (A); selenate (B) C – control, A2 – selenate 2 mg.m^{-2} Se, A4 – selenate 4 mg.m^{-2} Se, A20 – selenate 20 mg.m^{-2} Se, B2 – selenite 2 mg.m^{-2} Se, B4 – selenite 4 mg.m^{-2} Se, B20 – selenite 20 mg.m^{-2} Se

In the experimental group treated with selenite, content of GSH in all sampling periods was significantly ($P<0.05$) higher, with the highest found at B4 group, which contained 44 % more GSH compared with the control group. Significantly ($P<0.05$) increased levels were also observed in the group with the highest used concentration of selenium (B20) in the form of selenate at all sampling dates and B2 group 42 days after foliar application (Fig. 2C). In the case of foliar application of selenite, the significant ($P<0.05$) increase in GSH content in all experimental groups (Fig. 2D) was observed. The highest value was reached at 42th day after treatment (A20 group). Besides GSH content, the yield of aboveground mass was not negatively affected by foliar application of selenium. The mass determined at groups B4 and A2 was even significantly ($P<0.05$) higher at 14th day after treatment (Figs. 3A and 3B).

3.4. Antioxidant activity

In the evaluation of antioxidant status by FRAP, a significant increase ($P<0.05$) in B20 group 14 days after foliar application of selenite was recorded. The significant ($P<0.05$) decrease in the antioxidant activity was detected at 28th day of sampling for all experimental groups (B2, B4, and B20) and at 42nd day of sampling for B20 group (Fig. 4A). After foliar application of selenium as selenate, the value of antioxidant status determined by FRAP was higher ($P<0.05$) in A20 (14th day of sampling) and A4 (42nd day of sampling). Conversely, the decrease ($P<0.05$) was observed in groups of A4 (14th day of sampling), A2 (28th day of sampling) and A20 (42nd day of sampling), which is shown in (Fig. 4B).

Analysis of antioxidant status by DPPH showed increase ($P<0.05$) scavenging of the radicals at 14th day after application of selenite with the highest used concentration of selenium (B20). The groups B2 and B20 showed decrease ($P<0.05$) in this indicator to 7 % or 16 %, respectively, compared with

the control group (Fig. 4C). In the case of the experimental group treated with selenite, significant increase ($P<0.05$) in A2 group after 14 days after the application was observed. Significantly ($P<0.05$) lower percentage of scavenging of the radicals relative to the control group was observed in all experimental groups at 42 days after application and at A20 after 28 days of the treatment (Fig. 4D).

In the evaluation of antioxidant status measured by ABTS, significant ($P<0.05$) increase at 14th day after treatment at B20 group was found. The decrease ($P<0.05$) of this value was observed at 28th and 42nd day of collection of aboveground mass at groups B4 and B20 (Fig. 5A). In comparison with the control group, 15% increase ($P <0.05$) in the antioxidant status measured using ABTS in A2 group 14 days after foliar application occurred. The A4 and A20 groups (28th day of sampling), and A2 and A20 groups (42nd day of sampling) showed significant ($P <0.05$) decrease in this value (Fig. 5B).

The significant ($P <0.05$) increase in the antioxidant status measured by FR method was found at 14th day after treatment for B4 group. In comparison with the control group, there was an increase by 36 %. The decrease ($P<0.05$) in this value was observed in B4 group at 42nd day after treatment (Fig. 5C). Increases ($P <0.05$) in the antioxidant status by 34 % and/or 43 % at 14th day of sampling for A2 and/or A20 group, respectively were also found. The decrease ($P<0.05$) of this value was observed in A20 group at 42nd day of sampling (Fig. 5D).

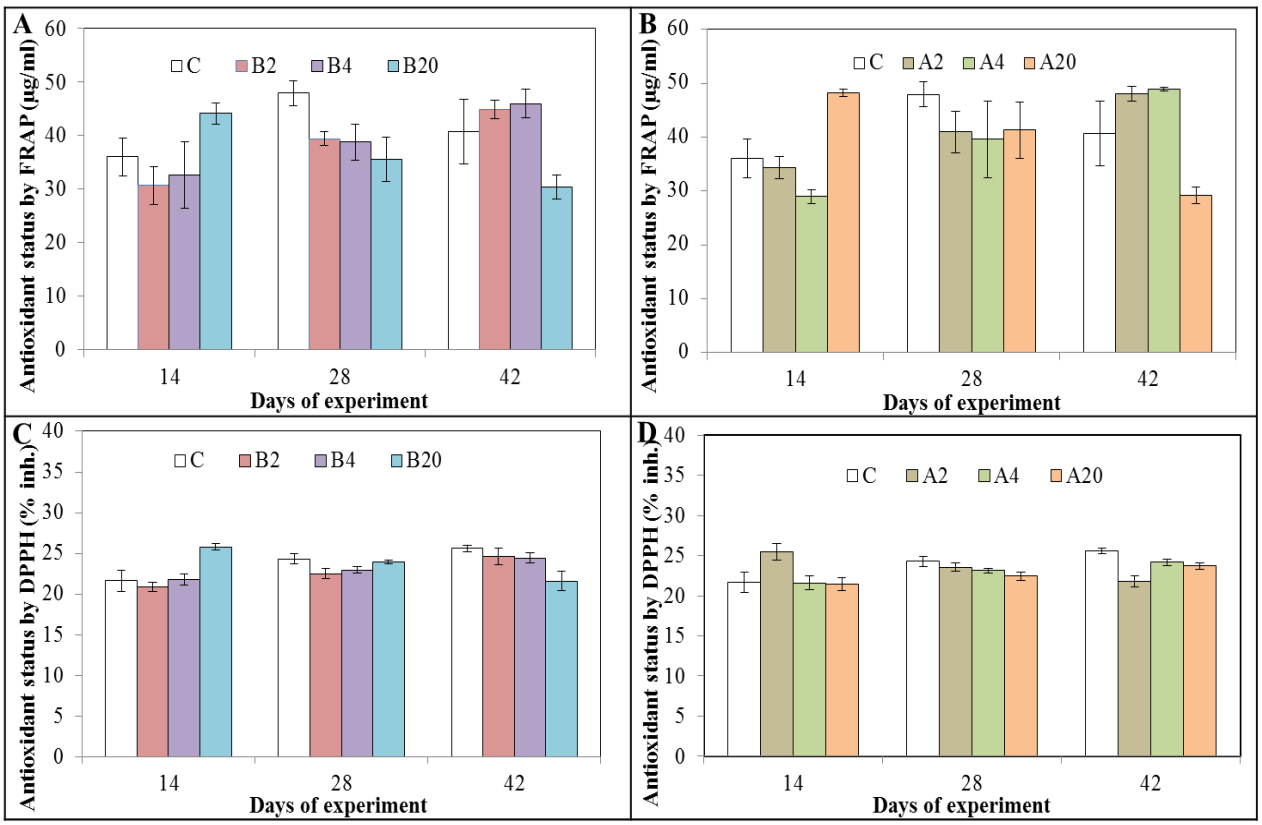

Figure 4. The influence of different doses and forms of selenium on antioxidant status of red clover measured by FRAP method: selenite (A); selenate (B) and by DPPH method: selenite (C), selenate (D) C – control, A2 – selenate 2 mg.m^{-2} Se, A4 – selenate 4 mg.m^{-2} Se, A20 – selenate 20 mg.m^{-2} Se, B2 – selenite 2 mg.m^{-2} Se, B4 – selenite 4 mg.m^{-2} Se, B20 – selenite 20 mg.m^{-2} Se

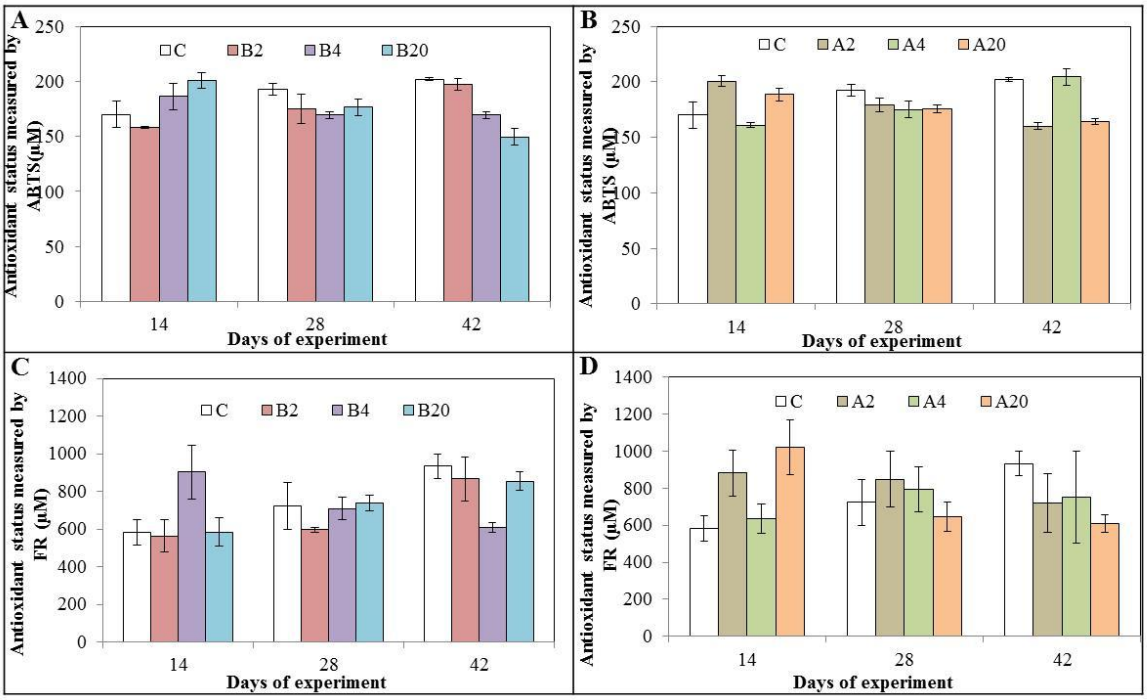

Figure 5. The influence of different doses and forms of selenium on antioxidant status of red clover measured by ABTS method: selenite (A); selenate (B) and by FR method: selenite (C), selenate (D) C – control, A2 – selenate 2 mg.m^{-2} Se, A4 – selenate 4 mg.m^{-2} Se, A20 – selenate 20 mg.m^{-2} Se, B2 – selenite 2 mg.m^{-2} Se, B4 – selenite 4 mg.m^{-2} Se, B20 – selenite 20 mg.m^{-2} Se

3.5. An evaluation of selenium effect

In our experiment, two different forms of selenium (selenite and selenate) and their different doses 0, 2, 4 and/or 20 mg.m^{-2} were used. It clearly follows from the results obtained that the content of selenium increased significantly in selenium-treated groups independently on the form of selenium. Similar results were obtained when adding selenate in grass silage with 50 mg of selenium per kilogram [24]. According to Ramos et al. [25], selenate dose of 6 mg.kg^{-1} significantly increased the content of selenium in plant biomass in comparison with sodium selenite. This fact was observed in our experiment, too. Additionally, this team of authors argue that this increased accumulation of selenium should result in higher antioxidant status of grasses. In our study, this trend has not been fully confirmed, because we have found the increase of GSH content in groups treated by selenite or selenate. Another group showed in the experiment, where selenium was dosed as selenite at dose of 0-60 g Se per ha on the crop of white clover (*Trifolium repens* L.), that the yield of vegetable matter was reduced at dose of 20 g per ha. At the same time, concentration of selenium and activity of antioxidative enzymes linearly increased depending on the content of applied selenium [26]. However, we did not reveal any decrease in the yield. Conversely, in the first days of the experiment, the higher growth in crops of clover, which were treated with selenium (even at a dose of 20 mg.m^{-2}) was documented. In the experiment, which was dedicated to fertilization of ryegrass (*Lolium perenne*), selenite or selenium at dose of 0-10 mg.kg^{-1} did not cause any difference between each other [19]. It

clearly follows from the results obtained that not only the form of selenium, but also the actual plant species influences its accumulation.

Application of selenium to crops of red clover, according to some authors, can reduce the impact of stress conditions (e.g. drought), keep constant level of GSH under these stress conditions and increase the activity of some antioxidant enzymes [27]. We found that the application of selenium in the form of selenite or selenate increased concentration of GSH. The antioxidant activity as measured by FRAP was also found higher compared to untreated plants. If we build on the results of Wang et al. [27,28], we can say that the plants were not exposed to stress conditions in our study, especially when drought reduces selenium content in plants. It follows that the application of selenium (sodium selenite) on leaf plants seems to be more effective from the viewpoint of absorption compared to selenate [29]. In addition to this, the application of selenium directly on leaf plant characterized by high efficiency, in particular the use of selenate as it was confirmed here [30], too. Selenite was compared to selenate approximately 50 % less efficient accumulation of selenium in plants. According to Kaur et al. [17], selenium dose of 1 $mg.kg^{-1}$ should have a positive impact in terms of plant health. Higher doses of selenium than this may have a negative effect. In this case, none of these signs of toxicity observed even when using high doses of selenium.

4. CONCLUSIONS

The foliar application of selenium proved to be a very effective method to increase antioxidant indicators and selenium content in aboveground mass of red clover. After successful foliar application of selenium, there was significant ($P < 0.05$) increase in the selenium content in aboveground mass of red clover. The maximum values for both forms (selenite and selenate) were achieved in groups treated with the highest concentration of selenium (20 $mg.m^{-2}$). In comparison with selenate, selenite showed approximately 50% less efficient accumulation of selenium in the aboveground mass. In antioxidants indicators, the positive effect on the level of GSH was observed. In the groups treated with selenate, content of PC3 increased ($P < 0.05$), too. Despite the use of high selenium concentration, no toxic effect on plants was observed.

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Conflict of interest:
The authors have declared no conflict of interest

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