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RESEARCH ARTICLE

Modulation of ascorbate-glutathione cycle by selenate and sulphate treatments in the seedlings of two rice (*Oryza sativa* L.) cultivars

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ABSTRACT

The trace element Selenium (Se) has a dual role in the growth and metabolism of plants. Low concentration of selenium (2 µM selenate) promotes growth and counteracts the detrimental effects of abiotic stress as opposed to higher levels (≥10 µM) where it acts as a pro-oxidant. We focused on both individual and interactive influence of selenate and sulphate on thiol metabolism in seedlings of rice cultivars, satabdi and khitish. Inhibition of ascorbate contents by about 17% on an average, in the test seedlings treated with Se correlated with increased activities of ascorbate peroxidase and ascorbate oxidase in the cultivars. The glutathione levels also increased significantly, on an average by about 102% in roots and 74% in shoots of cv. satabdi compared to a rise, by about 49% in roots and 56% in shoots of cv. khitish. The elevated level of glutathione coincided with the stimulatory influence of Se on its regulatory enzymes. Concomitantly the levels of α-tocopherol and phytochelatins were also induced in both the test cultivars. Increase in α-tocopherol activity reached a maximum by about 47% in roots and 80% in shoots of cv. satabdi whereas it increased by about 36% in roots and about 64% in shoots of cv. khitish. Substantive increase in the levels of PC4 followed by PC2 and PC3 was also noted. The effects were found to be less conspicuous in shoots than in roots. Rice seedlings exposed to combined Se and 10mM sulphate treatments showed improved growth and development as a result of better thiol metabolism due to amelioration of the adverse effects caused by selenium alone on all the parameters tested.

Introduction

Selenium (Se) displays metalloid characteristics and predominantly occurs in four oxidation states (selenide, elemental selenium, selenite and selenate). It acts as a micronutrient under low concentrations (1) while at higher concentrations it becomes toxic (2). It has been documented that 1 mg kg^{-1} Se protects rye grass against heavy metals induced damages by decreasing lipid peroxidation and increasing glutathione peroxidase (GPx) activity (3). Selenium also helps to alleviate stress in several plants *viz*., olive (4), sunflower (5), cucumber (6), rice (7, 8) and wheat (9) by regulating the production of antioxidants and thiol metabolism. The inherent defence system present in plants may be enzymatic and non-enzymatic. Stress supposedly influences the protein moiety in cells leading to the formation of various important molecules like organic acids and amino acids. Se-amino acids play a significant role in the synthesis of chlorophyll precursors (10) and also help to maintain membrane integrity and stability of the photosynthetic apparatus by reducing the accumulation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in plant cells (1, 11). Selenosis or Se toxicity occurs in plants when Se concentration exceeds its critical level (12). Excess Se restricts growth and inhibits the absorption of essential elements like N, P, S, Mn, Zn and Cu and causes chlorosis (1, 13). Increased lipid peroxidation and elevated activity of antioxidant enzymes leads to ROS accumulation evidently in *Triticum aestivum* L. and *Vicia faba* L. under Se stress (14, 15). Selenosis is also responsible for the interpolation of selenocysteine, and selenomethionine instead of cysteine and methionine in plant and animal cells. As

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a result the tertiary structure of proteins is distorted by the development of larger diselenide bridge causing detrimental alterations and improper functioning of the bio-enzymes (12).

Glutathione (GSH) in both oxidized (GSSG) and reduced (GSH) states acts as an indicator of oxidative stress and prevents the accumulation of ROS in plant cells under various stresses. (8, 16). GSH related to the thiol group of essential amino acid, cysteine possesses the ability to donate an electron and form glutathione disulfide (GSSG) along with another GSH molecule. GSH is mainly regenerated from GSSG by the activity of GR or GPx (15). The non-enzymatic antioxidants including reduced ascorbate (AsA) and glutathione (GSH) form an integral part of the ascorbate-glutathione cycle that effectively combat oxidative stress in plants (17, 18). Activities of other anti-oxidant enzymes *viz*., ascorbate peroxidase (APx), ascorbate oxidase (AOX),glutathione reductase (GR), glutathione peroxidase (GPx) and Glutathione-S-transferase (GST) also plays significant role in eliminating cellular ROS production and protects the plants from lethal damages (18, 19).

Heavy metal toxicity is prevalent abiotic stress having fatal consequences in both animals and plants. Complex coordination of physiological, biochemical and molecular processes helps the plant cells to resist and survive under such stressful conditions (20). Abiotic or biotic stresses can either be avoided or tolerated by extracellular precipitation, biosorption or by intracellular chelation through the synthesis of amino acids, organic acids and phytochelatins among others in living organisms especially plants (21). Phytochelatins (PCs) belong to a family of small, cysteine-rich polypeptides synthesized from GSH with the help of enzyme PC synthase in plants under oxidative stresses (22). The PCs have a common structure (γ-Glu-Cys)-n-Gly, where n=2-11. Among the different forms of PCs, the most stress-responsive PCs are PC2, PC3 and PC4 (23). The PCs are actively involved in accumulation, chelation, metabolism and sequestration of toxic ionic elements (24). Metal stresses principally induce a concomitant rise in PC2 and PC3 levels in plants (25, 26).

Selenate uptake in plants predominantly occurs via the sulphur assimilation pathway through sulphate transporters present in the root plasma membrane. Accumulation and its distribution depend on the chemical species, concentration and availability of the element in the soil solution (27). However, physical and chemical similarities between selenate and sulphate help to govern the relationship between selenate and sulphate metabolism in plants. It has been reported that high sulphate concentration restricts the entry and uptake of selenate since sulphate transporters have a higher selectivity for sulphate over selenate (28).

Rice (*Oryza sativa* L.), a staple food for half the world population is a member of the grass family (Poaceae) grown in both irrigated areas as well as rain-fed areas with high rainfall. Among the rice-producing states in India, West Bengal is in the leading position accounting for about 15 per cent of the total production. In this study we aimed to elucidate the effects of selenate on ascorbate-glutathione cycle and thiol metabolism in two high-yielding irrigated cultivars of rice *viz*., satabdi (IET 4786) and khitish (IET 4094). We further attempted to repair the selenate induced adverse responses with sulphate treatments in the mentioned cultivars.

Materials and Methods

Experimental material and treatments

The seeds of rice (*Oryza sativa* L.) cultivars, satabdi (IET4786) and khitish (IET4094) were procured from the State Rice Research Station, Chinsurah, Hooghly, West Bengal. The seeds were superficially disinfected in sodium hypochlorite (NaOCl) (5% w/v) solution and then thoroughly rinsed with sterilized water. Each test set up consisted of petridish (diameter-10 cm) with 50 seeds arranged carefully on moist filter paper. The petridishes were then placed carefully in a germinator (LCGC Bio Analytic Solutions LPP) for 72 hr at 30 ± 2 °C. The germinated rice seedlings were exposed to varying grades of sodium selenate ($NaSeO_4.7H_2O$; Loba-Chemie, India) solution (2 µM, 10 µM, 20 µM and 50 µM) either alone or in combination with 10mM sodium sulphate (Na_2SO_4; Merck, India) in a hydroponic set up with modified Hoagland's solution, pH 7.2 (29) in 16 hr photoperiod for 18 days. The mentioned concentration of sodium sulphate was found to be most effective after screening experiments with different concentrations of sodium sulphate and was therefore selected. After a total of 21 days the seedlings were harvested, washed extensively with distilled water. Roots and shoots were separated and stored at -20 ºC until further analysis.

Estimation of ascorbate contents

One gram tissue was extracted in 6 ml of chilled trichloroacetic acid (TCA) (6%w/v) and centrifuged (Remi C 23) at 11500 gm for 15 mins at 4°C. The method of Mukherjee and Chaudhuri (30) was followed with minor modifications to determine the ascorbate contents. The reaction mixture (4 ml of supernatant, 2 ml of 0.2% 2,4-dinitrophenyl hydrazine (DNPH) in 0.5(N) HCl and 0.01 ml 10% thiourea in 70% ethanol) was incubated in boiling water bath for 15 mins and then cooled. Finally, the reactions were terminated by adding 5 ml H_2SO_4. The absorbance of the reaction mixture was noted at 530 nm using a Hitachi U-2000 spectrophotometer. With the help of a standard curve, ascorbate contents were estimated and expressed as milligram ascorbate per gram fresh weight (mg ascorbate g^{-1}FW).

Assay of ascorbate peroxidase (APX) activity

The assay of APX (EC 1.11.1.11) was performed according to Nakano and Asada (31). The supernatant obtained from centrifuged pulverized test tissue (1 gm) was used for the assay. The alterations in absorbance of the reaction mixture containing 700 µl enzyme extract, 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 0.1 mM ethylene diamine tetra acetic acid (EDTA) were noted after 1 min at 290 nm. The extinction coefficient of

2.8$mM^{-1}cm^{-1}$ was applied to measure oxidation of ascorbate by the said enzyme at 290 nm and expressed as milligram ascorbic acid decomposed per mg protein per min (mg ascorbic acid decomposed mg^{-1}protein min^{-1}).

Assay of ascorbic acid oxidase (AOX) activity

The activity of AOX (EC 1.10.3.3) was measured according to Olliver (32). The reaction mixture containing 1ml enzyme extract, 3 ml sodium phosphate buffer 0.1 M (pH 7.0) and 1 ml of 0.025% ascorbic acid (w/v) was incubated for 30 min. After the stipulated time of incubation, the reaction was terminated by adding 5 ml of 10% TCA (w/v). The test set ups were then titrated with 2,6-dichlorophenol indophenol (DCPIP) and the alterations in absorbance readings in blank and sample helped to calculate enzyme activity expressed as mg ascorbic acid decomposed mg^{-1} protein min^{-1}.

Estimation of α-tocopherol contents

The α-tocopherol contents of the sample were estimated (33). 1g fresh tissue was chopped and placed in 20ml 0.1 N H_2SO_4, kept and filtered. A mixture of 1.5 ml supernatant and 1.5 ml ethanol was centrifuged at 5000 gm for 5 min to mix thoroughly. In the mixture 1.5 ml xylene was added and vortexed. The upper xylene fraction containing tocopherol was taken in a tube, and to it 1 ml 2, 2-dipyridyl reagent (Merck) was added and mixed. The absorbance of the mixture was recorded at 460 nm. Then 0.33 ml ferric chloride ($FeCl_3$) was added to each tube and incubated for 15 min to develop colour and absorbance was recorded at 520 nm. A standard curve was prepared using DL-α tocopherol and amount of tocopherol present in the sample was expressed as μg g^{-1} FW.

Estimation of total glutathione contents

Standard procedure was followed for total glutathione assay (34). Sample tissue homogenised in a solution containing sulphosalicylic acid (SSA) (5%w/v) and 10 mM EDTA were centrifuged at 10000 gm for 20 min. The final reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 6U ml^{-1} glutathione reductase (GR), 10 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 0.16 mg ml^{-1} NADPH to a total volume of 1 ml. The absorbance of the reaction mixtures was measured using a Hitachi U-2000 spectrophotometer measured at 412 nm. The total thiol contents were estimated and expressed as micromole GSH per gram fresh weight (μmol GSH g^{-1} FW).

Assay of glutathione reductase (GR) activity

The GR (EC 1.6.4.2) activity was assayed spectrophotometrically (35). 3 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA was used during homogenization of plant tissue (1 gm) which was then subjected to centrifugation at 4°C, 15000 gm for 20 min. The reaction solution consisted of enzyme extract, 100 mM phosphate buffer (pH 7.5), 0.5 mM EDTA, 0.75 mM DTNB, 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 1 mM glutathione (oxidized) (GSSG) in a final volume of 1 ml. On completion of incubation at 35 °C, the absorbance of the test solutions was measured at 412 nm for about 5 min. The extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was applied to measure the GR activity ultimately expressed as μmol min^{-1} mg^{-1} protein.

Assay of glutathione peroxidase (GPx) activity

The activity of GPx (E.C. 1.11.1.9) was estimated (36). 500 mg of tissue samples were pulverized in 1 ml of 50 mM chilled KH_2PO_4 buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. The supernatant (20 μl) collected from centrifugation of the homogenised tissue at 11500 gm for 10 mins was mixed with KH_2PO_4 buffer 100 mM (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.12 mM NADPH, 2mM GSH, 1 unit GR, and 0.6 mM H_2O_2 to initiate the reaction. The rate of NADPH oxidation at 340 nm for 1 min was measured using a Hitachi U-2000 spectrophotometer. The extinction coefficient of 6.62 mM^{-1} cm^{-1} was required to calculate the enzyme activity. Data expressed as nanomole of NADPH oxidized per min per mg protein (nmol min^{-1} mg^{-1} protein).

Assay of glutathione-S-transferase (GST) activity

Glutathione-S-transferase (EC 2.5.1.18) activity was measured as per the protocol (37). 100 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 14 mM β-mercaptoethanol and 7.5% poly vinyl pyrollidone (PVP) (w/v) was used during homogenisation of 1 gm of test tissue which was then centrifuged for 15 min at 15000 gm. The reaction mixture consisted of the required amount of the supernatant in 100 mM potassium phosphate buffer (pH 6.5) containing 5 mM Total glutathione (reduced) (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). At 340 nm the absorbance was measured and an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$was used to calculate the enzyme activity expressed as micromol per min per mg protein (μmol $min^{-1}mg^{-1}$protein).

Phytochelatin isolation and analysis

The phytochelatins were estimated (38) with minor modifications. The plant samples were homogenized in liquid nitrogen. 8 ml of chilled Diethylene triamine penta acetic acid (DTPA) (6.3 mM) containing 0.1% (v/v) trifluoro acetic acid (TFA) was used in the homogenisation of 1.5 gm pulverized plant tissue. Centrifugation of the homogenised samples was done at 13800 gm for 20 mins at 2°C. The supernatants were collected and filtered through a syringe filter (0.22 μM). Derivatization involved incubation at 45°C for 30 min of the test set ups consisting of 10 μl monobromobimane (mBBr) and 250 μl of the crude extract mixed thoroughly with 450 μl of 200 mM 4-(2-Hydroxyethyl)-piperazine-1-propanesulfonic acid (HEPPS) buffer 200 mM, (pH 8.2) containing 6.3 mM diethylene triamine penta acetic acid (DTPA) in the dark. 300 μl of 1(M) methane sulfonic acid (MSA) was used to terminate the reactions and the samples were later subjected to high performance liquid chromatography (HPLC) analysis. The derivatives (5 μl) were injected and separated by Shimadzu Nova Pak C18 analytical column following an autocratic gradient of 0.1% TFA and 100% acetonitrile (ACN) as 12–30% ACN in 22 mins, 30–55% ACN from 22–45

mins and 55–100% ACN from 45–60 min. The flow rate was 0.7 ml min^{-1}. A detector (Shimadazu fluorescence and refractive index detector) was used to monitor the fluorescence utilising the excitation wavelength at 380 nm and emission wavelength at 470 nm. The total duration of analysis was 60 min. Phytochelatin contents were expressed in terms of microgram per gram fresh weight (µg g^{-1} FW).

Estimation of protein contents

Estimation of protein contents was carried out using bovine serum albumin (BSA, Sigma) as standard (39).

Statistical analysis

Experiments were carried out thrice in a completely randomized design (CRD). All the parameters were tested three times with two replicates in each time. Thus, six sets of data for every treatment in each parameter were obtained. Each treatment comprised of a single petridish with an average of 50 seeds. The data point was expressed as mean ± SE of three independent replicates. One way analysis of variance (ANOVA) was used to compare the data and significant differences among the mean values at $p \leq 0.05$ was considered statistically significant.

Results

Influence on ascorbate contents

In the rice seedlings of both test cultivars, low concentrations of selenate increased the total ascorbic acid contents, which decreased at its higher concentrations (Fig. 1). The level of ascorbate increased by about 34% and 32% in roots and by about 36% and 33% in shoots respectively of cv. satabdi and cv. khitish over control. The ascorbate contents were further enhanced by about 11% and 18% in roots and by about 12% in shoots of 10 µM selenate treated cv. satabdi and cv. khitish seedlings respectively. However, in 20 and 50 µM selenate treated rice seedlings the ascorbate level declined by about 10% and 15% in roots and by about 10% and 17% in shoots of cv. satabdi respectively. In cv. khitish the total ascorbate contents initially increased very little in roots and then decreased by about 6% under 20 and 50 µM selenate treatments respectively. In shoots of cv. khitish the ascorbate level decreased by about 4%, and 11% treated with said concentrations of Se respectively compared to water control. However, the increment observed was less in the tolerant variety cv. khitish compared to the sensitive cultivar cv. satabdi.

The level of ascorbate contents induced in test seedlings of both cv. satabdi and cv. khitish subjected to the joint application of selenate and sulphate was more with respect to water control but less compared to selenate treatment only. The ascorbate contents increased significantly by about 44% in roots and by about 49% in shoots of 2 µM selenate and 10 mM sulphate treated test seedlings of cv. satabdi. In cv. khitish seedlings, exposed to same selenate and sulphate doses, the ascorbate contents also increased significantly by about 35% in roots and by about 38% in shoots over control. Combined doses of 10, 20, 50 µM selenate and 10 mM sulphate increased the said contents in descending order by about 37%, 15% and 11% in roots and by about 32%, 16% and 11% in shoots of cv. satabdi respectively. In cv. khitish, the ascorbate level similarly increased by about 26%, 10% and 6% in roots and by about 16%, 5% and very little in shoots under familiar selenate and sulphate treatment respectively over control.

Influence on ascorbate peroxidase (APX) (EC 1.11.1.11) activity

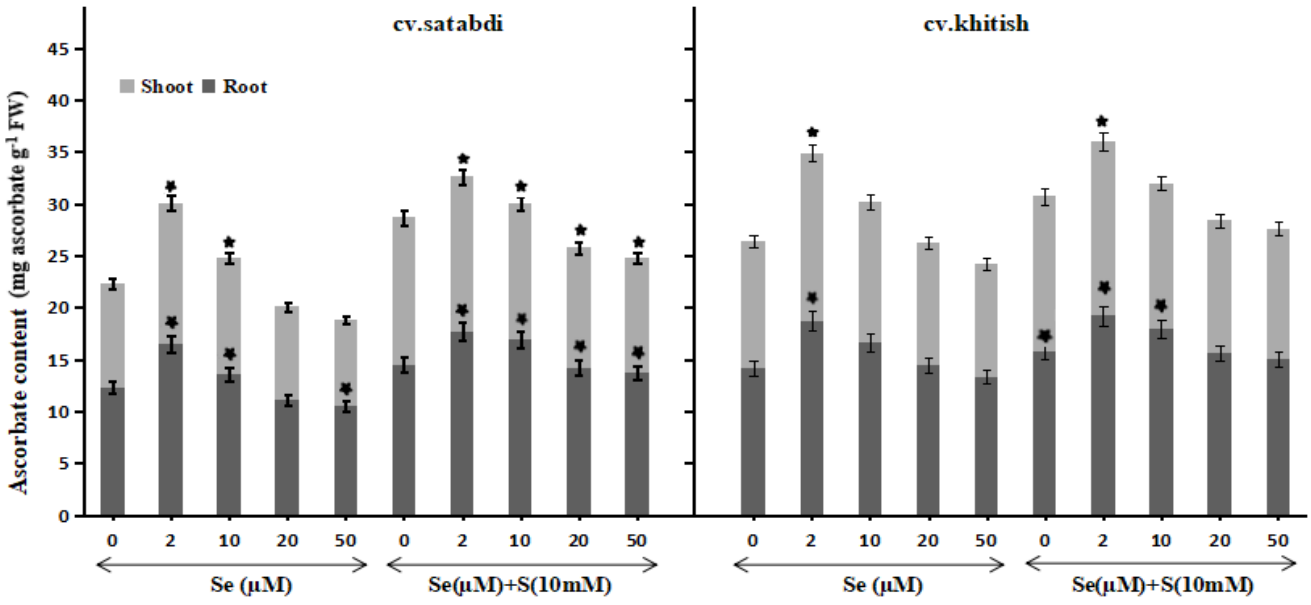

Fig. 1. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on the ascorbate contents of 21 days old cv. satabdi and cv. khitish seedlings.In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ with respect to control.

Peroxidases are enzymes that transfer a single e^- to H_2O_2 with the help of ascorbic acid. In our study the APX activity increased considerably with increase in selenate concentrations in both the test cultivars (Fig. 2). APX activity increased by about 9% in roots and by about 13% and 10% in shoots of cv. satabdi and cv. khitish respectively under 2µM selenate treatment

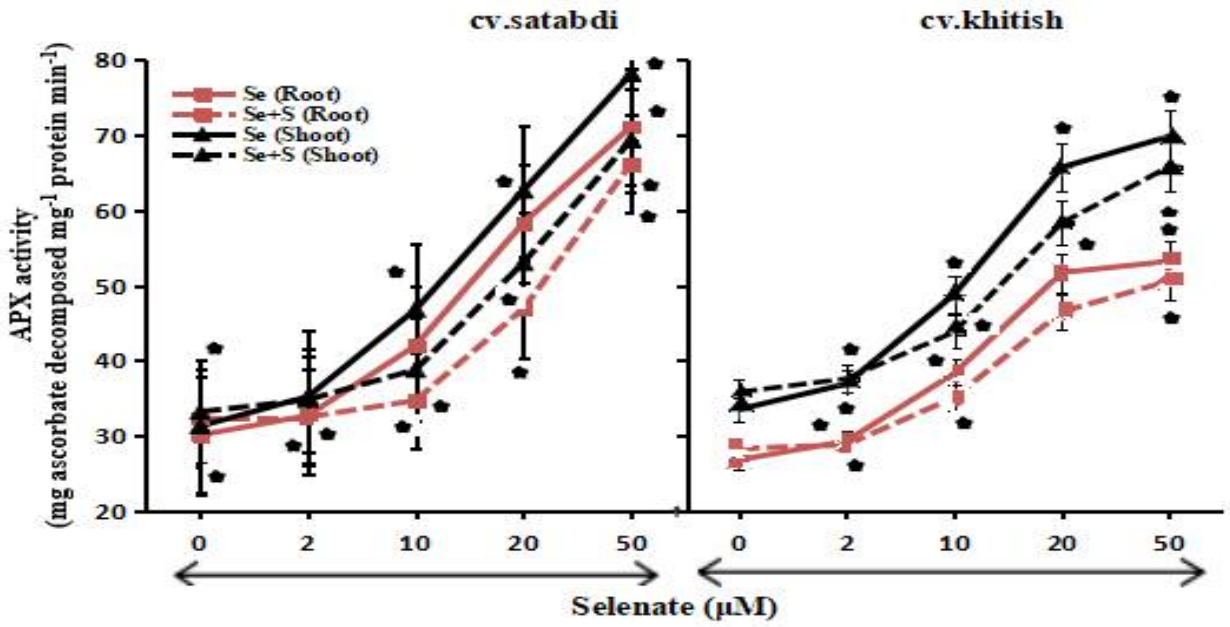

Fig. 2. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on ascorbate peroxidase (APX) activity of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

over control. The enzyme activity was significantly promoted by about 40%, 94% and 136% in roots and by about 50%, 100% and 150% in shoots of cv. satabdi seedlings under 10, 20, 50 µM selenate treatment respectively. In cv. khitish, the enzyme activity similarly increased by about 42%, 91% and 142% in roots and by about 77%, 90% and 157% in shoot under said concentrations of selenate over control.

Application of combined 10 mM sulphate and selenate, increased the enzyme activity in both cultivars but to a lesser degree compared to test seedlings treated with selenium alone. Under 2 µM selenate and sulphate treatment, the APX activity increased by about 8% in roots and 11% in shoots of cv. satabdi and by about 8% in roots and 12% in shoots of cv. khitish over control.

Further, an increment of about 15%, 56% and 120% in roots and about 24%, 69% and 121% in shoots were recorded in cv. satabdi seedlings treated simultaneously with 10, 20, 50 µM selenate and 10 mM sulphate respectively over water control. In cv. khitish, the APX activity was also induced by about 31%, 74% and 89% in roots and by about 31%, 74% and 96% in shoots of test seedlings treated with similar factorial combinations of selenate and sulphate.

Influence on ascorbic acid oxidase (AOX) (EC 1.10.3.3) activity

The activity of AOX showed a dose-dependent increase with the increasing concentrations of selenate in both the test cultivars (Fig. 3). Under 2 µM selenate treatment, the AOX activity increased by about 10% and 6% in roots and shoots of cv. satabdi respectively compared to an average increase of about 7% in cv. khitish seedlings over water control. The AOX activity further increased by about 14%, 23% and 31% in roots and by about 9%, 11% and 12% in shoots of 10, 20 and 50 µM selenate treated rice seedlings of cv. satabdi respectively. In cv. khitish, the AOX activity also increased by about 12% 14% and 15% in roots and by about 8%, 9% and 10% under 10, 20 and 50 µM selenate treatment respectively over water control.

The joint application of selenate and 10 mM sulphate promoted AOX activity but also narrowed the effect of selenium used singly in both test cultivars. The enzyme activity increased by about 4% and 6% in roots and by about 2% and 5% in shoots of 2 µM selenate treated test seedlings of both cultivars with respect to control. Further, the said activity increased by about 6%, 11% and 15% in roots and by about 8%, 9% and 10% in shoots of 10, 20 and 50 µM selenate and sulphate treated test seedlings of cv. satabdi respectively .The AOX activity similarly increased by about 7%, 11% and 13% in roots and by about 7%, 9% and 10% in shoots of 10, 20 and 50 µM selenate and sulphate treated cv. khitish test seedlings respectively over control.

Influence on α-tocopherol contents

Total α-tocopherol contents were significantly increased in the test seedlings by selenate treatment (Fig. 4). Compared to control, the level of α-tocopherol increased by about 2% in roots and 5% in shoots of 2 µM selenate treated rice seedlings of cv. satabdi while in cv. khitish α-tocopherol increased by about 1% in roots and 2% in shoots respectively over control. After that, the α-tocopherol contents steadily

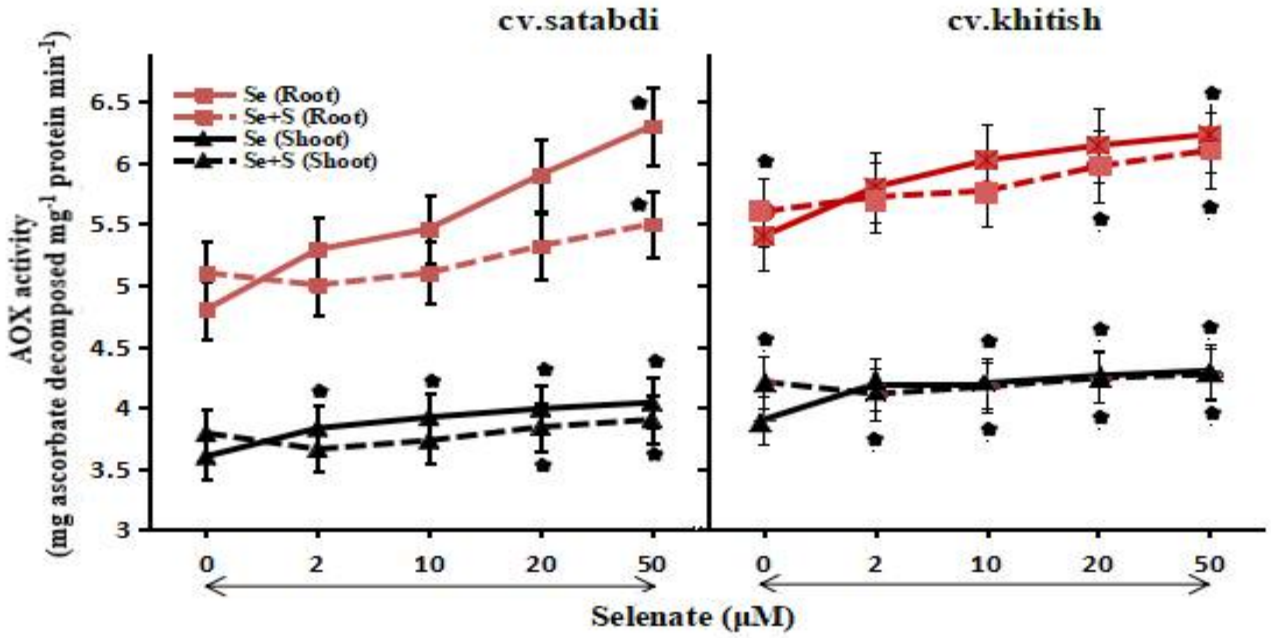

Fig. 3. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on ascorbate oxidase (AOX) activity of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

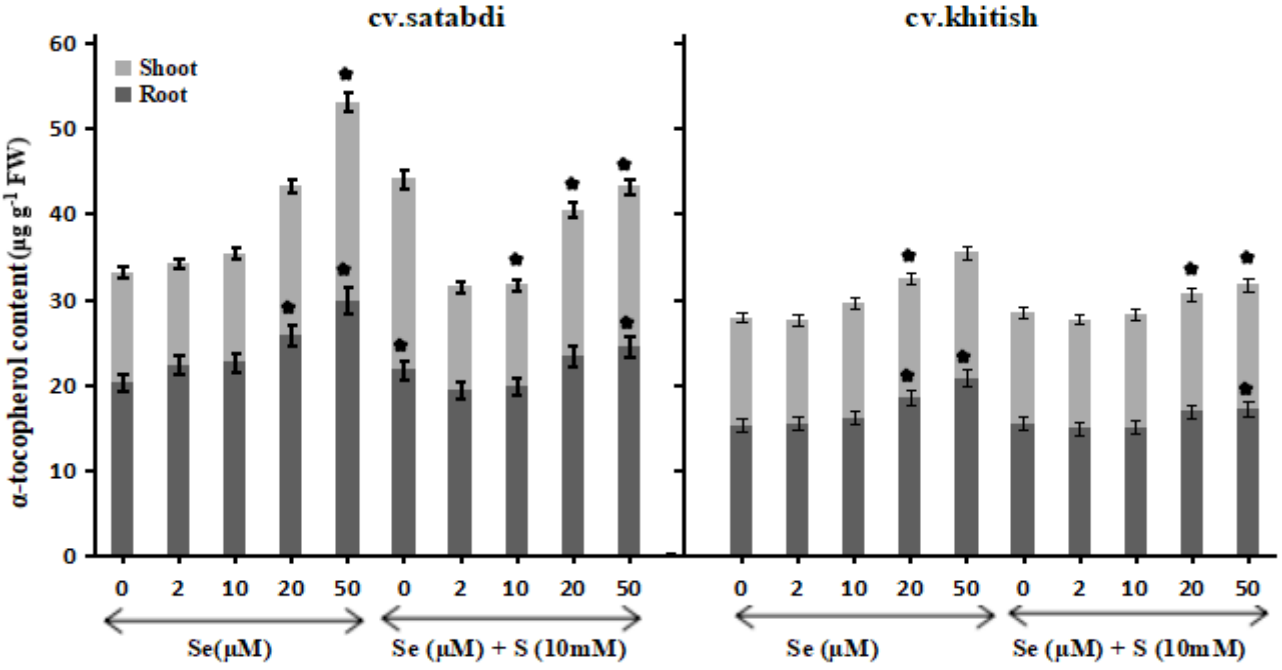

Fig. 4. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on α-tocopherol contents of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

increased by about 12%, 27% and 47% in roots and by about 11%, 36% and 80% in shoots of 10, 20 and 50 µM selenate treated seedlings of cv. satabdi. This trend was reflected in cv. khitish where the said contents were elevated by about 6%, 22% and 36% in roots and by about 7%, 25% and 64% in shoots under 10, 20 and 50 µM selenate treatments with respect to control.

Combined application of sulphate with selenate showed variable effect on the α-tocopherol contents in all treated seedlings compared to control. The α-tocopherol contents under 2 µM selenate treatment registered very little increase in both roots and shoots of cv. satabdi and cv. khitish under 2 µM selenate-sulphate treatment with respect to control. In cv. satabdi, the margin of increment in α-tocopherol contents was narrowed down by about 7%, 15% and 21% in roots and by about 8%, 33% and 44% in shoots under 10 µM, 20 µM and 50 µM selenate-sulphate treatments. In cv. khitish the said

contents increased by about 4%,11% and 13% in roots and by about 4%,18% and 46% in shoots respectively under combined 10, 20, 50 µM selenate-sulphate treatments over water control. It may be noted that cv. satabdi being sensitive to salt stress showed higher accumulation of α-tocopherol contents than cv. khitish which could withstand selenium stress more comparatively.

Influence on total glutathione contents

The total glutathione contents were significantly increased in the test seedlings by selenate treatment in both cv. satabdi and cv. khitish seedlings (Fig. 5). In 2 µM selenate treated test seedlings the total glutathione contents increased significantly by about 60% and 14% in roots and shoots respectively of cv. satabdi and by about 17% and 8% in roots and shoots respectively of cv. khitish seedlings. Compared to control, in cv. satabdi, the level of total glutathione significantly increased by about 66%, 107% and 133% in roots and by about 49%, 80% and 95% in shoots respectively under 10, 20, 50 µM selenate treatments. In cv. khitish test seedlings treated with said concentrations of selenate, the total glutathione level was also enhanced by about 35%, 64% and 79% in roots and by about 43%, 56% and 68% in shoots respectively over water control.

During the combined application of selenate with 10 mM sulphate, the promotive effect on total glutathione contents was narrowed in all treated seedlings compared to those treated with selenate only. The glutathione contents increased in roots and shoots by about 49% and 11% in cv. satabdi and by about 17% and 37% in cv. khitish respectively treated with 2 µM selenate and sulphate solution. An increment of about 49%, 87% and 107% occurred under combined 10, 20, 50 µM selenate and sulphate treatments in roots of cv. satabdi while in of cv. khitish roots the increment was by about 35%, 64% and 72% respectively. Similarly in shoots the increment was by about 23%, 60% and 88% in cv. satabdi and by about 31%, 55% and 60% in cv. khitish respectively under said treatments over control.

Influence on glutathione reductase (GR) (EC 1.6.4.2) activity

In the selenate treated test seedlings, the activity of GR was significantly increased (Fig. 6) in both cultivars with respect to control. Under 2 µM selenate treatment, the GR activity increased by about 4% and 9% in roots and by about 5% in shoots of cv. satabdi and cv. khitish respectively. The activity of GR increased significantly compared to control by about 34%, 50% and 55% in roots and by about 34%, 42%, and 62% in shoots of 10, 20, and 50 µM selenate treated cv. satabdi seedlings respectively. In cv. khitish, the GR activity was also promoted by about 23%, 34% and 43% in roots and by about 35%, 40% and 45% in shoots respectively under similar treatments over control.

Co-application of selenate with 10 mM sulphate also stimulated the activity of GR but at a lesser degree than that under selenium treatment alone. The enzyme activity increased on an average by about 5% and 12% in roots and by about 5% in shoots of cv. satabdi and cv. khitish respectively under 2 µM selenate and sulphate treatment. Further it increased in cv. satabdi roots by about 29%, 36% and 46% and in shoots by about 27%, 37% and 48% under 10, 20 and 50 µM selenate-sulphate treatments respectively over control. In cv. khitish, the GR activity increased by about 14%, 37% and 39% in roots and by about

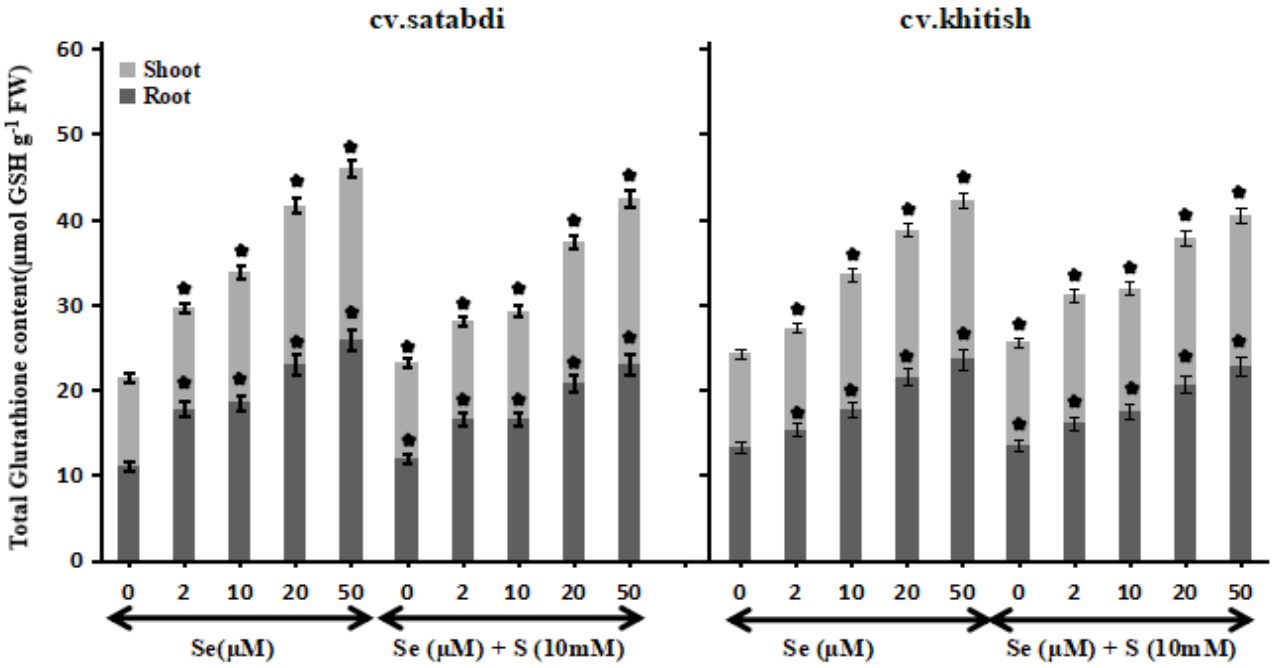

Fig. 5. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on total glutathione contents of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

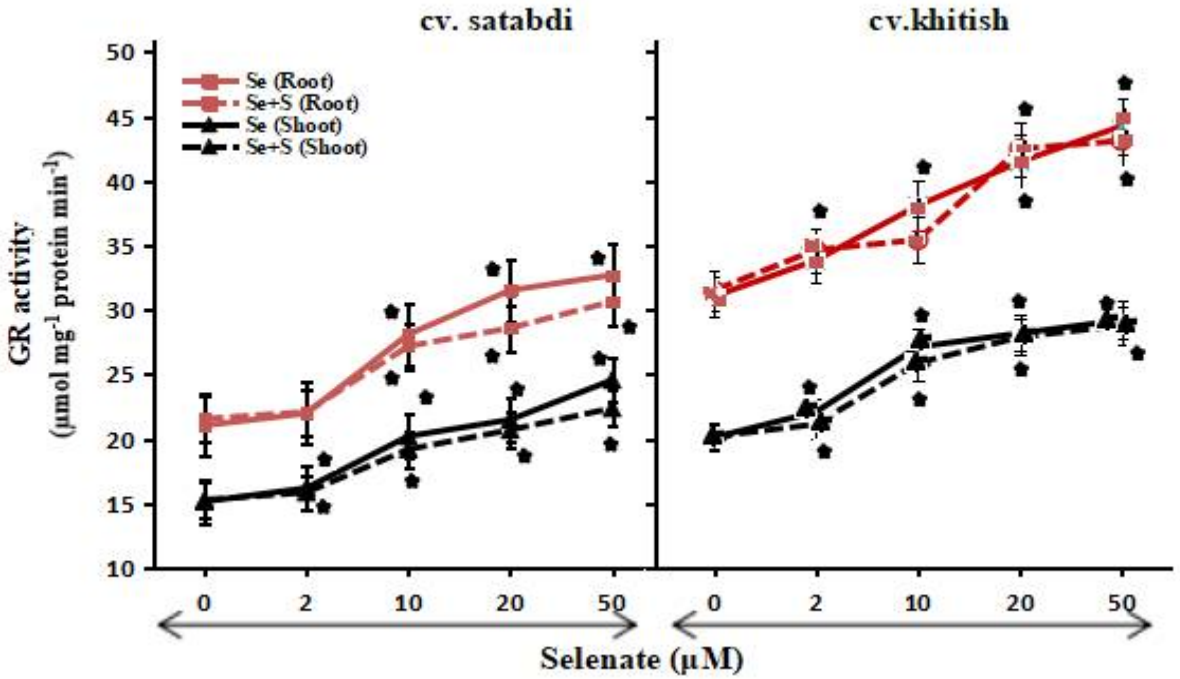

Fig. 6. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on glutathione reductase (GR) activity of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

29%, 39% and 43% in shoots under a similar combination of test doses over control.

Influence on glutathione peroxidase (GPx) (EC 1.11.1.9) activity

The activity of GPx in the test seedlings was increased by selenate treatment in both test cultivars (Fig. 7). The activity of GPx increased by about 14% and 15% in roots and by about 8% and very little in shoots in 2 µM selenate treated test seedlings of cv. satabdi and cv. khitish respectively over control. This promotive effect increased considerably by about 73%, 107%, and 139% in roots and by about 87%, 115% and 130% under 20, 50, 100 µM selenate treatment respectively in cv. satabdi test seedlings. Considerable increment also occurred in cv. khitish where the GPx activity was enhanced by about 59%, 87% and 123% in roots and by about 53%, 70% and 124% in shoots under said treatments over water control.

Application of combined selenate with 10 mM sulphate increased the activity of GPx in all test seedlings but at a lesser degree compared to selenate treatment singly. The activity of GPx increased by about 24% and 9% in roots and by about 5% and very little in shoots of cv. satabdi and cv. khitish respectively under 2 µM selenate and sulphate treatment. A gradual but significant increment by about 67%, 78% and 81% in roots and by about 36%, 86% and 144% in shoots of cv. satabdi occurred when treated with stipulated concentrations of selenate and sulphate solutions. In cv. khitish, the enzyme activity was also up-regulated by about 48%, 67% and 102% in roots and by about 28%, 55% and 91% in shoots under similar treatments over control.

Influence on glutathione-S-transferase (GST) (EC 2.5.1.18) activity

The activity of GST showed a dose-dependent increase in both the test seedlings with an increase in selenate treatment over water control (Fig. 8). Under 2 µM selenate, the GST activity increased on an average by about 11% in roots and very little in shoots of cv. satabdi and cv. khitish respectively. After that, in cv. satabdi the activity of GST increased considerably by about 28%, 61% and 87% in roots and by about 19%, 23% and 34% in shoots treated with 10, 20, 50 µM selenate solutions respectively. This pattern was reflected in cv. khitish where the enzyme activity also increased by about 25%, 37% and 76% in roots and by about 15%, 22% and 31% in shoots under 10, 20, 50 µM selenate treatment respectively over control.

Application of selenate and sulphate together narrowed the promotive effect of GST activity in all treated seedlings compared to control and also at a lesser degree than selenium treatment alone. The joint application of 2 µM selenate and 10 mM sulphate increased GST activity by about 5% and very little in roots and shoots respectively of cv. satabdi. On the contrary, it registered an increase of about 14% in roots and 5% in shoots of cv. khitish under similar treatment. The GST activity also increased by about 15%, 20% and 73% in roots and by about 2%, 6% and 16% in shoots treated with 10, 20, 50 µM selenate and 10 mM sulphate doses respectively in cv. satabdi. In cv. khitish, the GST activity similarly increased by about 16%, 18% and 20% in roots and by about 7%, 10% and 15% in shoots under said conditions of selenate and sulphate treatments respectively over control.

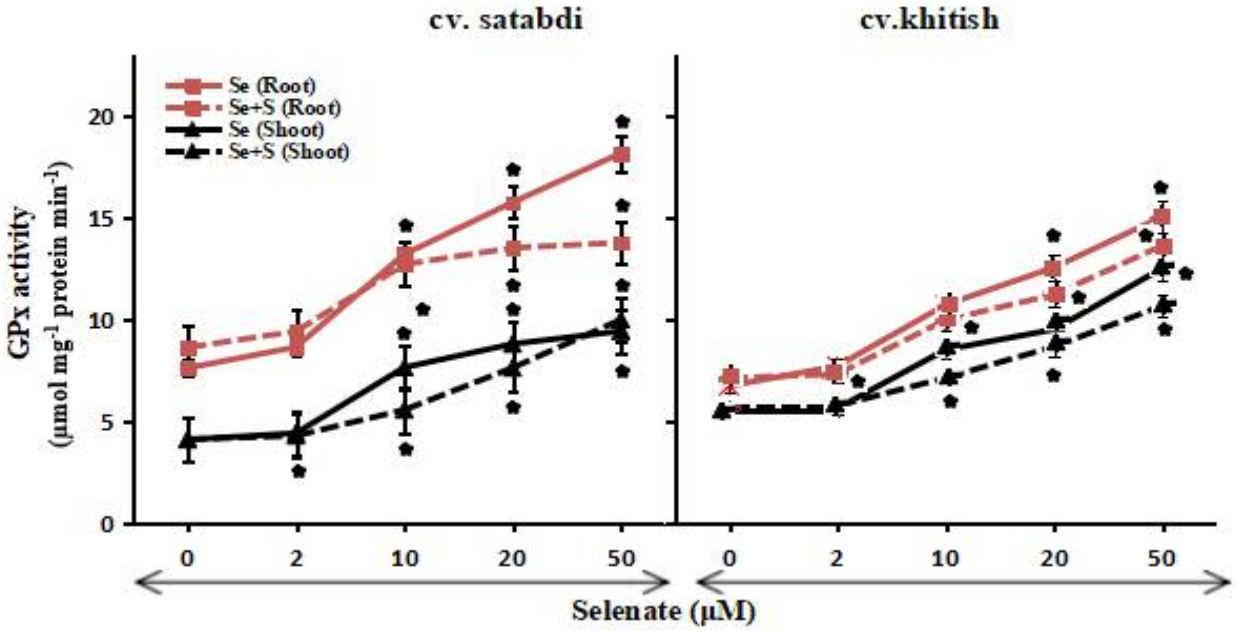

Fig. 7. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on glutathione peroxidase (GPx) activity of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

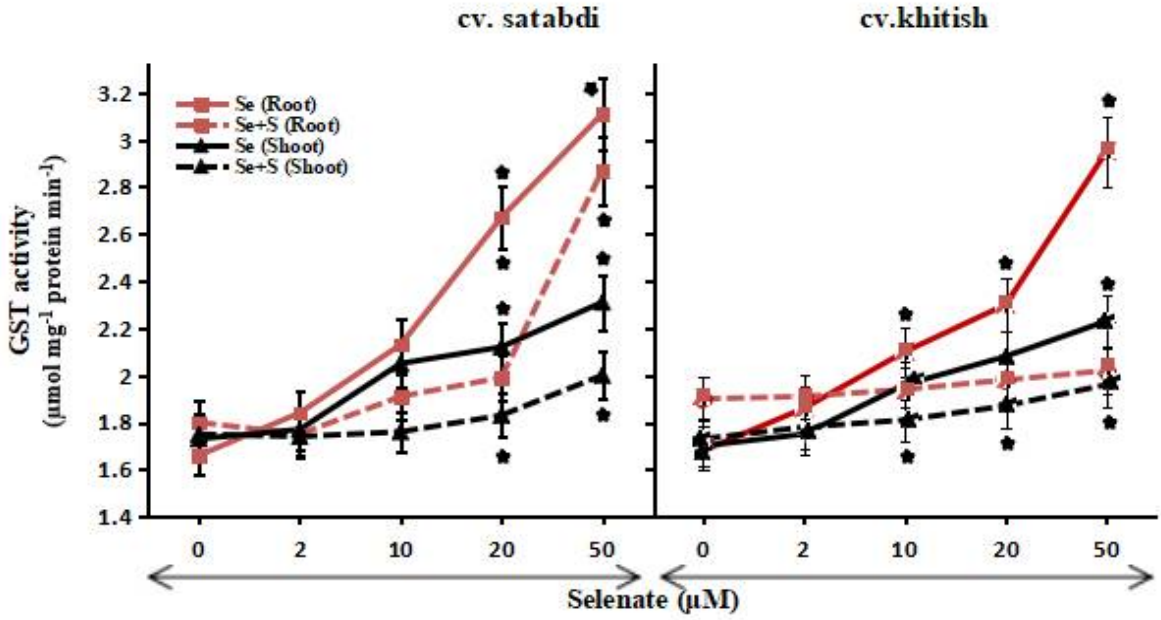

Fig. 8. Effect of different concentrations of selenate applied either alone or in their combination with sulphate (10 mM) on the glutathione-S-transferase (GST) activity of 21 days old cv. satabdi and cv. khitish seedlings. In each treatment values are the mean ±SE of three repeats with two replicates.*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

Influence on phytochelatin production

HPLC chromatogram peaks showed that selenate exposure on 21 days old rice seedlings lead to elevation of PCs varying in chain lengths (n = 2-4) (Fig. 9-12). The inductions in phytochelatin contents were found to be more in case of cv. satabdi as compared to that of cv. khitish, a probable indication of more sensitivity of cv. satabdi towards selenate. At 50 µM selenate treatments, the accumulation of PC's was found to be maximum in both the cultivars. PC2 increased on an average by about 378% and by about 210% in roots and by about 601% and by 228% in shoots of cv. satabdi and cv. khitish seedlings respectively over their controls. Increase in PC3 occurred on an average by about 158% and by about 150% in roots and by 74% and 55% in shoots of selenate treated cv. satabdi and cv. khitish seedlings

respectively over their controls. The accumulation of PC4 was escalated on an average by about 353% and by 302% in roots whereas in shoots, it increased by about 386% and by 295% in cv. satabdi and cv. khitish over their respective controls.

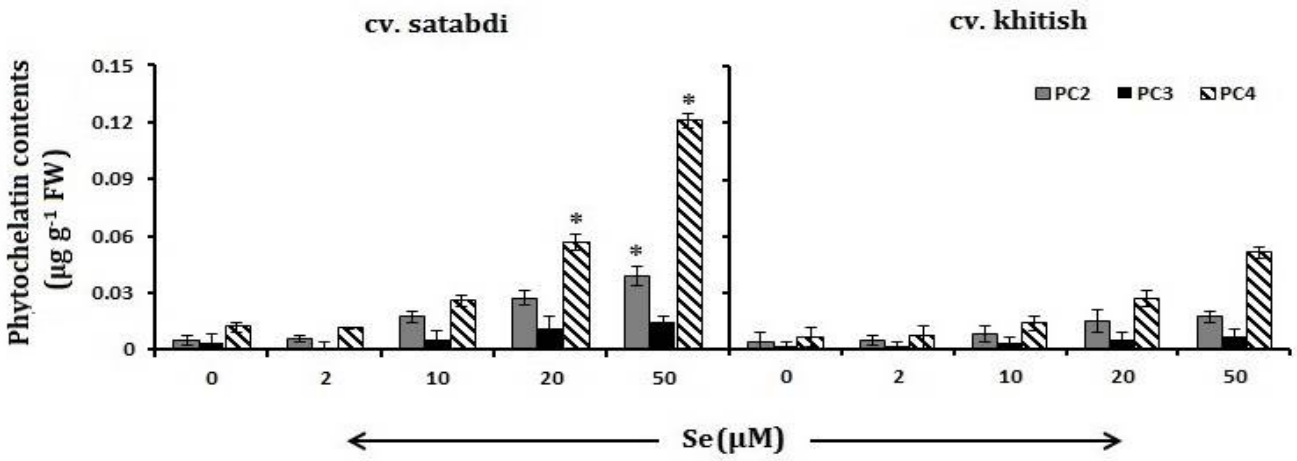

Fig. 9. Effect of selenate on phytochelatins (PC2, PC3 and PC4) in the root of 21 days old cv. satabdi and cv. khitish seedlings. Values are the mean ± SE of three repeats with two replicates in each treatment. .*indicates statistically significant at $p \leq 0.05$ respectively with respect to control.

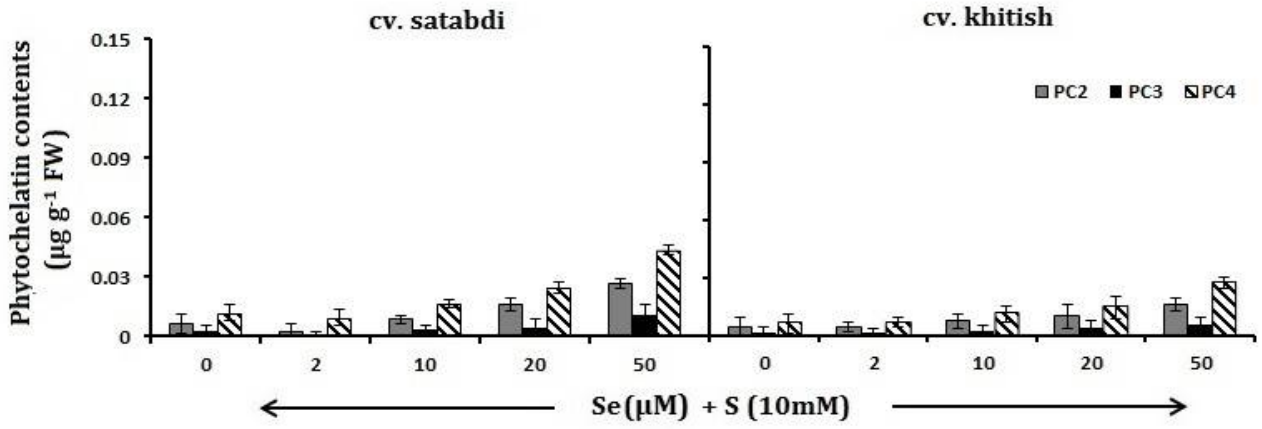

Fig. 10. Effect of selenate and sulphate on phytochelatins (PC2, PC3 and PC4) in the root of 21 days old cv. satabdi and cv. khitish seedlings. Values are the mean ± SE of three repeats with two replicates in each treatment.

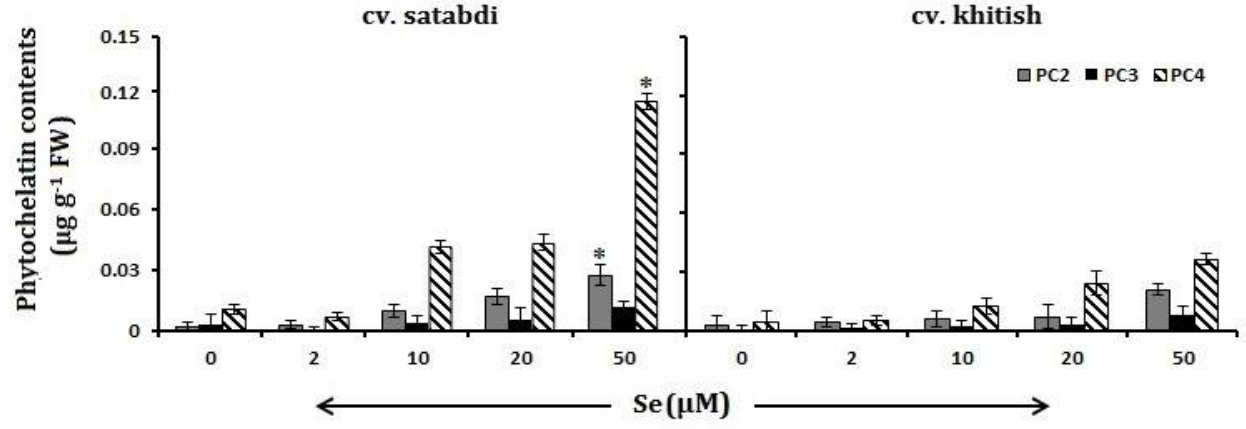

Fig. 11. Effect of selenate on phytochelatins (PC2, PC3 and PC4) in the shoot of 21 days old cv. satabdi and cv. khitish seedlings. Values are the mean ± SE of three repeats with two replicates in each treatment. *indicates statistically significant compared to control.

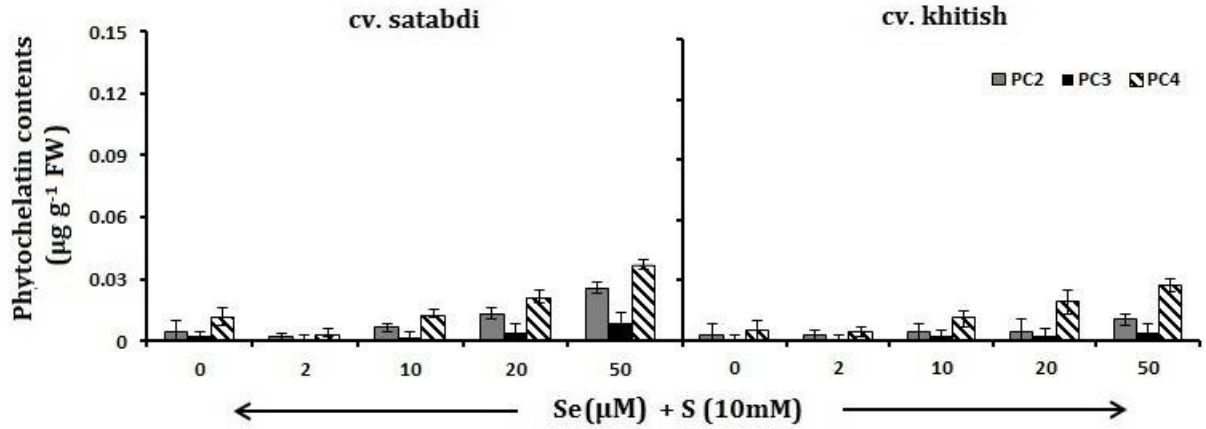

Fig. 12. Effect of selenate and sulphate on phytochelatins (PC2, PC3 and PC4) in the shoot of 21 days old cv.satabdi and cv. khitish seedlings. Values are the mean ± SE of three repeats with two replicates in each treatment

The elevated levels of PC2, PC3 and PC4 declined on an average by about 159%, 36% and 80% respectively in roots and by nearly 409%, 14% and 65% respectively in shoots of cv. satabdi seedlings raised under sulphate supplemented conditions. For cv. khitish the increments in PC2, PC3 and PC4 were also reduced to about 129%, 83% and 113% respectively in roots and to about 71%, 32% and 173% respectively in shoots on an average over their respective controls.

Overall induction of phytochelatins production was higher in cv. satabdi amounting to about 325% compared to that of cv. khitish, where it was about 173% indicating that cv. satabdi suffered more under selenate treatments as compared to that of khitish, showing its sensitivity towards selenate. In both the cases, accumulation of PC4 was maximum followed by PC2. Least accumulation of PC3 was observed under selenate treatments (Supplementary Figs. 2, 3, 4, 5).

Discussion

The present study showed that a low concentration of sodium selenate treatment had positive effects on growth. Contrastingly, high selenate levels induced variable responses in both the cultivars relative to water control (Supplementary Figs. 1a, 1b). It has been well documented that low selenate concentrations stimulates plant growth while at higher levels, plant growth is diminished due to reduction in biomass and photosynthetic pigments (1, 40, 41). Selenium provoked oxidative stress at higher concentrations leads to reactive oxygen species (ROS) production. Plants possess an efficient antioxidant defence system to deal with such ROS under diverse stresses and protect themselves from cellular damages (1, 42). Antioxidants such as glutathione (GSH), ascorbic acid (ASA), α-tocopherol, phenolic compounds, carotenoids (lipid-soluble compounds) along with specific enzymes such as ascorbate peroxidase (APX) catechol peroxidase (CPX) and ascorbate oxidase (AOX) effectively function in quenching ROS and restore cellular homeostasis through the ascorbate-glutathione cycle.

Ascorbic acid (vitamin C) synthesized in the mitochondria is considered as a powerful antioxidant and scavenger of oxygen radicals ($O_2^{\bullet -}$ and $OH^{\bullet}$) that regenerates α-tocopherol which synergistically acts with other antioxidants to reduce oxidative damages and provides membrane stability (43). Our results reveal enhancement in ascorbate contents under 2 µM selenate in both the cultivars, however, with higher doses of selenate treatments ascorbate contents declined in a dose-dependent manner in both the cultivars showing greater variations in cv. satabdi than cv. khitish and coincides with the study conducted in rice cultivar MTU 1010 under salinity stress (44). Increase in ascorbate contents under 2 µM selenate occurred due to the antioxidative nature of selenate under mentioned dose. Again, enhancement of ascorbate due to addition of sulphate in the selenate stressed seedlings lowered the levels of ROS and contributed to alleviation of selenate toxicity in the test seedlings.

Ascorbate peroxidase reduces H_2O_2 to water using ascorbate as an electron donor (45). In the study activity of APX increased in both cv. satabdi and cv. khitish with an increase in selenate concentrations. This is in line with results obtained in rocket tops and strawberry seedlings (42, 46). Greater increments in APX activity in both root and shoot of cv. satabdi with respect to cv. khitish was a response of the former cultivar to withstand stress due to its partial sensitive nature in comparison to that of cv. khitish. Increase in said enzyme activity also reduced the levels of its substrate, ascorbate in both the cultivars. Modifications induced in redox and cell signalling processes due to stress have been shown to boost APX activity that enhances its ability to scavenge ROS (47). Sulphate treatments along with selenate on the other hand, narrowed down increments in APX activity that lowered ascorbate accumulation in both the tested cultivars.

The oxidation of ascorbic acid by AOX produces dehydroascorbic acid (DHAR) and water (H_2O). Ascorbic acid oxidase reduces accumulated H_2O_2 in plant tissues and protects cells from ROS induced damages. Application of selenate enhanced AOX activity in both root and shoot of cv. satabdi and cv.

khitish. Stimulation of enzyme activity to a greater extent might be a response to withstand stress. However, such increments were relatively higher in cv. satabdi than cv. khitish indicating its sensitivity to stress. Sulphate treatment under selenate stress however, had a significant effect on cv. satabdi and lowered AOX activity than cv. khitish.

Low molecular weight antioxidant α-tocopherol interacts directly with oxidizing radicals and limits the chain propagation reaction of lipid peroxidation thus protecting the cells from ROS (48). Selenate treatments enhanced α-tocopherol levels in both the cultivars but enhancements in cv. khitish were lesser in comparison to that of cv. satabdi, possibly due to its superior ability to endure stress. On the other hand, the joint application of sulphate with selenate lowered α-tocopherol levels to a greater extent in cv. satabdi indicating a better recovery of stress than that of cv. khitish.

The most abundant thiol glutathione (GSH) conserves the cellular redox by maintaining the protein thiol groups in the reduced state. Glutathione scavenges ROS and an increase in GSH contents may be ascribed to induction of the sulphur assimilation pathway in the study. Irrespective of varietal differences, the level of GSH was higher in root than shoot. Moreover, enhancement in GSH contents occurred to a greater extent in cv. satabdi compared to that of cv. khitish due to its susceptibility to selenate. Significant increase in GSH under selenate exposure provides protection from oxidative damage as demonstrated in *Azolla caroliniana* Kaulf (49). It was reported that enhanced ROS production due to high concentrations of selenate leads to an imbalance in the production of GSH, thiols (-SH), ferredoxins (Fd.red) and/or NADPH, that play vital role in selenate assimilation (50). GSH not only participates in the regeneration of ascorbate via dehydroascorbate reductase, it also reacts with singlet oxygen and OH^- radicals to protect the thiol groups of proteins (51). Ascorbic acid and GSH provide reducing power necessary to quench excess ROS produced in plants due to stress (52).

The enzyme glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) to its reduced state (GSH) and mainly operates in the chloroplasts (53). GR has an essential role in cellular defence by maintaining the reduced status of glutathione. Dose dependant increment in the activity of GR was prominent in both the cultivars with higher increment in cv. satabdi compared to cv. khitish, pointing its sensitivity to selenate treatments. It has been documented that enhanced GR activity results in more GSH production and better protection against oxidative stress (54). Similar to our study an increase in GR activity with increased GSH contents were noted in *Azolla_caroliniana* Kaulf and *Pteris vittata* L (49, 50). Co-application of sulphate along with selenate lowered GR activity due to less uptake of Se in the presence of S and ultimately lowered ROS production.

Glutathione peroxidase (GPx) localized in the cytoplasm or bound to the cell wall reacts with H_2O_2 forming GSSG which is then reduced to GSH by GR. Evidence of a positive relationship between selenate concentration and GPx activity suggests the existence of a selenate-dependent GPx in plants (55). Moreover, selenate supplementation causes an increase in the activity of GPx which shows this enzyme functions to antagonise ROS formation in plants (49). Our study registered elevation in GPx activity in both the tested cultivars exhibiting greater variations in cv. satabdi than cv. khitish. Such significant increase in the level GSH in selenate-treated rice seedlings may be correlated to the high activity of GR which allowed the reducing agent (GSH) to subdue ROS production with the help of GPx or APX. Our study is in line with another report where GPx has been reported to be activated by selenate and acts as a powerful scavenger of H_2O_2 in ryegrass (55). The joint application of sulphate along with selenate however, lowered selenate uptake, decreased ROS production and consequently down-regulated GPx activity in both the cultivars.

Glutathione-S- transferases catalyze the addition of GSH to electrophilic substrates and sequester them to the vacuoles, thus protecting cells from oxidative damage due to environmental stresses (56). Increments in GST activity with increase in selenate concentrations in both the cultivars corroborates with the earlier studies (57, 58). The elevation in GST activity with selenate treatment was prominent in cv. satabdi than in cv. khitish possibly because of cv. satabdi succumbed more to the stress induced by selenate treatments. The joint application of sulphate along with selenate lowered selenate uptake and thereby lowered GST activity in both the cultivars.

Presence of sulphate in selenium solution acts as a limiting factor and hinders uptake of selenate in plant tissues due to the chemical similarity between selenate and sulphate (59). Selenate forms analogues with sulphate and causes structural changes in proteins especially in methionine and cysteine forming non- functional proteins and enzymes (60). The wrong incorporation of SeCys/SeMet in place of Cys/Met in protein chain resulting in malformed selenoproteins are detrimental to protein functioning and alter redox potential that affects enzyme kinetics (61). Increase in selenate uptake raised H_2O_2 contents and coincided with an increase in GPx and GST activities due to oxidation of GSH by seleno-products. Our study showed that increments in the total glutathione contents and activities of its related enzymes under selenate treatment were narrowed down with the application of 10 mM sulphate with selenate. Similar observations were already reported in rocket plants (*Eruca sativa* L.) (62). Presence of sulphate in treatment solution helped to counteract the oxidative stress generated by selenate by enhancing the production of GSH and other antioxidants (14).

Literature states that the stimulatory effects on the enzymes of the sulfur reduction pathway due to metal stress regulate sulfur uptake and transport in the cells (63, 64). In the present study similarly, selenate stress boosted up the enzyme activities of the ascorbate glutathione cycle, although differentially in the tested cultivars. Selenate treatments also enhanced the levels of GSH, the

substrate necessary for PC synthesis and corresponded with significant increments in the levels of phytochelatins (PCs) that have an exclusive role in heavy metal detoxification to maintain cellular homeostasis (65). Selenium exposure in both the cultivars stimulated PC synthesis in roots as well as in shoots for detoxification of selenate that enhanced the tolerance of the test seedlings. Greater accumulation of PC2, PC3 and PC4 occurred in cv. satabdi revealing its sensitivity to selenate. Conversely, corresponding increments in PC accumulation in cv. khitish were lesser as indicated by the lower PC2, PC3 and PC4 peak heights suggesting its relatively tolerant nature. Similar studies of PC induction in rice seedlings have been addressed under arsenic and chromium toxicity that enabled vacuolar sequestration of heavy metals (66, 67). However, induction of PC3 synthesis as in the present study was not noted under arsenate stress in cv. khitish (66). Such enhancements in PC production helped to bind the heavy metal via sulphydrl and carboxyl groups rendering survival under metal stressed environment (68). S treatments on the other hand, lowered PC accumulation as indicated by the decreased peak heights of PC2, PC3 and PC4 in both the varieties. This could be probably due to repression of sulphate/selenate transporters in the presence of competing sulphate under sulphate supplementation. Such sulphate enrichment has been documented to prevent the negative impacts of high selenate accumulation in *Diplotaxis tenuifolia* L. (69).

Conclusion

The dualistic nature of selenate is apparent from our present work that is reflected by Se-induced promotive effects at 2 µM concentration as opposed to the higher concentrations (10 µM to 50 µM) where selenate induced changes in the ascorbate-glutathione cycle hindered growth. Although exogenous selenate had similar influences on roots and shoots of the cultivars satabdi and khitish still a partial tolerant nature of the latter cultivar has been detected. Selenate treatments not only altered the activities of the ascorbate-glutathione cycle enzymes but also stimulated phytochelatins production in a differential manner in the tested cultivars. Co-application of sulphate along with selenate however, helped to restore the damages induced by selenate alone in both the cultivars and improved growth. Therefore, our study would be helpful for devising future strategies where application of sulphate enriched fertilizer in selenate-prone soils might serve to counter the adverse effects of selenate in growth and metabolism of the tested rice cultivars.

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Authors' contributions

DD carried out the experiments, acquired data and prepared the manuscript. PS performed the statistical analyses helped in preparing graphs and drafted the manuscript. SB also participated in preparing graphs and helped in drafting the manuscript. AKB conceived the study and finalized the manuscript. The final manuscript was approved by all authors.

Conflict of interests

The authors declare that they have no conflict of interests.

Supplementary File

Supplementary File (Fig. 1 to 5)

References

1. Das D, Seal P, Biswas AK. Influence of selenium on growth, antioxidants production and physiological parameters or rice (*Oryza sativa* L.) seedlings and its possible reversal by coapplication of sulphate. Am J Plant Sci. 2019;10 (12):2236-78. https://doi.org/10.4236/ajps.2019.1012158
2. Habibi G. Selenium ameliorates salinity stress in *Petroselinum crispum* by modulation of photosynthesis and by reducing shoot Na accumulation. Russ J Plant Physiol. 2017;64:368-74. https://doi.org/10.1134/S1021443717030086
3. Hartikainen H, Xue T, Piironen V. Selenium as an anti-oxidant and pro-oxidant in ryegrass. Plant Soil. 2000;225(1):193-200. https://doi.org/10.1023/A:1026512921026
4. Proietti P, Nasini L, Buono DD, D'Amato R, Tedeschini E, Businelli D. Selenium protects olive (*Olea europaea* L.) from drought stress. Sci Hortic. 2013;164:165-71. https://doi.org/10.1016/j.scienta.2013.09.034
5. Saidi I, Chtourou Y, Djebali W. Selenium alleviates cadmium toxicity by preventing oxidative stress in sunflower (*Helianthus annuus*) seedlings. J Plant Physiol. 2014;171(5):85-91. https://doi.org/10.1016/j.jplph.2013.09.024
6. Hawrylak-Nowak B. Beneficial effects of exogenous selenium in cucumber seedlings subjected to salt stress. Biol Trace Elem Res. 2009;132:259-69. https://doi.org/10.1007/s12011-009-8402-1
7. Ding YZ, Feng RW, Wang RG, Guo JK, Zheng XQ. A dual effect of Se on Cd toxicity: evidence from plant growth, root morphology and responses of the antioxidative systems of paddy rice. Plant Soil. 2014;375(1-2):289-301. https://doi.org/10.1007/s11104-013-1966-8
8. Kumar A, Dwivedi S, Singh RP, Chakrabarty D, Mallick S, Trivedi P K, Adhikari B, Tripathi RD. Evalution of amino acid profile in contrasting arsenic accumulation rice genotypes under arsenic stress. Biol Plant. 2014;58(4):733-42. https://doi.org/10.0007/s10535-014-0435-4
9. Manojlović MS, Lončarić Z, Cabilovski RR, Popović B, Karalić K, Ivezić V, Arsim A, Singh BR. Biofortification of wheat cultivars with selenium. Acta Agr Scand. 2019;69(8):715-24. https://doi.org/10.1080/09064710.2019.1645204
10. Feng R, Wei C, Tu S. The roles of selenium in protecting plants against abiotic stresses. Environ Exp Bot. 2013;87:58-68. https://doi.org/10.1016/j.envexpbot.2012.09.002
11. Han D, Xiong S L, Tu SX, Liu JC, Chen C. Interactive effects of selenium and arsenic on growth, antioxidant system, arsenic and selenium species of *Nicotiana tabacum* L. Environ Exp Bot. 2015;117:12-19. https://doi.org/10.1016/j.envexpbot.2015.04.008
12. Gupta M, Gupta S. An overview of selenium uptake, metabolism, and toxicity in plants. Front Plant Sci. 2017;7:2074. https://doi.org/10.3389/fpls.2016.02074

13. Garousi F. The essentiality of selenium for humans, animals, and plants, and the role of selenium in plant metabolism and physiology. Acta Univ Sapientiae Aliment. 2017;10(1):75-90. https://doi.org/10.1515/ausal-2017-0005

14. Łabanowska M, Filek M, Koscielniak´ J, Kurdziel M, Kulis´ E, Hartikainen H. The effects of short-term selenium stress on Polish and Finnish wheat seedlings—EPR, enzymatic and fluorescence studies. J Plant Physiol. 2012;169(3):275-84. https://doi.org/10.1016/j.jplph.2011.10.012

15. Mroczek-Zdyrska M, Wojcik M. The influence of selenium on root growth and oxidative stress induced by lead in *Vicia faba* L. minor plants. Biol Trace Elem Res. 2012;147:320–28. https://doi.org/10.1007/s12011-011-9292-6

16. Ahsan N, Lee DG, Alam I, Kim PJ, Lee JJ, Ahn YO, Kwak SS, Lee IJ, Bahk JD, Kang KY, Renaut J, Komatsu S, Lee BH. Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. Proteomics. 2008;8(17):3561-76. https://doi.org/10.1002/pmic.200701189

17. Diao Y, Xu H, Li G, Yu A, Yu X, Hu W, Zheng X, Li S, Wang Y, Hu Z. Cloning a glutathione peroxidase gene from *Nelumbo nucifera* and enhanced salt tolerance by overexpressing in rice. Mol Biol Rep. 2014;41(8):4919-27. https://doi.org/10.1007/s11033-014-3358-4

18. Singh VP, Singh S, Kumar J, Prasad SM. Investigating the roles of ascorbate-glutathione cycle and thiol metabolism in arsenate tolerance in ridged *Luffa* seedlings. Protoplasma. 2015;252(5):1217-29. https://doi.org/10.1007/s00709-014-0753-6

19. Lv SW, Wang XG, Mu Y, Zang TZ, Ji YT, Liu JQ, Shen JC, Luo GM. A novel dicyclodextrinyl diselenide compound with glutathione peroxidase activity. FEBS J. 2007;274(15):3846-54. https://doi.org/10.1111/j.1742-4658.2007.05913.x

20. Dubey RS. Metal toxicity, oxidative stress and antioxidative defense system in plants. In: Gupta SD, editor. Reactive oxygen species and antioxidants in higher plants. Boca Raton: Florida: CRC Press; 2011. p. 177-203.

21. Yadav SK. Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S Afr J Bot. 2010;76(2):167-79. https://doi.org/10.1016/j.sajb.2009.10.007

22. Grill E, Mishra S, Srivastava S, Tripathi RD. Role of phytochelatins in phytoremediaton of heavy metals. Environ Biorem Tech. 2007;101-46. https://doi.org/10.1007/978-3-540-34793-4_5

23. Cobbett CS. Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr Opin Plant Biol. 2000;3(3):211-16. https://doi.org/10.1016/S1369-5266(00)80067-9

24. Dago A, Gonzalez I, Arino C, Martinez-Coronado A, Higueras P, Diaz-Cruz JM, Esteban M. Evaluation of mercury stress in plants from the Almaden mining district by analysis of phytochelatins and their Hg complexes. Environ Sci Technol. 2014;48(11):6256-63. https://doi.org/10.1021/es405619y

25. Iglesia-Turiño S, Febrero A, Jáuregui O, Caldelas C, Araus JL, Bort J. Detection and quantification of unbound phytochelatin 2 in plant extracts of *Brassica napus* grown with different levels of mercury. Plant Physiol. 2006;142(2):742-49. https://doi.org/10.1104/pp.106.085068

26. Mendoza-Co´zatl DG, Zhai Z, Jobe TO, Akmakjian GZ, Yong Song W, Limbo O, Russell MR, Kozlovskyy VI, Martinoia E, Vatamaniuk OK, Russell P, Schroeder JI. Tonoplast-localized Abc2 transporter mediates phytochelatin accumulation in vacuoles and confers cadmium tolerance. J Biol Chem. 2010;285(52):40416-26. https://doi.org/10.1074/jbc.M110.155408

27. Terry N, Zayed AM, De Souza MP, Tarun AS. Selenium in higher plants. Ann Rev Plant Physiol Plant Mol Biol. 2000; 51:401–32. https://doi.org/10.1146/annurev.arplant.51.1.401

28. White PJ. Selenium accumulation by plants. Ann Bot. 2016;117(2):217-35. https://doi.org/10/1093/aob/mcv180

29. Zhu YG, Geng CN, Tong YP, Smith SE, Smith FA. Phosphate (Pi) and arsenate uptake by two wheat (*Triticum aestivum*) cultivars and their doubled haploid lines. Ann Bot. 2006;98:631-36.

30. Mukherjee SP, Chaudhuri MA. Implications of Water Stress-Induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Physiol Plant. 1983; 58(2):166-70. https://doi.org/10.1111/j.1399-3054.1983.tb04162.x

31. Nakano Y, Asada K. Hydrogen Peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 1981;22:867-80.

32. Olliver M. Ascorbic Acid Estimation. In: Sebrell, W H and Harris, R S., editors. The Vitamins VI. New York: Academic Press; 1967. p. 338.

33. Backer H, Frank O, De Angelis, B and Feingold S. Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. Nutrition Reports International. 1980;21:531-36.

34. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue by Ellman's reagent. Anal Biochem.1968;25:192-208. https://doi.org/10.1016/0003-2697(68)90092-4

35. Smith IK, Vierheller TL, Thorne CA. Assay of glutathione reductase in crude tissue homogenates using 5, 5-Dithiobis (2-Nitrobenzoic Acid). Anal Biochem. 1988;175(2):408-13. https://doi.org/10.1016/0003-2697(88)90564-7

36. Elia AC, Galarini R, Taticchi MI, Dorr AJM, Mantilacci L. Antioxidant responses and bioaccumulation in *Ictalurus melas* under mercury exposure. Ecotoxicol Environ Saf. 2003;55:162-67. https://doi.org/10.1016/S0147-6513(02)00123-9

37. Ando K, Honma M, Chiba S, Tahara S, Mizutani JK. Glutathione transferase from *Mucor javamicus*. Agric Biol Chem 1988;52(1):135-39. https://doi.org/10.1271/bbb1961.52.135

38. Sneller EFC, Van-Heerwaarden LM, Schat H, Verkleij JAC. Toxicity, metal uptake, and accumulation of phytochelatins in *Silene vulgaris* exposed to mixtures of cadmium and arsenate. Environ Toxicol Chem. 2000;19(12):2982-86. https://doi.org/10.1002/etc.5620191219

39. Lowry OH, Rosenbrough NJ, Farr A, Randall RJ. Protein measurement by Folin phenol reagent. J Biol Chem. 1951;193:265-67.

40. Djanaguiraman M, Devi DD, Shanker AK, Sheeba JA, Bangarusamy U. Selenium - an antioxidative protectant in soyabean during senescence. Plant Soil. 2005;272:77-86. https://doi.org/10.1007/s11104-004-4039-1

41. Chen TF, Zheng WJ, Wong YS,Yang F. Selenium induced changes in activities of antioxidant enzymes and content of photosynthetic pigments in *Spirulina platensis*. J Integr Plant Biol.2008;50:40-48. http://dx.doi.org/10.1111/j.1744-7909.2007.00600.x

42. Khattab H. Metabolic and oxidative responses associated with exposure of *Eruca sativa* (rocket) plants to different levels of selenium. Inter J Agricul Biol. 2004;6(6):1010-06.

43. Ellouzi H, Hamed KB, Cela J, Müller M, Abdelly C, Munné-Bosch. Increased sensitivity to salt stress in tocopherol-deficient *Arabidopsis* mutants growing in a hydroponic system. Plant Signal Behav. 2013;8:e23136.. http://dx.doi.org/10.4161/psb.23136

44. Das P, Manna I, Biswas AK, Bandyopadhyay M. Exogenous silicon alters ascorbate-glutathione cycle in two salt stressed indica rice cultivars (MTU 1010 and Nonabokhra). Environ Sci Pollut Res Int. 2018;25(26):26625-42. https://doi.org/10.1007/s11356-018-2659-x

45. Sharma P, Dubey RS. Involvement of oxidative stress and role of antioxidative defence system in growing rice seedlings exposed to toxic concentrations of aluminium. Plant Cell Rep. 2007;26(11):2027-38. https://doi.org/10.1007/s00299-007-0416-6

46. Huang C, Qin N, Sun Li, Yu M, Hu W, Qi Z. Selenium Improves Physiological Parameters and Alleviates Oxidative Stress in Strawberry Seedlings under Low-Temperature Stress. Int J Mol Sci. 2018;19(7):1913. https://doi.org/10.3390/ijms19071913

47. Dai HP, Jia GL, Shan CJ. Jasmonic acid-induced hydrogen peroxide activates MEK1/2 in upregulating the redox states of ascorbate and glutathione in wheat leaves. Acta Physiol Plant. 2015;37(10):200. https://doi.org/10.1007/s11738-015-1956-y

48. Halliwell B. Free Radicals and Antioxidants: A Personal View. Nutr Rev. 1994;52(8):253-65. https://doi.org/10.1111/j.1753-4887.1994.tb01453.x

49. Mostafa EM, Hassan AMA. The ameliorative effect of selenium on *Azolla caroliniana* grown under UV-B stress. Phytoprotection. 2015;95:20-26. http://dx.doi.org/10.7202/1031954ar

50. Feng R, Wei C, Tu S. The roles of selenium in protecting plants against abiotic stresses. Environ Exp Bot. 2013;87:58-68. https://doi.org/10.1016/j.envexpbot.2012.09.002

51. Asada K. Production and action of active oxygen in photosynthetic tissue. In: Foyer CH, Mullineaux PM, (editors). Causes of photooxidative stress and amelioration of defence system in plants. Boca Raton Florida: CRC Press;1994. p.77-104.

52. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2000; 7(9):405-10. https://doi.org/10.1016/s1360-1385(02)02312-9

53. Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Gracia B, Queval G, Foyer CH. Glutathione in Plants: an integrated overview. Plant Cell Environ. 2012;35(2):454-84. https://doi.org/10.1111/j.1365-3040.2011.02400.x

54. Gratao PL, Polle A, Lea PJ, Azevedo RA. Making the life of heavy metal-stressed plants a little easier. Funct Plant Biol. 2005;32(6):481-94. https://doi.org/10.1071/FP05016

55. Hartikainen H, Xue T, Piironen V. Selenium as an anti-oxidant and pro-oxidant in ryegrass. Plant Soil. 2000;225(1/2):193-200. https://doi.org/10.1023/A:1026512921026

56. Edwards R, Dixon DP. Plant glutathione transferases. Methods Enzymol. 2005;401:169-86.https://doi.org/10.1016/S0076-6879(05)01011-6

57. Dixon DP, Cummins I, Cole DJ, Edwards R. Glutathione-mediated detoxification systems in plants. Curr Opin Plant Biol.1998;1(3):258-66. https://doi.org/10.1016/S1369-5266(98)80114-3

58. Csiszar J, Horvath E, Vary Z, Galle A, Bela K, Brunner S, Tari I. Glutathione transferase supergene family in tomato: Salt stress-regulated expression of representative genes from distinct GST classes in plants primed with salicylic acid. Plant Physiol Biochem. 2014;78:15-26. https://doi.org/10.1016/j.plaphy.2014.02.010

59. Mackowiak CL, Amacher MC. Soil sulfur amendments suppress selenium uptake by alfalfa and western wheatgrass. J Environ Qual. 2008;37(3):772-79. https://doi.org/10.2134/jeq2007.0157

60. Germ M, Stibilj V, Kreft I. Metabolic importance of selenium for plants. Eur J Plant Sci Biotechnol.2007;1:91-97.

61. Hondal RJ, Marino SM, Gladyshev VN. Selenocysteine in thiol/disulfide-like exchange reactions. Antioxid Redox Signal. 2012;18:1675-89. https://doi.org/10.1089/ars.2012.5013

62. Hassan AM, Saber NE, Ali AS, El-Hosary G. Interactive effect between sulphate and Se on glutathione pool in garden rocket (*Eruca sativa* L.) leaves. J Plant Nutr. 2017;41(14):1834-41.https://doi.org/10.1080/01904167.2018.1462384

63. Rausch T, Wachter A. Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci. 2005;10(10):503-09. https://doi.org/10.1016/j.tplants.2005.08.006

64. Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette MLM, Cuine S, Auroy P, Richaud P, Forestier C, Bourguignon J, Renou JP, Vavasseur A, Leonhardt N. Genomewide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. Biochimie. 2006;88(11):1751-65. https://doi.org/10.1016/j.biochi.2006.04.018

65. Rauser WE. Phytochelatins and related peptides. Plant Physiol. 1995;109:1141-49. https://doi.org/10.1104/pp.109.4.1141

66. Saha J, Majumder B, Mumtaz B, Biswas AK. Arsenic-induced oxidative stress and thiol metabolism in two cultivars of rice and its possible reversal by phosphate. Acta Physiol Plant. 2017;39:263-82. https://doi.org/10.1007/s11738-017-2562-y

67. Mukta RH, Khatun MR, Nazmul Huda AKM. Calcium induces phytochelatin accumulation to cope with chromium toxicity in rice (*Oryza sativa* L.). J Plant Interact. 2019;14(1):295-302. https://doi.org/10.1080/17429145.2019.1629034

68. Emamverdian A, Ding Y, Mokhberdoran F, Xie Y. Heavy metal stress and some mechanisms of plant defense response. Sci World J. 2015;2015:1-18. https://doi.org/10.1155/2015/756120

69. Dall'Acqua, Ertani A, Pilon-Smits E.A.H., Fabrega-Prats M, Schiavon M. Selenium biofortification dfferentially affects sulfur metabolism and accumulation of phytochemicals in two rocket species (*Eruca sativa* Mill. and *Diplotaxis tenuifolia* L.) grown in hydroponics. Plants. 2019;8(3):68-87. https://doi.org/10.3390/plants8030068