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Effect of salinity on *Brassica rapa* var. *toria* (BRSRT) under selenium defence: A trial to assess the protective role of selenium

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ABSTRACT

The present study assesses the role of selenium, an antioxidant in salt-stressed plants. A hydroponic trial of sodium selenate (Na_2SeO_4) on the growth, oxidative stress and antioxidant protection system of *Brassica rapa* var. *toria* (BRSRT) plant was studied. 40 µmol and 100 µmol of Na_2SeO_4 were hydroponically applied to BRSRT roots with 50 mmol and 100 mmol sodium chloride (NaCl) for 12 days. Plant growth, biomass production and photosynthetic pigments at 100 mmol salt stress was inhibited while oxidative stress indicators, for example, hydrogen peroxide and lipid peroxidation were stimulated. Supplementation of 40 µmol Na_2SeO_4 with 50 mmol and 100 mmol NaCl improved growth, photosynthetic pigments and acted as an antioxidant by inhibiting lipid peroxidation and increasing superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, glutathione reductase activities. The in-gel assays also showed enhanced activities of these enzymes. At 100 µmol concentration, selenium under salt stress, repressed growth and expression of antioxidant enzymes and stimulated oxidative stress with enhanced glutathione peroxidase activity. Under consolidated stress treatment, an addition of 40 µmol Na_2SeO_4 was the most effective for both NaCl concentrations. The finding reveals that the optimal selenium supplementation presents a promising potential for use in conditions of relatively high levels of NaCl stress for BRSRT seedlings.

Key words: *Brassica rapa*; antioxidants; salinity stress; sodium selenate

IZVLEČEK

UČINEK SLANOSTI NA VRSTO *Brassica rapa* var. *toria* (BRSRT) OB PRISOTNOSTI SELENA: POSKUS OVREDNOTENJA ZAŠČITNE VLOGE SELENA

V raziskavi je ovrednotena vloga selena kot antioksidanta pri rastlini v slanostnem stresu. Vpliv natrijevega selenata (Na_2SeO_4) na rast, oksidacijski stres in antioksidacijsko zaščito vrste *Brassica rapa* var. *toria* (BRSRT) je bil preučevan v hidroponskem poskusu. 40 µmol in 100 µmol Na_2SeO_4 je bilo 12 dni hidroponsko dodajano koreninam rastline, ki so rastle v 50 mmol in 100 mmol raztopini natrijevega klorida (NaCl). Rast rastlin, produkcija biomase in vsebnost fotosinteznih pigmentov so bili zavrti pri rastlinah pod 100 mmol solnim stresom, aktivirani pa so bili indikatorji oksidativnega stresa kot sta vodikov peroksid in peroksidacija lipidov. Dodajanje 40 µmol Na_2SeO_4 pri 50 mmol in 100 mmol NaCl je izboljšalo rast, vsebnost fotosinteznih barvil in delovalo kot antioksidant z inhibicijo peroksidacije lipidov in povečanjem aktivnosti superoksid dismutase, askorbat peroksidaze, katalaze, glutation peroksidaze in glutation reduktaze. Povečano aktivnost teh encimov je pokazala tudi njihova gelska anliza. Selen je pri koncentraciji 100 µmol v razmerah solnega stresa zavrl rast in tvorbo antioksidativnih encimov in vzpobudil oksidacijski stres s povečano aktivnostjo glutation peroksidaze. V danih stresnih obravnavanjih je bilo dodajanje 40 µmol Na_2SeO_4 najbolj učinkovito pri obeh koncentracijah NaCl. Rezultati kažejo, da predstavlja optimalno dodajanje selena dober obet za njegovo uporabo v razmerah relativno velikega NaCl stresa pri sadikah obravnavane rastline.

Ključne besede: *Brassica rapa*; antioksidanti; slanostni stres; natrijev selenat

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1 INTRODUCTION

A major challenge toward world agriculture involves production of 70 % more food crop for an additional 2.3 billion people by 2050 (FAO, 2009). Up to 20-25 % of the world's irrigated land, which produces one third of the world's food, is subjected to salt stress. Salinization is dispersing more rapidly in irrigated lands because of improper management of irrigation and drainage (Aliu et al., 2015). The negative effect of salt stress has been endorsed to increase in Na^+ and Cl^- ions in diverse plants where these ions produce crucial conditions for plant survival by intercepting different plant mechanisms (Tavakkoli et al., 2010).

On the basis of adaptive evolution plants can be classified roughly into two major types: first are the halophytes that can survive in salinity and second are glycophytes that cannot survive in salinity. Majority of crop species belong to this second category. Salt stress is one of the leading abiotic stresses that affect both qualitative and quantitative behaviour of many crop species such as cabbage (*Brassica oleracea* L.ssp. *oleracea* convar. *capitata* (L.) Alef.), mustard (*Brassica juncea* L.), rapeseed (*Brassica napus* L. ssp. *napus*), and turnip rape (*Brassica rapa* L.). The diploid species of brassica (*Brassica rapa* L.) is more susceptible to salt stress then other polyploidy species (Kumar, 1995). *Brassica* is the third most common oil plant in the world often cultivated in arid and semiarid regions in different countries, where salt stress warns to become or already is a problem.

Salinity builds up the assembly of oxidative stress in plant cells. These reactive oxygen species (ROS) like hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and superoxide radicals ($O_2^{\bullet-}$), are involved in the degradation of membrane components, the oxidation of protein sulphydryl groups, and the loss of membrane function (Feng et al., 2013). To shield cellular membranes and organelles from the destructive effects of reactive oxygen species, plants develop various non-enzymatic and enzymatic antioxidant defence systems. Jaleel et al. (2009) reported that the non-enzymatic antioxidants include lipid soluble (e.g., α-tocopherol and β-carotene) antioxidant, and water soluble reductants (e.g., reduced glutathione (GSH) and ascorbic acid (AsA)). Facts suggest that membranes are the principal sites of salinity damage to cells and organelles (Janmohammadi et al., 2012) because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasma membrane or in the membranes of organelles. The antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), peroxidase (POX), superoxide dismutase (SOD), were considered as a self-protective team, whose pooled purpose is to protect cells from oxidative damage. AsA is considered the most powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions. SOD catalyzes the dismutation of superoxide into H_2O_2 and O_2 and is one of the most effective antioxidant enzymes in limiting oxidative damage. The physiological role of GPX is to maintain low levels of H_2O_2 within the cell, thus decreasing potential damage from free radicals. GR catalyzes the reduction of GSH, a molecule involved in many metabolic, regulatory, and antioxidative processes in plants (Apel & Hirt, 2004).

In recent years articles have been published describing selenium (Se) as an essential element, counteracting various stress factors in animals and humans. Although it has not been confirmed to be a vital micronutrient in higher plants, there is increasing evidence that Se functions as an antioxidant in plants (Avila et al., 2014). Recent studies on the mitigating effect of Se on environmental stress have mostly aimed on the Se-mediated activation of antioxidative defense (Hajiboland et al., 2014). High Se concentration is shown to provoke oxidative stress responses. Growth stimulating effect of trace amounts of Se has been often reported in some plant species such as ryegrass (Hartikainen et al., 2000), lettuce (Xue et al., 2001), potato (Seppänen et al., 2003), soybean (Djanaguiraman et al., 2005), sorghum (Djanaguiraman et al., 2010), rice (Wang et al., 2012), and different varieties of *Brassica oleracea* L. (Hajiboland & Amjad, 2007). Previous studies have indicated that adequate Se concentration can lessen the harmful impact of excessive salinity in some plant species, mainly by enhancing the antioxidant response of plants [Hawrylak-Nowak, 2009 (cucumber); Walaa et al., 2010 (cucumber); Hasanuzzaman et al., 2011 (rapeseed) ; Diao et al., 2014 (tomato)]. *Brassica* plants are special crops that have high potential to create and accumulate monomethylated forms of Se, such as Se-methylselenocysteine and selenomethionine (Terry et al., 2000). Se affects plants by (1) improving plant growth and defending plant against abiotic stresses (heavy metal) at low dosage, and (2) as a pro-oxidant, which is toxic to plants at high doses (Kaur et al., 2014). Therefore, the present experiment was conducted as a trial assessment to evaluate the possible protective role of Se (Na_2SeO_4) with an emphasis on enzymatic and non-enzymatic antioxidative defence response on *Brassica rapa* var. *toria* (BRSRT) plants against salt stress.

2 MATERIALS AND METHODS

2.1 Chemicals

All reagents like sodium selenate (Na_2SeO_4), thiobarbituric acid (TBA), reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB), L-cysteine, nicotinamide adenine dinucleotide (NADPH), bovine serum albumin (BSA), Baker's yeast glutathione reductase (GR), O-dianisidine dihydrochloride, 3'(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), 2,6 dichlorophenolindophenol (DCPIP) were purchased from Sigma Life Sciences (U.S.A.). Sulfosalicylic acid (SSA) was purchased from Hi-Media, India.

2.2 Phytotron trial

The mature seeds of *Brassica rapa* var. *toria* (BRSRT) were provided by the Directorate of Rapeseed Mustard Research (formerly, NRCRM), Sewer, Rajasthan, India. BRSRT seeds were first treated with 5 % sodium hypochlorite solution for 5 min. for surface sterilization and continuous shaking with doubled distilled water for 5-10 minutes. The seeds were then sown on the top of filter paper moistened with doubled distilled water inside the 15 cm plastic petri dishes. On the fifth day uniform-sized seedling were selected and transferred into hydroponic medium in the pots (one plant in each pot), which were acid-washed (2 % nitric acid). Each pot contained half strength Hoagland nutrient solution (Hoagland & Arnon, 1950). Sodium selenate was preferred as it was one of the most effective form of Se for increasing plant growth and accumulation (Poblaciones et al., 2014) and NaCl was used for visualizing salinity toxicity in plants. The pots were arranged in complete randomized design.

The conditions in each phytotron were closely monitored to ensure that the four groups and nine treatments of plants grew under identical conditions, which was as follow: the first group include two treatments of NaCl (50 mmol and 100 mmol), second group include two treatments of Se (40 µmol and 100 µmol Na_2SeO_4), and third group include four treatment of Se and NaCl (50 mm NaCl + 40 µmol Na_2SeO_4 , 50 mmol NaCl + 100 µmol Na_2SeO_4, 100 mmol NaCl + 40 µmol Na_2SeO_4 and 100 mmol NaCl + 100 µmol Na_2SeO_4) together against fourth group of control treatment (only half strength Hoagland solution). NaCl was gradually added to the growth medium in five divided dose at every alternate day to avoid osmotic shock and Se was applied on third day, sixth day and when NaCl was added as last dose into the growth medium. The growth conditions were as follows: photoperiod of 14/10 h light/dark cycle, temperature 23/15 °C (day/night), 75 % mean relative humidity, and the maximal photosynthetic photon flux density of 270 µmol $m^{-2}s^{-1}$. The plants were harvested from each phytotron on 12th day after commencement of the experiment. For each treatment, three replicates were taken for analysis.

2.3 Stress tolerance index (STI) percentage (%)

Root length was considered as an indicator for stress tolerance and the ratio of average root length with Se and average root length of control was calculated. Tolerance index percentage was calculated by multiplying the obtained ratio by 100 (Pilon-Smits et al., 1999).

$$\text{STI (\%)} = \frac{\text{Average root length (cm) + Treated (Se or NaCl)} \quad \text{X 100}}{\text{Average root length control (cm)}} \text{.....(i)}$$

Tolerance index was also calculated for shoot length and leaf area after 12 days of plant growth under different experimental conditions.

2.4 Chlorophyll and carotenoids content

Chlorophyll content was estimated following Arnon (1949) method with some modifications. Fresh leaves (100 mg) from each of the sample were homogenized in 1.5 ml of 80 % (v/v) acetone and the reaction was incubated in dark for 1 h at 25 °C. The mixture was centrifuged at 10,000 g for 5 min and absorbance of the supernatant was recorded at 645 nm, 663 nm (for chl) and 470 nm (for carotenoids) against 80 % acetone which served as blank. The chlorophyll and carotenoid content was determined as follows:

Chl *a* (mg g^{-1} FM) = 12.7 (A_{663}) – 2.29 (A_{645}) × (volume/1000) × mass of tissue..... . (ii)

Chl *b* (mg g^{-1} FM) = 22.9 (A_{645}) – 4.68 (A_{663}) × (volume/1000) × mass of tissue..... .. (iii)

Carotenoids (mg g^{-1} FM) = (1000X A_{470}) – (1.29 Chl *a* – 53.798 Chl *b*) / 220..... ..... (iv)

2.5 Measurement of ROS generation

H_2O_2 was assayed according to the method described by Yu et al. (2003). The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content using the extinction co-efficient of 0.28 μmol^{-1} cm^{-1} and expressed as µmol g^{-1} fresh mass (FM). Lipid peroxidation of leaf was estimated by the level of malondialdehyde (MDA) production using TBA method as described by Hodges et al. (1999). The supernatant (0.5 ml) was mixed with 20 % TCA (2.5 mL) containing 0.5 % TBA and heated in a boiling water bath for 30 min and immediately allowed to cool in an ice bath, the

absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for nonspecific absorption. The MDA-TBA complex was quantified using the extinction coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as μmol g^{-1} FM.

2.6 Measurement of non-enzymatic antioxidant

Leaves (0.5 g FM) were homogenized in 3 ml ice-cold acidic extraction buffer (6 % meta-phosphoric acid containing 1 mmol EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500×g for 15 min at 4 °C, and the supernatant was collected for analysis of ascorbate and glutathione while for NPT, 1:6 ratio of 5 % sulfosalicylic acid (SSA) was used instead of acidic extraction buffer and the supernatant was stored at -20 °C.

Ascorbic acid estimation was done by hydrazine method, according to Mukherjee & Choudhuri (1983). The absorbance was recorded at 530 nm. Unknown concentration of ascorbic acid was estimated by plotting standard curve with known concentration of ascorbic acid. GSH and GSSG content were determined by the recycling method according to Anderson (1985). Aliquot (0.5 ml) was taken in a microfuge tube, to which 0.5 ml reaction buffer (0.1 mol PBS pH 7.6), 0.5 mmol EDTA and 50 μl of 3 mM DTNB were added in a final volume of 1.2 ml. After 5 min, absorbance of GSH was read at 412 nm. To the same tube 100 μl of NADPH (0.4 mmol) and 2 μl GR was added for the determination of total glutathione; the reaction was allowed to run for 20 min. The amount of GSSG was calculated by subtracting GSH from total glutathione concentrations. A standard curve was prepared with varying concentration of reduced glutathione. NPT assay, were done according to method described by Israr et al. (2006). Absorbance were taken twice, initial absorbance was recorded at 412 nm while final absorbance recorded after addition of 300 μl DTNB solution (6 mmol DTNB dissolved in 5 mmol EDTA in reaction buffer PBS pH 7.6). Strength of NPT content in the sample was calculated by L-cysteine standard curve.

2.7 Measurement of enzymatic antioxidant

Fresh leaves (1g) were homogenized in 1.5 ml of chilled reaction buffer (100 mmol potassium phosphate buffer pH 7.0, 1mmol EDTA and 1 % (w/v) PVP) in pre-cooled mortar. The homogenate was filtered and then centrifuged (pre-cooled) at 10,000 x g for 20 min. Experiments were done at 4 °C, supernatant collected and stored at -20 °C for further enzyme assay. Total protein concentration of antioxidative enzymes was assayed according to Lowry et al. (1951) using BSA (bovine serum albumin) as standard and specific activity of antioxidative enzyme is expressed as μmol min^{-1} mg^{-1} protein.

The SOD (EC 1.15.1.1) activity was monitored by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). One unit of SOD was defined as the amount of enzyme necessary to cause 50 % inhibition of the rate of NBT reduction at 560 nm. The reaction mixture was placed on a shaker under light at 80 μmol photons s^{-1} m^{-1} for 15 min and absorbance was recorded. A non-irradiated reaction mixture that did not develop colour served as the control and its absorbance was subtracted from A_{560} of the reaction solution (Giannopolitis & Ries, 1977). CAT (EC 1.11.1.6) activity was monitored by measuring the reduction in absorbance due to consumption of H_2O_2 at 240 nm (Aebi, 1984). The reaction was initiated with enzyme extract and the activity was calculated using the extinction co-efficient of 39.4 mol^{-1} cm^{-1}. APX (EC 1.11.1.11) activity was determined following the method of Nakano and Asada (1981). The enzyme activity was measured by observing the decrease in absorbance at 290 nm for 1 min using the extinction co-efficient of 2.8 $mmol^{-1}$ cm^{-1}. GPX (EC 1.11.1.9) activity was measured as described by Elia et al. (2003) with minor modification. The oxidation of NADPH was observed at 340 nm for 1 min and the enzyme assay activity was calculated using the extinction co-efficient of 6.62 $mmol^{-1}$ cm^{-1}. GR (EC 1.6.4.2) activity was assayed according to the method of Cakmak et al. (1993). The reaction was started with oxidized GSH and the reduction in absorbance at 340 nm due to NADPH oxidation was recorded for 1 min. The activity was calculated using extinction co-efficient of 6.2 $mmol^{-1}$ cm^{-1}.

2.8 Activity staining of antioxidant enzymes

In-gel assays was performed to visualize the change in enzyme activities of the extracts from the control, NaCl treated and Se induced plants by One-dimensional Native-PAGE according to the method of Lammeli (1970). The amount of protein in supernatant was measured according to Lowry et al. (1951). Hundred μg protein equivalents were loaded in the gel. Native-PAGE After the run, the gels was zymographed for the presence of the oligomeric form as follows.

Activity staining for SOD was carried out following the method of Yen et al. (1996). The gel was equilibrated in 50 mmol K-PO_4 buffer pH 7.8 containing 2.3 mmol NBT, 0.028 mmol riboflavin and 280 mmol TEMED for 15 min in dark. The gel was washed twice with distilled water and illuminated in light until achromatic bands appeared against purple-background. CAT activity was determined by pre incubating the gel in 3.27 mmol H_2O_2 for 25 min, rinsed in distilled water and stained in solution of 1 % $FeCl_3$ and $K_3Fe[CN]_6$ until achromatic bands on a Prussian blue background appeared (Woodbury et al., 1971). POX activity was detected by

incubating the gel in 50 mmol $NaPO_4$ buffer pH 7.0 containing 2 mmol ascorbate for 15 min and further incubation for 20 min in presence of 4 mmol H_2O_2 plus 20 mmol pyrogallol as described by Mittler & Zilinskas (1993). For GPX activity, firstly the gel was washed with 2.5 % triton X-100 for 15 min and washed twice with distilled water followed by incubation in 10 mmol K-PO_4 buffer pH 7.5 containing 2 mmol O-dianisidine dihydrochloride for 1 hr in darkness. The gel was then incubated for 15 min in the same buffer containing 0.1 mmol H_2O_2 to develop bands against pale yellow background (Kankofer, 2002). For GR gels were immersed in 0.25 mol tris/cl, pH 7.5 containing 0.24 mmol MTT, 0.4 mmol NADPH, 10 mg DCPIP, 3.4 mmol oxidized glutathione and incubated for 1hr in dark to develop purple bands (Kang et al., 1999).

2.9 Statistical Analysis

The results of in vitro study were given as mean ± standard deviation (SD) obtained from three independent experiments and analyzed with two-way analysis of variance ANOVA and a "*p*" value less than 0.05 ($p < 0.05$) was considered as significantly different from each other.

3 RESULTS AND DISCUSSION

3.1 Growth parameters

Noteworthy reduction was seen in BRSRT seedling development (shoot length, root length and leaf area) under salt stress. Plants responded to Se treatment in dose dependent manner; 40 µmol Na_2SeO_4 have a stimulatory impact on development when compared with control plants, while 100 µmol Na_2SeO_4 alone altogether significantly reduce the tolerance index of root length, shoot length and leaf area by 26.23 %, 50.09 % and 61.5 %, respectively over control (Table 1). The maximum tolerance index was obtained in plants treated with 40 µmol Na_2SeO_4 alone. 40 µmol Se supplementation with 50 mmol and 100 mmol NaCl concentrations promoted BRSRT seedling growth (108 %, 101.1 %, 110.3 % and 52.87 %, 69.20 %,79.36 %) respectively. Our results suggest that 40 µmol Se has better protective response to different salinity level as compared to control.

Table 1: Stress tolerance Index (STI) of selenium in the form of Na_2SeO_4 induced growth parameters (root length, shoot length, leaf area) under salinity stress

Selected biometric parameters				
Treatments		Root length TI	Shoot length TI	Leaf area TI
Salt (mmol)	Se (µmol)	(%)	(%)	(%)
0	0	0	0	0
	40	116a	111.2a	114.2a
	100	26.23e	50.09e	61.50e
50	0	86.78c	86.06c	104.7b
	40	108.0b	101.1b	110.3a
	100	84.57c	83.60c	81.71c
100	0	50.33d	51.63e	65.15e
	40	52.87d	69.20d	79.36c
	100	23.91e	54.05e	70.02d

Values are the mean ± SD of three replicates in column, and the values in the same column with different letters are significantly different from each other ($p < 0.05$)

3.2 Photosynthetic pigments

A significant decrease of 0.17 fold & 0.40 fold in chlorophyll *a* (Figure 1a), 0.40 fold & 0.63 fold in chlorophyll *b* (Figure 1b) and 0.55 fold & 0.40 fold in carotenoids content (Figure 1c), was seen in leaves of BRSRT grown in the medium containing 50 mmol and 100 mmol NaCl respectively. Whereas the leaves when grown in 40 µmol Na_2SeO_4 alone increased the content of chl *a* by 0.46 folds, chl *b* by 0.26 fold and carotenoids by 0.85 fold (Figure 1). However, the addition of 40 µmol Na_2SeO_4 to BRSRT leaves grown in 50 mmol and 100 mmol NaCl stressed seedlings showed a significant increase of 0.071 fold & 0.100 fold in chlorophyll *a* (Figure 1a), 0.25 fold & 0.31 fold in chlorophyll *b* (Figure 1b) and 0.68 fold & 0.14 fold in carotenoids content (Figure 1c) as compared to salt treatment alone.

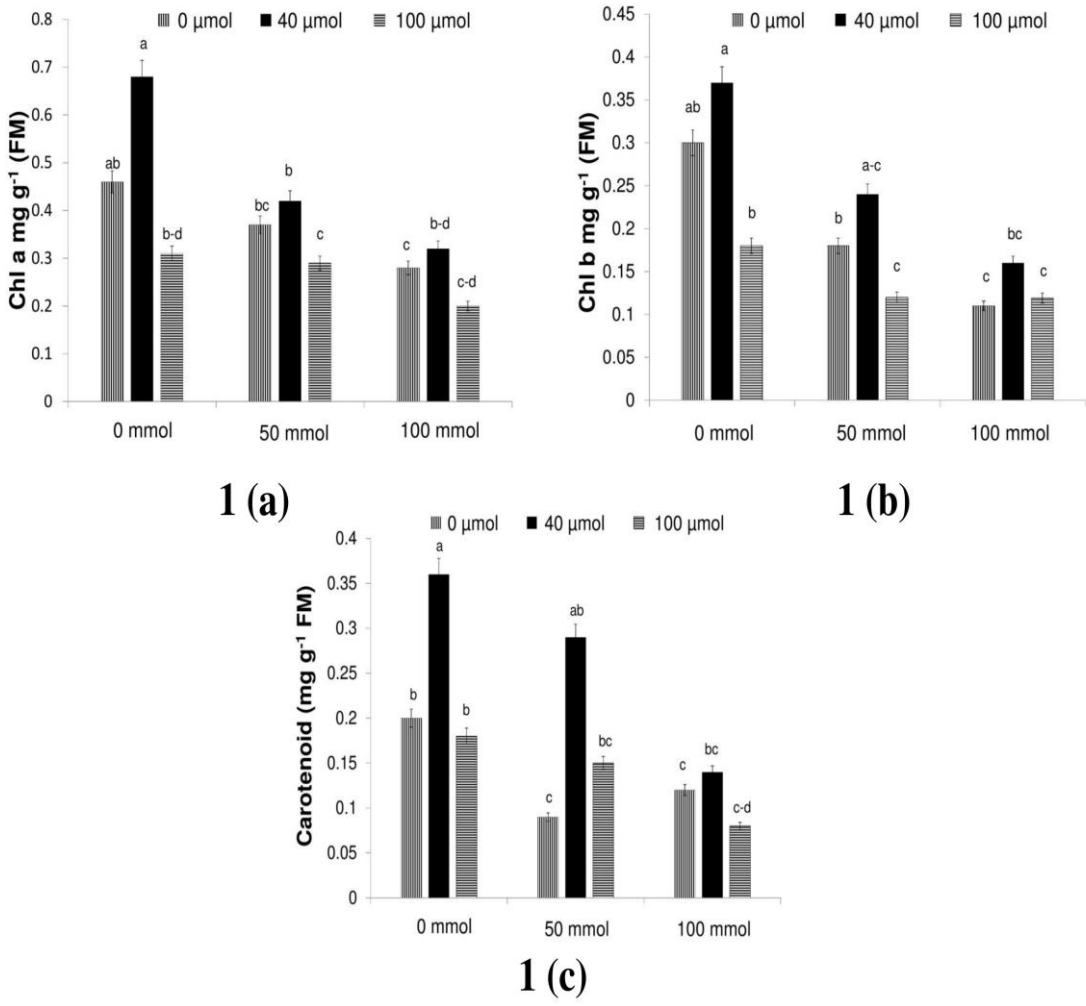

Figure 1: Chlorophyll and carotenoids contents in rapeseed seedlings induced by selenium (Na_2SeO_4) under salt stress conditions: [a] chlorophyll *a* [b] chlorophyll *b* [c] carotenoids content. The mean values (n = 9) with different letters across treatments are significantly different at $p < 0.05$.

3.3 Measurement of ROS generation

Salt stress prompts accumulation of ROS, which aggravates cell redox homeostasis and results in oxidative harm. H_2O_2 content and lipid peroxidation (MDA content) are the oxidative stress indicators in plants. There was an increase in H_2O_2 (1.41 fold & 2.99 fold) and MDA content (0.70 fold & 1.5 fold) in 50 mmol and 100 mmol NaCl stressed plant over control. A 40 µmol Na_2SeO_4 in combination with 50 mmol and 100 mmol salt in plant demonstrated a decrease in H_2O_2 and MDA content (1.21 fold & 2.0 fold and 0.19 fold & 0.96 fold) which is less than the control and salt stressed plant (Figure 2). These result demonstrated that 40 µmol Na_2SeO_4 supplementation shielded BRSRT seedlings from harm by salt stress.

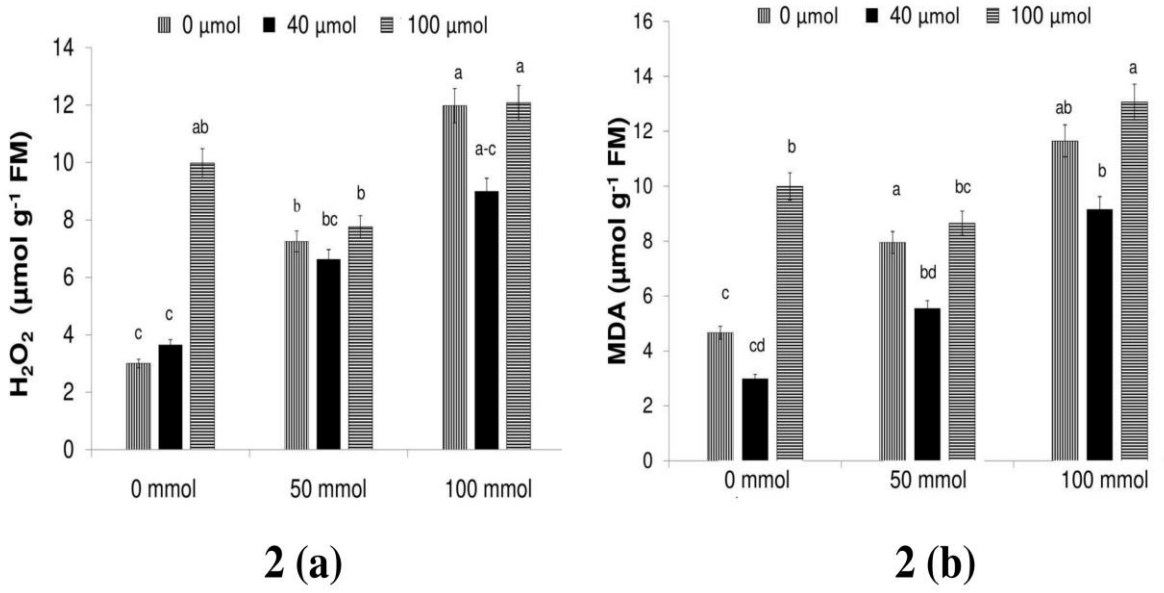

Figure 2: Oxidative stress in BRSRT seedlings: [a] H_2O_2 content [b] MDA content. The mean values (n = 9) with different letters across treatments are significantly different at $p < 0.05$.

3.4 Measurements of non-enzymatic antioxidant activity

Significant reduction (0.20 and 0.42 fold) in the AsA content of BRSRT leaves grown in 50 mmol and 100 mmol salt was seen. 40 µmol Na_2SeO_4 supplementation increases the ascorbic acid content by 0.15 and 0.27 fold (Figure 3a), while 100 µmol Na_2SeO_4 addition significantly increased the AsA content. Se alone is not capable to enhance the AsA content but under salt stress it proves to be beneficial.

Salt stress resulted in increase levels of GSH (0.68 and 0.44 fold at 50 and 100 mmol NaCl, respectively) compared to control (Figure 3b). Addition of Se to non treated seedlings did not change the GSH content significantly. However, the addition of 40 µmol Na_2SeO_4 to BRSRT leaves grown in 50 mmol and 100 mmol salt stressed seedlings resulted in significant increase (0.40 and 0.10 fold, respectively) of GSH as compared to the seedling grown without Se. 100 µmol Na_2SeO_4 did not change the GSH content in both the salt concentration.

Salinity treatments markedly increased the GSSG content in rapeseed seedlings. In our experiment, GSSG level increased by 0.85 and 1.42 fold at 50 and 100 mmol NaCl, respectively (Figure 3c). Se alone did not increase the GSSG content, but, under salt stress, in both 40 µmol and 100 µmol Na_2SeO_4 the levels of GSSG was lower than salt alone.

The GSH/GSSG ratio remained almost unchanged at 50 mmol NaCl, and 100 mmol NaCl (Figure 3d). However, supplement of 40 µmol Na_2SeO_4 showed a significant (0.98 and 0.21 fold) increase in the GSH/GSSG ratio, compared to seedlings treated with salt alone.

NPT level at 50 and 100 mmol NaCl increased by 0.67 and 0.43 fold as compared to control (Figure 3e). Se alone did not change the NPT content. However, under salt stress (50 and 100 mmol), the seedlings with 40 µmol Na_2SeO_4 supplement showed a significant (0.40 and 0.10 fold) increase in the NPT content, compared to seedlings treated with salt alone.

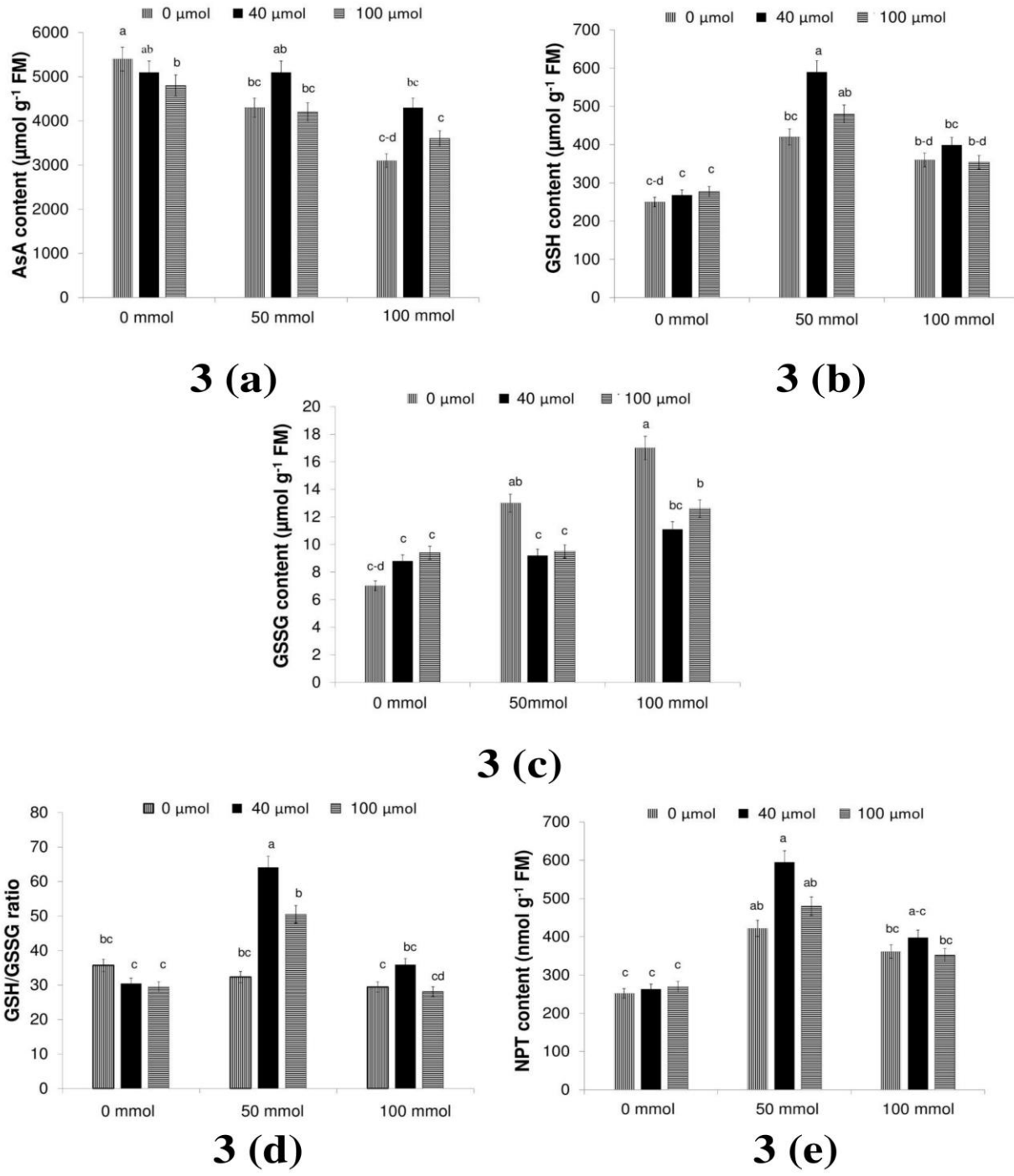

Figure 3: Non enzymatic antioxidant content in rapeseed seedlings induced by selenium (Na_2SeO_4) under salt stress conditions [a] Reduced Ascorbate (AsA), [b] Reduced Glutathione (GSH), [c] Oxidized Glutathione (GSSG), [d] GSH/GSSG ratio, [e] Non Protein Thiol (NPT). The mean values (n = 9) with different letters across treatments are significantly different at $p < 0.05$.

3.5 Measurements of enzymatic antioxidant activity

The effect of different concentrations of Na_2SeO_4 treatments on SOD, CAT, APX, GPX & GR activities in BRSRT leaves, exposed to salt stress are shown in (Figure 4 & 5).

SOD activity of BRSRT leaves slightly increased at 50 mmol salt stress while at 100 mmol NaCl stress the

activity decreased (Figure 4a). 40 and 100 µmol Na_2SeO_4 alone is incompetent to increase the SOD activity whereas 40 µmol Na_2SeO_4 supplementation showed a remarkable increase in the SOD activity (0.87 and 0.67 fold) at both the salt stress (50 mmol and 100 mmol). Activity staining of SOD gels shows three oligomeric forms at 40 µmol Na_2SeO_4 and in combination of 40 µmol Na_2SeO_4 and 50 mmol NaCl stress (Figure 5a).

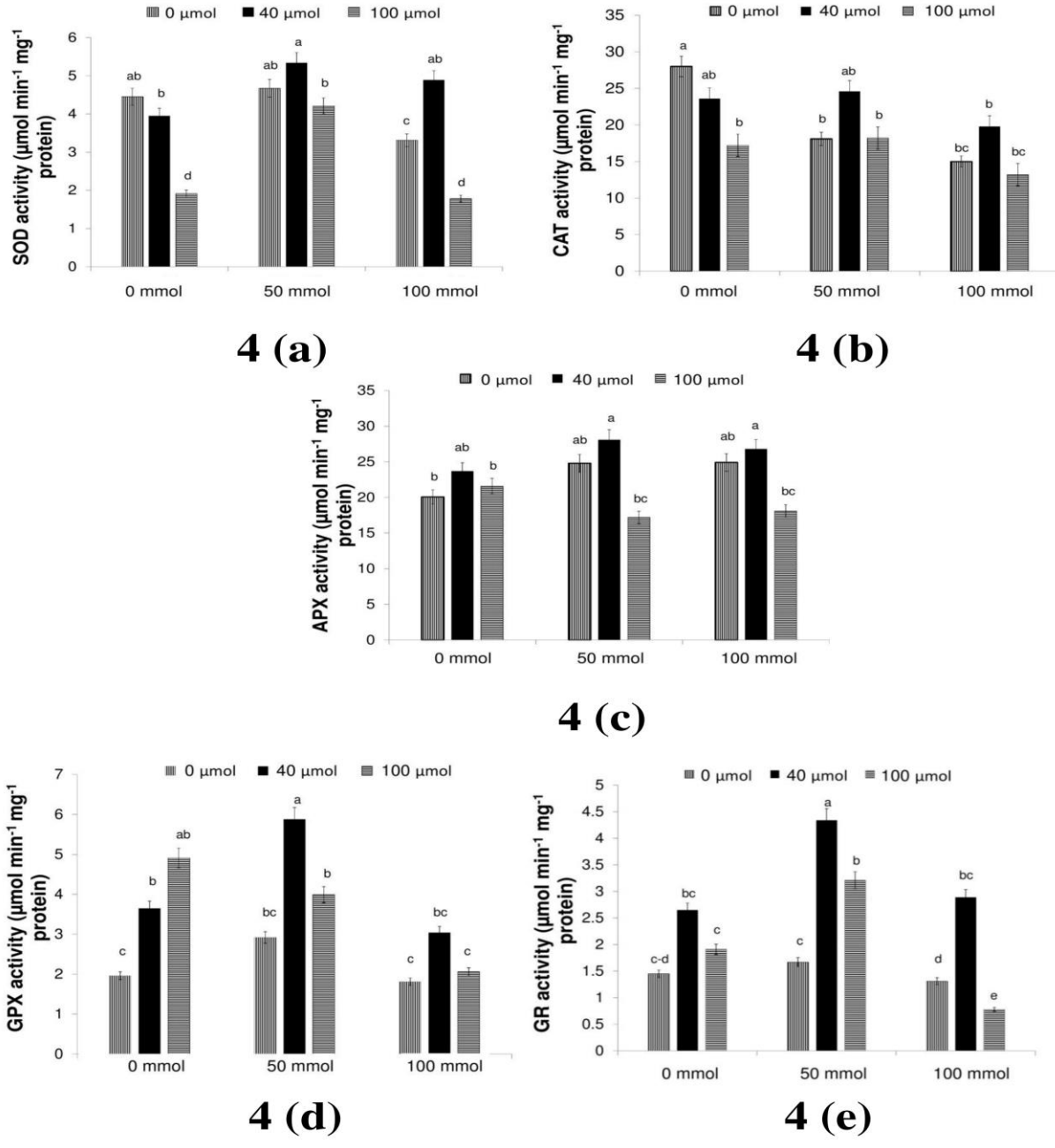

Figure 4: Spectrophotometric analysis of antioxidant enzymes in rapeseed seedlings induced by selenium (Na_2SeO_4) under salt stress: [a] Superoxide dismutase (SOD), [b] Catalase (CAT), [c] Ascorbate Peroxidase (APX), [d] Glutathione Peroxidase (GPX) [e] Glutathione reductase (GR). The mean values (n = 9) with different letters across treatments are significantly different at $p < 0.05$.

CAT activity of BRSRT leaves was 0.35 and 0.46 fold lower at 50 and 100 mmol NaCl stress (Figure 4b) and impeded with Se treatments alone. However, under 50 and 100 mmol salt stress, the seedlings with 40 µmol Na_2SeO_4 supplementation demonstrated an increment (0.36 and 0.32 fold) in the CAT activity, compared to seedlings treated with NaCl alone. 100 µmol Na_2SeO_4 did not change the CAT activity in both the salt concentration. In-gel assays for detecting CAT activity, revealed three isozymes (CAT 1, CAT 2 and CAT 3) (Figure 5b). BRSRT leaves treated with Na_2SeO_4 and NaCl alone demonstrated a reduction in total CAT activity as judged by intensities of bands. Salt stress plus 100 µmol Se treatments did not create any remarkable effect on total CAT activity. Notwithstanding, both densitometric (Figure 4b) and total enzyme analyses (Figure 5b) showed that after 40 µmol Se in addition to osmotic stress, the BRSRT leaves had a significant increase in CAT activity as compared to separately applied stresses.

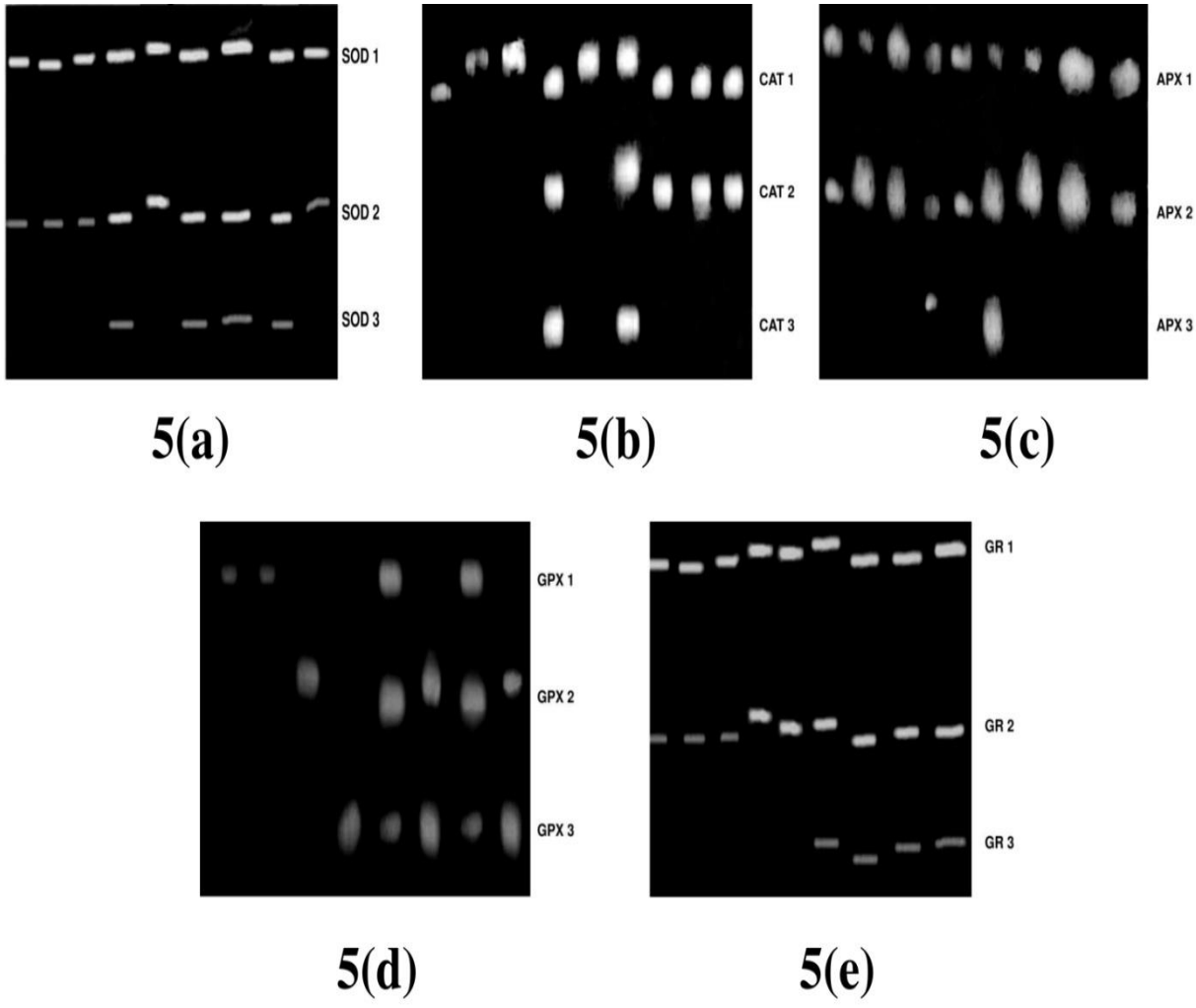

Figure 5: In-gel visualisation of antioxidant enzymes in rapeseed seedlings induced by selenium (Na_2SeO_4) under salt stress: [a] Superoxide dismutase (SOD), [b] Catalase (CAT), [c] Ascorbate Peroxidase (APX), [d] Glutathione Peroxidase (GPX), [e] Glutathione reductase (GR). Lane 1 - Control; Lane 2 – 50 mM NaCl concentration; Lane 3 – 100 mM NaCl concentration; Lane 4 – 40 µmol Na_2SeO_4 concentration; Lane 5- 100 µmol Na_2SeO_4 concentration; Lane 6 - 50 mmol NaCl concentration + 40 µmol Na_2SeO_4 concentration; Lane 7 - 50 mmol NaCl concentration + 100 µmol Na_2SeO_4 concentration; Lane 8 - 100 mmol NaCl concentration + 40 µmol Na_2SeO_4 concentration; Lane 9 - 100 mmol NaCl concentration + 100 µmol Na_2SeO_4 concentration.

In BRSRT seedlings, APX activity increased by 0.26 and 0.24 fold, at 50 and 100 mmol NaCl, respectively, compared to the activity in control (Figure 4c). The application of Se alone did not significantly influence the APX activity but salt-stressed (50 and 100 mmol NaCl) seedlings with 40 µmol Na_2SeO_4 supplement showed elevated APX activity (0.15 and 0.06 fold). High intensity bands of APX (APX2 and APX3) were found in presence of 40 µmol Se treated plants as shown in Figure 5(c). These results corroborated with the spectrophotometric analysis. However, 100 µmol Na_2SeO_4 significantly decreased the APX activity and the intensity of bands reduced (Figure 5c) at both high salt and selenium combination.

As compared to control seedlings, the activity of GPX in 50 mmol NaCl increased (by 0.49 fold), although at 100 mmol, it remained unaltered (Figure 4d) as in control plants. However, 50 and 100 mmol salt-stressed seedlings supplemented with 40 µmol Na_2SeO_4 showed a significant (1.01 and 0.68 fold) rise in GPX activity in contrast to seedlings treated with NaCl alone. 100 mmol salt in addition to 100 µmol Na_2SeO_4 treatments did not make any impact on total GPX activity. The in-gel assay showed high intensity of bands of GPX (GPX1, GPX2, and GPX3) in Figure 5(d) of salt-stressed (50 and 100 mmol) seedlings supplemented with 40 µmol Na_2SeO_4.

The activity of GR in 50 mmol NaCl treated leaves did not increase at 100 mmol (decreases by 0.1 fold) as compared to control plants. Application of 40 µmol Na_2SeO_4 increases (1.67 and 1.05 fold) at both the salt stressed plants (50 mmol and 100 mmol NaCl) respectively (Figure 4e). 100 mmol salt in addition to 40 µmol Na_2SeO_4 treatments showed remarkable increment in the GR activity (0.98 fold) as compared to 50 mmol NaCl treated plants. In-gel assays for detecting GR activity, revealed three isozymes (GR1, GR 2 and GR 3) (Figure 5e) in presence of salt and selenium whereas two bands were appeared on the control, salt and Na_2SeO_4 treated plants.

4 DISCUSSION

Among the diverse abiotic stresses restraining the crop productivity, salinity stress is a foremost problem which needs to be addressed. High salt concentration decreases the osmotic potential of soil solution creating water stress in plants and cause severe ion toxicity (Shabala, 2013). Reduction of photosynthetic pigments during salt stress could be activation of photosynthetic destructive enzymes chlorophyllase (Hashem et al., 2013). It was observed that the chlorophyll content increased significantly with 40 µmol Na_2SeO_4 application which somewhat reduces the damage to the chloroplasts (Figure 1), this is in agreement with the positive effects of Se treatment in delaying the loss of chlorophyll in senescing *Vicia faba* L. plants (Moussa et al., 2010). Findings of the previous studies have indicated that exogenously applied Se can be favourable for some species of salt-stressed crops (Hawrylak-Nowak, 2009; Walaa et al., 2010; Hasanuzzaman et al., 2011). In our study, high concentration of Na_2SeO_4 with 50 mmol and 100 mmol salt lead to reduction of chlorophyll content. Jiang et al. (2017) also observed that high Se concentration aggravated the damage to the photosynthetic system in *Zea mays* and resulted in decrease in net photosynthetic rate.

H_2O_2 performs dualistic role in plants: at low concentrations, it acts as an indicator molecule involved in adaptation to various forms of biotic and abiotic stress, and at high concentrations, it causes oxidative stress in plants (Hu et al., 2012). Tsai et al. (2004) have observed a progressive H_2O_2 accumulation in salt-treated (150 mmol NaCl) roots of rice. A sharp increase in the level of H_2O_2 and lipid peroxidation in salt stressed BRSRT seedlings resulted in increased oxidative damage probably due to impairment of the antioxidant defense. However, the presence of 40 µmol Na_2SeO_4 in salt-stressed seedlings significantly lowered the levels of H_2O_2 and MDA compared to salt-stress alone (Figure 2). Se retarded MDA formation also in experiments performed by Djanaguiraman et al. (2005). The growth stimulating effect of Se may be related to its antioxidative function as demonstrated by diminished H_2O_2 content lipid peroxidation. This finding is in accordance with Hartikainen et al. (2000) in ryegrass, Xue et al. (2001) in lettuce and Seppanen et al. (2003) in potato.

It is suggested that Se compounds can control production and quenching of reactive oxygen species either directly or indirectly through regulation of antioxidants level (Kaur et al. 2014). AsA can protect membranes by directly scavenging $O_2^{\cdot-}$and $OH^{\cdot-}$. It has been reported that plants with high AsA contents show improved tolerance to oxidative stress (Foyer et al., 1995). In this study, we observed that the level of AsA is decreased when plant is exposed to 50 mmol and 100 mmol NaCl stress (Figure 3a). However, addition of 40 µmol Na_2SeO_4 significantly increased the AsA content in salt-stressed seedlings. In the nonexistence of stress, tissues such as leaves usually maintain measurable GSH/GSSG ratios of approximately 20:1 (Foyer & Noctor, 2011). Addition of 40 µmol Na_2SeO_4

significantly increased the GSH content in salt-stressed seedlings (Figure 3b). This increased level of GSH in Se-treated leaves might be attributable to Se boosting GSH synthesis. This study is in accordance with Xue et al. (2001), who reported that Se accelerated efficient recycling of GSH, which is based on GR activity. The relationship between Se and GSH synthesis was also reported by Anderson & McMahon (2001). Similarly, Hasanuzzaman & Fujita (2011) reported that the GSH content was also enhanced by Se both in drought-stressed rapeseed seedlings. As shown in Figure 3(c), GSSG levels increased in salt-stressed seedlings. This increase might partly attribute to a decrease in the rate of GSH recycling or to an increased rate of degradation (Foyer & Noctor, 2009). Sumithra et al. (2006) reported that chickpea plants with lower GSSG concentrations possessed a strong antioxidative system and were better protected against oxidative damage during salt-stress conditions. In our experiment, the Se-treated salt-stressed seedlings showed lower GSSG level than the seedlings treated with salt alone (Figure 3c). This increase in the GSH/GSSG ratio (Figure 3d) in Se-supplemented salt stressed BRSRT seedlings also provides a clear demonstration of the role of Se towards salt tolerance. Tolerance toward metals correlates well with the level of non-protein thiols which include glutathione, phytochelatins and other SH-rich compounds (Kafel et al., 2010). In the present work treatment of BRSRT leaves with 40 µM Na_2SeO_4 in salt stress caused a significant increase in proline content compared to untreated plants. This could be due to the effect of Se on proline metabolism enzymes. This was analogous to the study of Nadgórska-Socha et al. (2013) who observed an increment in non-protein –SH group content in leaves of *Silene vulgaris* (Moench) Garcke populations.

The enhanced SOD activity under salt stress (Figure 4a) in our study is corroborated with the finding of Meloni & Martińez (2009) in *Prosopis ruscifolia* Griseb. With the increase in total SOD activity, a concomitant increase in the activities of isoform of SODs (three bands) was observed in response to Se under both the salt stress (Figure 5a). According to Hernandez et al. (2010), *Brassica oleracea* revealed three bands of SODs under salt stress. The decline in CAT activity is regarded as a general response to many stresses (Liu et al., 2008). It may also be linked with dreadful conditions caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme. On the other hand, CAT activity decreased under salinity stress, similarly as in the experiments of Hasanuzzaman et al. (2011) on rapeseed plants also stated that Se supplementation induced an increase in CAT activity, which indicated a protective role of Se in scavenging H_2O_2 under salt stress. However, the CAT activity was restored to the control level after Se supplementation of salt- stressed plants with 40µmol Se in our study (Figure 4b). APX activity increased in accordance with earlier observation of Mittal et al. (2012) in *Brassica juncea* (L.) Vassiliĭ Matveievitch Czernajew. The addition of 40 µmol Se to the medium containing 50mmol and 100 mmol salt increased APX activity as compared to the salt exposed plants without selenium (Figure 4c). In accordance with Salekjalali et al. (2012), six bands of APX were present in the drought stressed barley plants. Moreover addition of 40 µmol Se to salt stressed BRSRT leaves, revealed three isozymes with enhanced intensity (Figure 5c).

One of the likely candidates responsible for H_2O_2 detoxification and eventually lowering of lipid peroxidation from cellular H_2O_2 pool in high salt and Se stress could be the GPX antioxidant system. In our study, since there is no significant increase in GPX activity in BRSRT leaves, subjected to salt stress, it was not sufficient to detoxify the H_2O_2 radicals, as is also reported in Yildiztugay et al. (2016). Moreover, the application of selenium to salt treated BRSRT leaves, brought about significant increase in the GPX activity (Figure 4d). In accordance with Diao et al. (2014), the amplification in enzyme activity may be due to Se-dependent GPX. GPX isoform pattern was strongly affected by salt stress in combination with 40 µmol Se, at which new isozymes designated as GPX 2 and GPX 3 appeared. The activities of other isoform GPX 1 were enhanced as compared to control (Figure 5d). GPX was more efficient in destroying H_2O_2 than was catalase under salt stress, since Se could not increase the catalase activity in a significant manner. The reason for this could be that GPX, which is present throughout the cell and has higher substrate affinity in the presence of glutathione as a reductant (Foyer & Noctor, 2009) and CAT is present only in the peroxisomes and has low substrate affinity, as it requires simultaneous access of two molecules of H_2O_2 (Filek et al., 2008). GR catalyses the NADPH-dependent reduction of the disulphide bond of GSSG, and is, therefore, important for maintaining the GSH pool. The present study showed high GR activity on Se addition under salt stress (Figure 4e) which is in accordance with the study of Hoque et al. (2007). This increase in the activity of GR contributes to the maintenance of a high GSH/GSSG ratio which is beneficial for GSH dependent enzymes involved in the antioxidant defense. The increase in the GR activity upon Se treatment also resulted in the accumulation of GSH, and ultimately, conferred salt tolerance in plants.

5 CONCLUSION

The present study provides an insight into the role of Se in regulating of biochemical responses to salt stress. It was observed that 40 µmol Na_2SeO_4 treatment in BRSRT seedlings enhanced protective role of Se in ROS detoxification system protection against high salt-stressed oxidative damage, as indicated by the down regulation of lipid peroxidation and H_2O_2 levels. The components of the antioxidant defense system machinery are upregulated in the presence of Se, which allowed the plants to cope better with salt stress. Based on our results and the results found in the literature, we conclude that the non-enzymatic and enzymatic antioxidant system are simultaneously regulated to control the levels of ROS during salt stress and to maintain redox homeostasis to increase plant survival.

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