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Metabolic and Oxidative Responses Associated with Exposure of *Eruca sativa* (Rocket) Plants to Different Levels of Selenium

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ABSTRACT

Rocket (*Eruca sativa* L.) plants grown in sand culture were exposed to different concentrations (0, 5, 10, 100, 1000, 2000 and 3000 μM) of sodium selenate for 10 days. Selenate up to 10 μM enhanced the growth and levels of chlorophylls, sugar and amino acids. However, high levels of selenate (100 μM and up) exert toxic effects. Selenium uptake by rocket plants reduced uptake of some nutrient elements (P, K, Ca, Mg, Fe and Se). Selenate significantly increased the uptake of Ca^{2+} ions. Proline level was significantly increased in the Se-treated plants. The levels of non-enzymatic antioxidants (glutathione, ascorbic acid and carotenoids) were increased by selenate. Low selenate (5 μM) diminished lipid peroxidation as measured by malondialdehyde. The activities of enzymatic antioxidants (superoxide dismutase, ascorbate peroxidase, ascorbate oxidase, guaiacol peroxidase) in rocket green tops were enhanced by low level of selenate. The results showed that both enzymatic and non enzymatic antioxidants as well as Ca^{2+} played significant roles in selenate detoxification. Rocket plants can survive in soil contaminated with low level of selenate up to 100 μM.

Key Words: *Eruca sativa* L.; Selenium; Metabolites; Antioxidants; Phytoremediation

INTRODUCTION

Selenium (Se) is a trace element which is vital to life since its deficiency is believed to be involved in several diseases in human (Zachara *et al*., 2004). Rocket plant belongs to family *Cruciferae* and might contain and tolerate high concentrations of selenium (Zayed & Terry, 1992).

Plants vary considerably in their physiological response to selenium. Some plants are Se tolerant and accumulate very high concentrations of Se (Se accumulators, e.g. members of *Cruciferea*), but most plants are Se non-accumulators and are Se sensitive (Terry *et al*., 2000). It was thought that selenium might be essential for growth of the accumulator species but there is no definitive supporting evidence (Hopkins, 1995).

Selenium was shown to affect several physiological and biochemical processes, in plant species (Baszynski *et al.*, 1980; van Assche, 1988; Moya *et al.*, 1993; Pennanen *et al*., 2002; Kinraide, 2003). Selenium can also regulate some ions absorption (e.g. phosphorus, Moore *et al.*, 1998). It was reported that heavy metals induces oxidative stress in plants through the production of reactive oxygen species (Briat & Lebrun, 1999). Plants respond to heavy metal toxicity in a variety of different ways, such responses include immobilization, exclution, chelation and compartmentalization of metal ions (Cobbett, 2000).

Exogenous selenium (low concentration) can reduce the intensity of peroxide processes of membrane lipids and affect the activity of redox enzymes and thereby change the oxidation-reduction status of the cell, thereby increasing stress tolerance of caucasian goat's rue leave (Vikhreva *et al*., 2002). Recently, selenium has been found to increase the antioxidative capacity and stress-defending ability of lettuce plants and enhanced the growth of UV stressed plants (Pennanen *et al*., 2002). Plants respond to oxidative stress through increasing the enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants may include superoxide dismutase (SOD), catalase (CAT), ascorbic acid peroxidase (APX) and guaiacol peroxidase (GPX), (Cao *et al.*, 2004). The non-enzymatic antioxidants may include glutathione, ascorbic acid and carotenoids (Smirnoff, 1996; Noctor & Foyer, 1998).

Selenium insensitive plants, which are selenium accumulators, accumulated non-protein selenoamino acids, which contain most of the accumulated selenium, which helps and act in selenium detoxification (Jackson *et al.*, 1990). It was postulated also that selenium accumulators are capable of excluding selenoamino acids from their proteins (Brown & Shrift, 1981).

This study was an attempted to use selenium as micronutrient to study the bioeffects of high levels of selenate on rocket plants, which can be used as phytoremdiator. This will help to use the Se-enriched shoot tops (edible parts) as a new source of mineral dietary supplements.

MATERIALS AND METHODS

Plant material and growth conditions. Seeds of rocket (*Eruca sativa*) were purchased from a local market. The seeds were planted in water washed - sand culture in a greenhouse under natural conditions of light, temperature and humidity (14 h photoperiod, 32°C and 70% humidity). Twenty days after emergence, the plants were watered every two days with 100 ml of 1/2-strength Hoagland's solution containing 0, 5, 10, 100, 1000, 2000 or 3000 μM sodium

selenate for further 10 days. After 10 days of selenium exposure, the plant were harvested and growth parameters (shoot and root fresh weight and dry weight, shoot and root length were determined). The rocket plants subjected to high levels of selenium greater than 1000 μM were wilted and died after the second irrigation. Four groups of the rocket plant subjected to 0, 5, 10 and 100 μM selenate were chosen for measuring growth parameters and pigment contents. Since 5 μM selenate had the most stimulatory effect and 100 μM had inhibitory effect, both concentrations were used in this study. All treatments were replicated five times.

Elemental Analysis

Extraction and analysis of minerals. The concentrations of phosphorus, potassium, calcium, magnesium and iron were extracted from the dried tissues of the green tops or roots by wet digestion method (Jones & Case, 1990), using acid mixture (nitric: sulphuric and perchloric; 2.5:0.5 : 1, v : v : v). These mineral ions were estimated by inductively Coupled Plasma (ICP) Emission Spectroscopy as described by Donohue and Aho (1992). Selenium was extracted from plant tissues and make complex with 2, 3 diaminonaphthalene and cyclohexan. Selenium concentration was determined spectrophotometerically according to Lott *et al*. (1963).

Determination of photosynthetic pigments. Chlorophyll a, chlorophyll b and total carotenoids were extracted in 85% acetone, measured spectrophotometrically and calculated according to the formula of lichtenthaler (1987).

Determination of carbohydrates. Soluble sugars were extracted in 80% hot ethanol. Sugar free residues were extracted with 1.5 N H_2SO_4 following the method described by Naguib (1963). Total soluble sugars and those resulting after polysaccharides hydrolysis were estimated by anthrone reagent (Fairbairn, 1953).

Determination of free amino acids and proline. Free amino acids were extracted from fresh plant tissues with 80% ethanol (4^{o}C, 15 min) according to the method described by Vartanian *et al*. (1992) and determined using a standard ninhydrin assay (Yemm & Cocking, 1955). Proline was determined colorimetrically according to the method of Bates *et al*. (1973).

Determination of water soluble antioxidants. Total ascorbic acid content was determined according to the method described by Kampfenkel *et al*. (1995) as modified by de Printo *et al*. (1999). Glutathione was assayed according to the method described by Griffith (1985).

Determination of antioxidant enzyme activities. The plant tissue was frozen in liquid nitrogen and homogenized in a prechilled morter using a pestle. A known volume of 100 mM phosphate buffer (pH 7) containing 5 mM 2-mercaptoethanol was added. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 10000 g for 20 min. The supernatant was used for enzyme assays. Cu-Zn superoxide dismutase (Cu-Zn SOD) was measured by the photochemical method described by Giannopolitis and Ries (1977). Guaiacol peroxidase (GPX) activity was determined according to the method adopted by Bergmeyer (1974). Ascorbate Peroxidase (APX) assay was performed using the method of Koricheva (1997) as modified by Cao *et al*. (2004). Ascorbic acid Oxidase (AO) activity was assayed according to the method of Maxwell and Bateman (1967). Catalase (CAT) activity was assayed according to the method of Chen *et al*. (2000).

Oxidation product estimation. The degree of lipid peroxide formation was assayed by measuring the accumulated malondialdehyde (MDA). MDA was measured by the method of Minotti and Aust (1987).

Protein Assay and Electrophoresis

Extraction, estimation and characterization of proteins. Soluble proteins were extracted from the green tops of rocket plant using a ratio of 1:1 tissue/extraction mixture. The extraction mixture contained tris-HCl buffer (100 mM tris, PH 7.5, 4 mL B-mercaptoethanol, 0.1 mM EDTA-Na_2, 10 mM KCl and 10 mM MgCl). The crude homogenate was centrifuged at 1000 g for 20 min. The supernatant was used for quantitative estimation of total soluble protein by the method described by Bradford (1976) with BIO-RAD protein assay dye reagent (BIO-RAD Chemical Company, Richmond, Ca), using bovine serum albumin (BSA) as a standard.

Electrophoretic protein profile of rocket green tops were characterized and identified by using one-dimensional sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gel (12.5%) was prepared according to the method of Laemmli (1970). The destained gel was analyzed by Gel Documentation System (UVP'S GDS 8000, California, USA. The band pattern, molecular weights and the relative concentration of each gel were analyzed.

Statistical analysis. Data are presented as mean with standard error. The significance of the data was assessed using paired student's t-test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of selenium on plant growth. The length, fresh weight and dry weight of rocket shoots and roots were increased in response to low concentration of selenate (5 and 10 μM). However, application of high selenate concentration (100 μM)) significantly reduced the length, fresh weight and dry weight of rocket shoots and roots (Table I). Our results are in agreement with those of Pennanen *et al.* (2002) who report a toxification effect of Se at high concentration and promotive effects at low Se level on lettuce plants in response to UV. Similar result was demonstrated in *Spirulina platensis* by Zhiyong *et al.* (2003). High Se may inhibit photosynthesis, impair nutrient uptake and transport (Kahl, 1988**).**

Table I. Effect of Se treatments on growth parameters of rocket plants Data are expressed as the means of five different replications ± SE.

Growth parameter	Selenate concentrations (μM)			
	0	5	10	100
Shoot length (cm)	13.83 ± 0.577	17.3 ± 0.58	15.6 ± 0.58	10.0 ±1.16
Root length (cm)	3.9 ± 0.115	4.9 ± 0.82	4.5 ± 0.15	2.6 ± 0.14
Fresh weight of shoot (g)	0.295 ± 0.13	0.79 ± 0.30	0.34 ± 0.11	0.13 ± 0.08
Fresh weight of root (g)	0.016 ± 0.006	0.018 ± 0.005	0.017 ± 0.09	0.0062 ± 0.004
Dry weight of shoot (g)	0.037 ± 0.017	0.098 ± 0.068	0.054 ± 0.01	0.023 ± 0.009
Dry weight of root (g)	0.0033 ± 0.001	0.0065 ± 0.002	0.0053 ± 0.002	0.0011 ± 0.001

Table II. Effect of Se treatments on photosynthetic pigments of rocket leaves The results are expressed as μg g^{-1} F wt. Each value is the mean of five different replications ± SE

Se concentration (μM)	Chl. a	Chl. B	carotenoids	Total pigments
0	7.82 ± 0.171	6.4 ± 0.23	1.18 ± 0.09	15.4 ± 0.23
5 M	12.1 ± 0.46	11.63 ± 5.8	0.34 ± 0.11	24.1 ± 0.58
10 M	11.7 ± 0.185	11.18 ± 0.104	0.311 ± 0.18	23.3 ± 0.115
100 M	7.4 ± 0.173	5.31 ± 0.4	1.418 ± 0.72	14.1 ± 0.32

Table III. Effect of Se treatments on the concentrations (%) of Phosphorus, Potassium, Calcium, Magnesium, Iron and Selenium content of rocket green tops and roots Each value is the mean of five different replications ± SE.

Element concentration	Treatments					
	0.0		5 μM		100 μM	
	Shoot	Root	Shoot	Root	Shoot	Root
Phosphorus	0.543 ± 0.23	0.524 ± 0.23	0.562 ± 0.11	0.351 ± 0.13	0.446 ± 0.17	0.286 ± 0.09
Potassium	1.911 ± 0.58	1.6 ± 0.12	2.048 ± 0.5	1.341 ± 0.7	1.6 ± 0.12	0.663 ± 0. 21
Calcium	0.175 ± 0.08	0.284 ± 0.07	0.398 ± 0.09	0.354 ± 0.11	0.551 ± 0.26	0.498 ± 0.13
Magnesium	0.253 ± 0.13	0.266 ± 0.15	0.29 ± 0.15	0.281 ± 0.08	0.076 ± 0.042	0.109 ± 0.081
Iron	0.075 ± 0.03	0.292 ± 0.17	0.085 ± 0.04	0.218 ± 1.04	0.071 ± 0.03	0.201 ± 0.08
Selenium	0.00	0.00	0.44 ± 0.15	0.023 ± 0.04	0.653 ± 0.21	0.135 ± 0.09

Effect of selenium on photosynthetic pigments. Exposure of rocket plants to low concentrations of selenate (5 and 10 μM) induced a significant increase in chlorophyll a and b contents (Table II). On the other hand, a significant reduction in carotenoid contents was observed in rocket leaves exposed to low levels of selenate. High concentrations of selenate (100 μM) significantly reduced the amount of chlorophyll a, chlorophyll b and total pigment content. However, it increased the carotenoids content in rocket (Table II). The increase in chlorophyll a and chlorophyll b contents of rocket leaves may be attributed to Se effect on protection of chloroplast enzymes and thus increasing the biosynthesis of photosynthetic pigments (Pennanen *et al.*, 2002). Selenate high concentration induced reduction in chlorophyll content observed in this study is consistent with the result of Panmaja *et al*. (1989) and Padmaja *et al*. (1995). The previous authors indicate that high Se has an adverse effect on the production of porphobilinogen synthetase required for chlorophyll biosynthesis and also inhibits biosynthetic enzymes through lipid peroxidation.

On the other hand, carotenoid was significantly increased in rocket leaves subjected to 100 μM selenate. Carotenoids can protect the photosystems by reacting with lipid peroxidation products (Burton & Ingold, 1984).

Effect of selenium on nutrient contents. Selenium modulated the element levels in either green tops or roots of rocket plants (Table III). The concentration of P, K, Mg and Ca were significantly increased in the rocket green tops exposed to 5 μM selenate. However, a significant decrease in P, K and Mg levels were observed at 100 μM selenate. Fe was non- significantly affected in green tops subjected to selenate treatments. In roots, P, K, Mg and Fe levels were markedly decreased at all Se levels. Changes in nutrient levels were most pronounced in roots (Gussarsson, 1994).

It was recorded that, potassium absorption is competitively inhibited by selenite and selenate (Salisbury & Ross 1992). Calcium is positively responses to selenate treatments in either green tops or roots of rocket plants. This perhaps attributed to the increase in the absorption of calcium from the external medium. Se had an adverse effect on phosphorus absorption (Moore *et al*., 1998). Kinraide (1994) observed an enhancement of selenate rhizotoxicity with increase in $CaCl_2$. Calcium in particular, plays many physiological roles such as signal transduction and the maintenance of cell wall and plasma membrane structural integrity. Also, play a role in activating antioxidant enzymes (Kinraide, 2001).

The level of endogenous Se in the treated roots was much lower than that in the green tops exposed to selenate. The Se levels in either green tops or roots significantly increase concomitantly with increasing the concentration of selenate in the soil. These results are in agreement with those obtained by Brown and Shrift (1982) and Zayed and Terry (1992). Heavy metals can be toxic above a certain

threshold level and plants have evolved a complex metal homeostasis network system, which regulates their uptake and distribution thereby inducing an effective protection of the metabolic processes (Clemens *et al*., 2002).

Effect of selenium on carbohydrates. The green tops of rocket plants exposed to 5 μM or 100 μM selenate induced significant increase in soluble sugar contents, more so at high Se concentration (Table IV). The low concentration of selenate (5 μM) significantly increased the total polysaccharide while the high concentration reduced it (Table IV). Similar results have also been reached by Arvy *et al*. (1995) who indicated that addition of selenate to selenium deficient subculture of *Catharanthus roseus* cells resulted in high cellular level of carbohydrate accumulation after 10 days of culture. Accumulation of soluble sugars is thought to play a role in adaptation of plants to salinity (Everard *et al.*, 1994). Our previous results suggested that low concentration of selenate may activate the photosynthetic machinery, as a result of its effect on increasing photosynthetic pigments (Table I, Konecna *et al.*, 1989). Polysaccharide decrease at high level of Se may be due to effect of Se on photosynthesis as it is reported that heavy metals may inhibit chlorophyll synthesis and thus affect photosynthesis (Ralph & Burchett, 1998; Zhang *et al*., 2003).

Effect of selenium on proline accumulation. Free proline content is significantly increased at high selenate treatment (Table IV). Proline is reported to be a universal osmolyte accumulated in response to several stresses (Öncel *et al.*, 1996; Mansour, 2000) it also plays a role in the protection of enzymes against denaturation and the stabilization of protein synthesis (Kuznetsov & Shevyakova, 1997). It could be postulated that rocket plants experienced Se stress at high concentration as it is indicated that heavy metals lead to proline accumulation (Alia-Saradhi, 1991). Öncel *et al.* (2002) recorded that lead applications have not stimulated proline accumulation but cadmium applications have stimulated proline accumulation considerably in wheat seedlings.

Effect of selenium on total free amino acids. The total free amino acids accumulated in green tops of rocket plants are shown in Table IV. The total free amino acids significantly increased in the tops exposed to 5 μM or 100 μM selenate. Similar results were reported by Hu *et al*. (2001) who found that the total amino acids of selenite-treated green tea were markedly increased compared with the tea produced in areas poor in selenium. It seems that Se caused disorder in amino acid metabolism (Gowily *et al*. 1996; Wu, 1998).

Effect of selenium on lipid peroxidation. Lipid peroxidation was determined in order to find out whether selenium induced oxidative stress. Low level of selenate significantly diminished lipid peroxidation which measured as malondialdehyde (product of lipid peroxidation). The high level of selenate exerted an increase in lipid peroxidation (MAD) as compared with low Se but less than that of the control plants (Table IV). The decrease in lipid peroxidation by selenate may be attributed to its effect on the activity of antioxidant enzymes and/or the increased levels of water soluble ascorbic acid and glutathione. Carotenoids may react with lipid peroxidation products (Burton & Ingold, 1984; Tables, IV, V). Similar results were recorded by Pennanin *et al*. (2002).

Selenium may act as antioxidant and reduced the level of reactive oxygen speies through its metabolization to selenite and then to volatile dimethylselenide (Pilon-Smits *et al*., 1998).

Effect of selenium on antioxidants. The antioxidant defense system of plants includes the low molecular weight (water soluble) antioxidants such as glutathione (GSH), ascorbic acid (ASA) phenolic compounds, carotenoids (lipid

Table IV. Effect of Se treatments on carbohydrates, free amino acids, proline, total soluble protein, ascorbic acid, glutathione (GSH) and lipid peroxidation Each value is the mean of five different replications ± SE.

Metabolite	Selenate concentrations (μM)		
	0	5	100
Soluble carbohydrates (mg/g F.wt.)	83.81 ± 1.73	104.16 ± 2.3	112.03 ±1.18
Polysaccharides (mg/g F.wt.)	5.23 ± 0.24	7.02 ± 0.21	4.6 ± 0.12
Proline (M mol)	1.19 ± 0.20	1.2 ± 0.115	1.52 ± 0.29
Free amino acids (mg/g F.wt.)	1.52 ± 0.115	2.37 ± 0.40	2.82 ± 0.34
Total soluble proteins (mg/g F.wt.)	1.088 ± 0.08	1.974 ± 0.17	1.9 ± 3.4
Malondoialdehyde (M mol/L)	3.9 ± 0.23	0.10 ± 0.05	2.4 ± 0.12
Ascorbic acid (mg/g F.wt.)	2.45 ± 0.31	7.48 ± 1.14	4.24 ± 0.81
Glutathione (G,SH) (mg/g F.wt.)	1.2 ± 0.25	1.48 ± 1.16	1.26 ± 0.06

Table V. Effect of Selenate treatments on the specific activity of some oxidative enzymes of rocket green tops. The enzyme activity expressed as unite g^{-1} Fwt. Each value is the mean of five different replications ± SE.

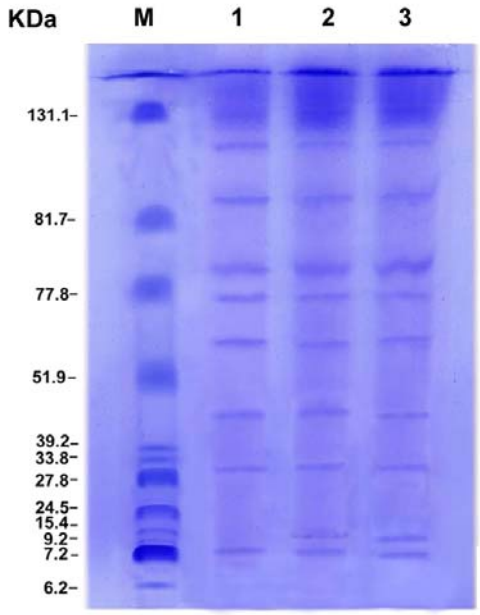
Enzyme	Specific enzyme treatments activity (unite g^{-1} Fwt)		
	(0.0)	5.0 μM	100 μM
SOD	153.5 ± 1.74	173.8 ± 2.0	160.3 ± 1.15
CAT	178.86 ± 4.65	56.6 ± 1.155	36.03 ± 0.8
APX	8.83 ± 0.50	12.88 ± 0.64	21.3 ± 0.75
AO	27.67 ± 1.20	22.79 ± 1.3	9.9 ± 1.2
GPX	6.7 ± 1.75	2.8 ± 0.12	7.1 ± 1.82

soluble compounds), which scavenge reactive oxygen radicals and they are substrates for the antioxidative enzymes such as ascorbate peroxidase (APX) and guaiacol peroxidase enzymes. Ascorbate oxidase enzyme also control the ascorbate-glutathione cycle.

Exogenous selenium can affect the activity of redox enzymes and thereby change the oxidation reduction status of the leaves increasing its stress tolerance (Vikhreva *et al*., 2002). Results of the present investigation showed that the amounts of ascorbic acid and glutathione (GSH) in rocket tops were markedly increased above those of the control tops (Table IV). The increase in either ascorbic acid or GSH was greater at 5 μM selenate than at 100 μM. It has been reported that glutathione and ascorbic acid may play a key role in antioxidant defenses (Smirnoff *et al*., 2001). The enhanced activities of Cu/Zn SOD and APX have been recorded in selenate-subjected-plants (Table V). The greatest activity of Cu/Zn SOD was recorded at 5 μM selenate, however, greatest APX activity was obtained at 100 μM selenate. The enhanced APX activity may have either increased the scavenging capacity for superoxide produced following stress injury or indirectly by modifying redox and cell signaling processes (Sandalio, *et al.*, 2001). On the other hand, selenate induced a significant reduction in the activities of catalase and ascorbate oxidase enzymes at both treatments (Table V). It can be proposed that the selenate-induced superoxide radicals in rocket tops are converted to H_2O_2 by SOD. The accumulated H_2O_2 seemed to be reduced by ASX and GPX to H_2O_2 but not through catalase enzyme. The enhanced effect of selenate on SOD and APX activities may buffer the free radical-mediated lipid peroxidation of the membrane (Table V). Moreover, guaiacol peroxidase activity was markedly increased at the high level of selenate but significantly decreased at 5 μM selenate as compared with those of the control. These obtained results indicated that the exposure of rocket plant (accumulator plant) to high concentration of selenate results in the generation of reactive oxygen species and the reduction of selenate to selenite may enhanced the synthesis of SOD and APX enzymes.

Effect of selenium on total soluble protein profiles. The changes in SDS-PAGE banding patterns of rocket leaves in response to different levels of selenate were shown in Fig. I. Quantitative and qualitative differences were observed in polypeptide patterns. Ten polypeptide bands are detected in the green tops of control plants but 11 polypeptide bands are shown in Se-treated leaves. Moreover, a new polypeptide with molecular weight 9.2 KDa is detected in the green rocket tops exposed to selenate. These new polypeptides may be related to metal binding polypeptides phytochelatins (cadystins). Similar results have been recorded by Steffens (1990) and Schopfer (1995) who stated that selenate anions induced the synthesis of phytochelatins (3- 10 K Da). Glutathione may form a complex with this ion and reduces it. Jackson *et al.* (1990) proposed that plant cells synthesize organic acids or polypeptides that chelate metal ions, which is required for some tolerance mechanisms. However, Rodrigo *et al*. (2002) reported that the substitution of selenium for sulfur alters the redox properties of these proteins.

Fig. 1. Electrograph of soluble protein patterns SDS_PAGE extracted from rocket leaves exposed to different levels of selenium. Each lane contains equal amounts of protein. Lane M: marker, lane 1: control, lane 2: 5 μ M selenate, lane 3: 10 μ M selenate



In conclusion, selenium, particularly high concentration, may induce the accumulation of active oxygen species, which cause disturbance in cellular calcium as an early signal. In turn, the activity of some antioxidant enzymes, non-enzymatic antioxidants as well as protective proteins may increase to scavenge such AOS. Low selenate level may be required by this accumulator plant as a micronutrient and may increase its antioxidative capacity.

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