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Antioxidative defense response of selenium by hyper accumulator plant *Brassica rapa* var. PS66 and Toria towards phytoremediation

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Abstract

Natural processes for instance volcanic eruptions and anthropogenic activities lead to emission of heavy metals in ecosystem. Brassica species have competency to absorb and sequester Se and harness to manage environmental Se contamination via phytoremediation. All the test species of Brassica were found to respond to Se by registering changes in the expression of antioxidative enzymes, and tolerance level showed significant inhibition at higher concentration. The raised value of oxidative stress determinants - lipid peroxidation and hydrogen peroxide, near 100µM signified build up of stress at this concentration. Brassica rapa showed greater Se tolerance as was evident from the increased expression of glutathione peroxidase (GPX) in the treated plants, results corroborated with in gel assays for the enzymes, whereas little or no basal activity was found in the control plants. The positive tie-up between Se enhancement and GPX activity is suggestive for the existence of Se-dependent GPX despite the fact that most plant GPX studied so far have not been reported to require Se for their function. One noteworthy attribute of Brassica rapa PS66 and Toria have adequacy to transform inorganic Se to volatile forms, principally dimethylselenide (DMSe), which is 500-600 times less toxic than the available form of selenium in the environment, thus a prospect benefit for selenium phytoremediation.

Keywords: Selenium, antioxidant, phytoremediation, hyper accumulator plant, *Brassica rapa*.

Introduction

Selenium (Se) has been demonstrated as an essential micronutrient for the maintenance of human health and animals and is required to enhance the activities of some selenozymes such as glutathione peroxidase, iodothyronine 5'-deiodinase and thioredoxin reductase[1]. Increasing scientific evidence manifest that selenium has many advantageous effects for plants as well as many other forms of life. Selenate begins to involve in the plants actively through transporters of sulfur in the plasma membrane of the root cells[2] while selenite bound up passively[3]. Selenate directly competes with sulfate for uptake by plants. Subsequent uptake, it has been suggested that selenate is primarily carried to the chloroplasts; where it is processed by the sulfate assimilation pathway[4]. Majority of the enzymes concerned in sulfur metabolism thus catalyze the analogous reactions with the corresponding Se substrates leading to the reductive incorporation of selenate. Selenate is first reduced to selenite and then followed by selenide, which is then enzymatically integrated into selenoprotein[5]. This reduction involves a number of key enzymes in the S assimilation pathway: ATP-sulfurylase (ATP-S), APS reductase (APR) and cysteine synthase represented in figure-1. Although Selenium has not been confirmed to be an essential micronutrient for higher plants but does emerge to be a beneficial nutrient substantially for hyper accumulator plants such as *Brassica juncea*, *Stanleya pinnata*, and *Arabidopsis thaliana* which can attain two-fold elevated biomass in the presence of Se. Thus, the efficient implication of Se hyperaccumulation may recommend for better growth, conceivably due to better oxidative stress conflict[6]. On the other hand at high concentration, selenium is recognized as an environmental contaminant because it is considerably found in the wastes of mining, agricultural, petrochemical, and industrial manufacturing operations. Superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are prominent reactive oxygen species (ROS) generated in higher plants, algae and cyanobacteria when exposed to environmental stresses. Accumulation of ROS can cause impair damages to macromolecules like DNA, proteins and lipids[7]. To combat with these oxidative stresses, photosynthetic organisms gradually develop an efficient enzymatic and non-enzymatic antioxidant system to protect cellular components from oxidative damages. Major antioxidant compounds include glutathione and ascorbate, whereas antioxidant enzymes include catalase, peroxidase, glutathione peroxidases (GPXs) and thioredoxin peroxidases (also named peroxiredoxins, Prxs) are indulged to fight with these oxidative stresses[8]. Glutathione peroxidases, also known as selenoproteins, are a family of antioxidant enzymes that pace up the reaction between glutathione and free radicals, principally toxic hydrogen peroxide, which selenium-containing glutathione peroxidases help to transform into harmless water. Glutathione Peroxidase has been categorized into two: the one containing selenocysteine (SeCys) at the catalytic site referred as selenium dependent GPX and that without SeCys referred as non-selenium dependent GPX (NS-GPX) [9]. Unlike higher plants that only possess NS-GPXs, a green alga, *Chlamydomonas reinhardtii*, may have both types of GPXs. The function of

GPXs in plants and *C. reinhardtii* appears to be elimination of phospholipid hydroperoxides and protection of cells from oxidative damages. In addition, NS-GPXs may perhaps act as ROS sensors in signal transduction pathways under stressful conditions[10]. Phytoremediation is an innovative utilization of green plants to clean up our environment. The Indian mustard *Brassica juncea* considered to be an ideal selenium phytoremediator plant due to its high selenium accumulating ability, rapid growth and excessive biomass. Here we the study the response of some of the *Brassica* species to Se stress with respect to the antioxidative defense mechanism of selenium accumulation and tolerance effects in plants.

Methodology

Plant Material and Treatment: Seeds of various *Brassica* species *Brassica juncea* var. Varuna, *Brassica napus* var. Gobhi sarson, *Brassica carinata* var. Karan rai, *Brassica rapa* var. Toria, *Brassica rapa* PS 66 var. Yellow sarson, *Brassica rapa* KBS var. Brown sarson, *Eruca sativa* var. Taramira graciously provided by The Directorate of Rapeseed-Mustard Research (formerly, NRCRM), Sewar, Rajasthan. Plants were grown hydroponically using Hoagland's plant growth medium. Filter paper layered plastic pots (10cm by 15cm) were used for growing the plants directly in the hydroponic solution amended with the desired concentrations of Na_2SeO_4. The filter paper worked as an anchor for the roots of the emerging seedlings. Once the seeds germinated, the solution was changed every 2 days to maintain the nutrient concentrations. The plants were grown under natural conditions of light and temperature away from direct sunlight. Samples collected from the filter paper sown seedlings at 10 days after germination. Two sets of pots were maintained – control and treated. Plants supplied with Hoagland solution alone are referred to as control. Plants exposed to different concentrations of Na_2SeO_4 are referred to as treated. Selenium tolerance was examined according to Pilon-Smits et al.[11]. In short, seeds were surface-sterilized (70% ethanol, 3 min) for seedling experiments and cultured for ten days on half-strength Hoagland solution with or without Se added as Na_2SeO_4. After 10 days, root length was deliberated as an indicator of tolerance and the ratio of average root length with Se and average root length without Se was calculated. Selenium tolerance index (STI) percentage was calculated by multiplying the obtained ratio by 100.

$$\text{Selenium tolerance index \%} = \frac{\text{Average root length}_{+\text{selenate}}}{\text{Average root length}_{\text{control}}} \times 100$$

Selenium tolerance index was also calculated for shoot length and fresh weight of the seedlings using the same method as well as growth parameter study using plant height, leaf area, and no. of leaves.

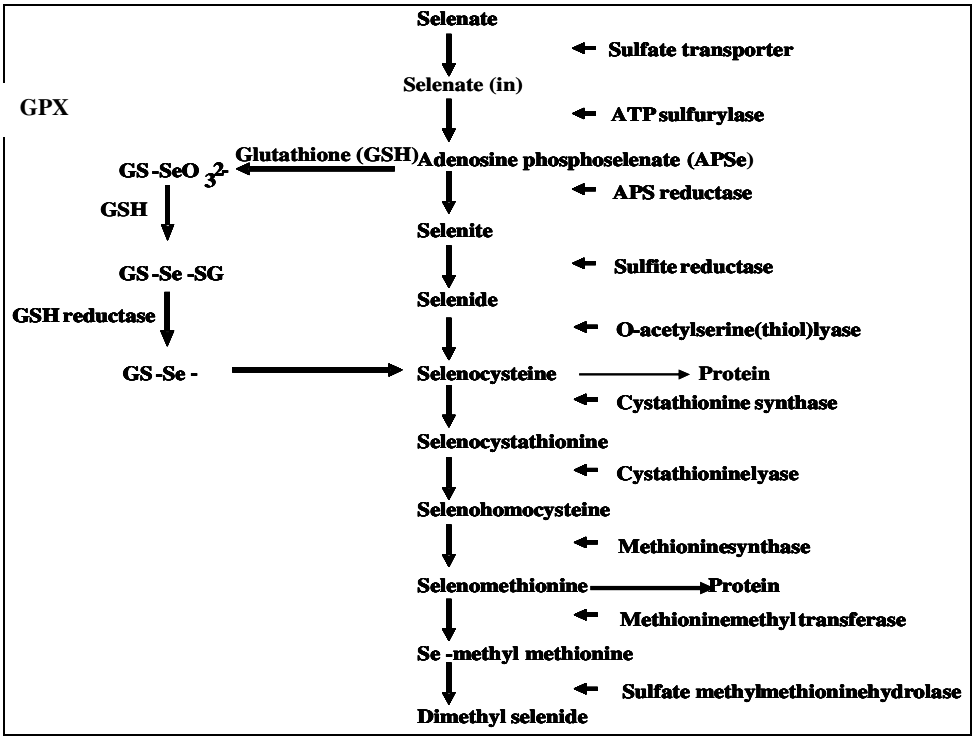


Figure-1
Scheme summarizing a proposed selenium assimilation and volatilization pathway in higher plants[2]

Determination of cellular ROS level: A general increase in ROS level in plants is attributed to H_2O_2 and MDA contents. According to Lee and Shin[12] for ROS measurements presumably H_2O_2, control and treated leaf samples were homogenized with 0.1 % trichloroacetic acid (TCA) under constant stirring at 4 °C for 5 min and harvested at 3,000×g. The extracted supernatant was incubated with 50 mM potassium phosphate buffer at pH 7 and absorbance was recorded at 390 nm after adding 1 M KI. Hydrogen peroxide content was estimated using a calibration curve of H_2O_2. A different method of ROS estimation was followed for measuring most likely lipid peroxidation in terms of malondialdehyde content by recording absorbance at 532 nm. Harvested TCA leaf extract were incubated with 0.25 % 2- Thiobarbituric acid in 10 % TCA at 95 °C for 30 min and cooled to stop the reaction. Absorbance of the aliquot was measured after centrifugation at 10,000×g for 15 min and malondialdehyde content was estimated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of antioxidants: Extraction procedure for Non-protein thiols (NPT) and glutathione were carried according to Israr et al.[13]. Leaves (0.5 gm FW) were homogenized in 1:6 ratio of 5% sulfosalicylic acid (SSA) under cold conditions and harvested at 10,000×g, 4˚C for 10 min to remove cell debris and supernatant was collected. 300µL of this supernatant was mixed with 0.1 M PBS (phosphate buffer saline pH- 7.6). After taking initial absorbance at 412nm, DTNB solution (6mM DTNB dissolve in 5mM EDTA, 0.1M PBS pH-7.6) was added further and increase in absorbance was recorded. A standard curve was prepared from varying concentrations of L-cysteine to calculate the non-protein thiols content in the samples. To estimate glutathione, 100 µL of the aliquot 0.5 mL reaction buffer [0.1 M phosphate buffer (pH 7.0), 0.5 mM ethylenediaminetetraacetic acid (EDTA)] and 50 µL 3 mM 5' dithio-bis-(2-nitrobenzoic acid) (DTNB) were added. Absorbance for determination of GSH was recorded at 412 nm and glutathione content was calculated from standard curve of reduced glutathione[14].

Evaluation of antioxidative enzyme activity and in-gel visualization: The measurements of the antioxidant enzyme activities were done on 10 days old leaf samples. For leaf extraction Fresh tissues (leaves) were homogenized in chilled 100 mM potassium phosphate buffer (pH 7.0) in addition to 1 mM EDTA and 1% (w/v) PVP in pre-cooled mortar and pestle and harvested at 10,000g for 20 min at 4˚C. The clear supernatant was used as the enzyme source[15]. Protein in the supernatant was measured according to Lowry et al.[16] and SDS-PAGE was carried according to Laemmli's[17]. CAT (EC 1.11.1.6) activity was assayed by monitoring the rate of decomposition of H_2O_2 at absorbance 240 nm[18] in a reaction mixture containing: 1.5 mL of 100 mM K-PO_4 buffer pH 7.0 and 1.2 mL of 150 mM H_2O_2. POX (EC 1.11.1.7) assay was performed in a reaction mixture containing 26 mM H_2O_2 (500 µL), freshly prepared 0.01M pyrogallol (1mL) and 0.1M K-PO4 buffer at pH 7.0 (1 mL) was determined as increase in absorbance at 420 nm for 120 s[19]. For estimating GPX (EC 1.11.1.9) the reaction mixture was comprised of 8.4 mM NADPH, 100 U glutathione reductase, 0.02 mM reduced glutathione and 50 mM K-PO_4, buffer at pH 7.0 and the reaction was started by adding 100 µL 22 mM H_2O_2 .Oxidation of NADPH was monitored at 340 nm[20].

The enzyme activities were also determined by in-gel assays. Briefly, 100µg protein equivalent extracts were applied to native PAGE using 7.5% running gel. Electrophoresis was carried out for 6-8 hrs at 4˚C and the gels were stained as follows: CAT - gel was incubated in 0.2 mM H_2O_2 for 15 min and rinsed with distilled water. After rinsing with distilled water the gel was immersed in a solution of 1% ferric chloride and 1% potassium ferricyanide solution until achromatic bands appeared on Prussian blue green background[21]. POX - Gel was pre-incubated in 50 mM Na-PO_4 buffer (pH 7.0) for 15 min, followed by an additional 20 min incubation in the presence of 4mM H_2O_2 and freshly prepared 20 mM pyrogallol until dark brown bands appeared on a pale background[22]. GPX - the gel was washed in queue with 2.5% Triton X -100 for 15 min and distilled water for 15 min. Gel was then placed in 10 mM K-PO_4 buffer (pH 7.2) containing 2mM O-diansidine dihydrochloride for 1 h and shifted for 15 min to another solution containing buffer and 0.1 mM H_2O_2. Brown bands appeared against a pale background[23].

Results and Discussion

Brassica juncea plants were subjected to a wide range of Na_2SeO_4 concentrations (0 - 400 µM). Growth parameters like plant height, leaf area and leaf number were recorded. The plants were also subjected to selenium tolerance assays using the same concentration range. The plants could tolerate 100 µM Na_2SeO_4 concentrations. Beyond this concentration, the toxic effects leading to Chlorosis and necrosis. The study of the antioxidative enzymes glutathione peroxidase, peroxidase and Catalase was conducted with 0 – 100 µM selenate. *Brassica juncea,* at Se concentrations of 80 and 100 µM, responded with an increase in the antioxidative enzymes especially glutathione peroxidase and peroxidase. The results were corroborated by the in-gel assays for the enzymes. The raised values of the malondialdehyde content and H_2O_2 content, near 100 µM signified build up of stress at this concentration. The role of antioxidative defense system in *Brassica juncea's* tolerance to high selenium accumulation was evident by the increase in antioxidative enzymes at the higher Se concentrations of 80 and 100 µM. On the basis of the above results, the remaining *Brassica* species, namely- *Brassica carinata*, *Brassica rapa* var. Toria, *Brassica rapa* var. PS66, *Brassica rapa* var. KBS and *Eruca sativa,* were tested for their Se stress response. A comparison was drawn among all the species by determining the growth attributes as for *B. juncea.* The Selenium tolerance index assays figure-2 revealed the vulnerability of *E.sativa* and KBS over others while *B.carinata* with its slow growth rate gave the opposite result. When cultivated at high concentration (100 µM), the only species' that survived long enough to complete life cycle were *Brassica juncea*, Toria and PS66. On the basis of

the various experiments conducted it became evident that *Brassica carinata, Brassica rapa* var. KBS and *Eruca sativa* were unable to grow at the concentrations in which *Brassica juncea*, Toria and PS66 survived.

Determination of ROS generation and non enzymatic antioxidants: Hydrogen peroxide content, MDA content, glutathione and NPT values were considered for only the higher concentration of 100 µM as at 40 µM the plants grew. The results for the determination of stress levels through MDA content and H_2O_2 content revealed very high values for KBS, and *E. sativa* at 100 µM and the values for Toria and PS66 along with *B. juncea* showed moderate increase in the MDA generated at this concentration as shown in figure-3. An increase in NPT levels has so far been associated with greater stress tolerance of the species that have been researched for Se tolerance. The values for NPT levels and glutathione of Toria and PS66 were higher than the control values at 100 µM and *B. carinata*, KBS and *E. sativa* were found to show decreased levels of NPT and glutathione at 100 µM as represented in Table-1. It has already been reported that at high concentration of Se there is a formation of reactive oxygen species which causes oxidative stress in plants[24, 25]. The optimal levels of ROS generation were needed for resistance to Se in *Arabidopsis thaliana* was suggested[26]. This was further developed into a hypothesis that the ROS molecules serve as signals for the activation of pathways needed to counter the stress[27]. Here, the NPT content of the species *Brassica juncea*, Toria and PS66 showing increase in GPX activity was also found to be considerably high while for the other species the concentrations were decreased. Going by the levels reported for the Se hyper accumulator *Stanleya pinnata* over the Se-sensitive *S. albescens*, increment in the values of antioxidants in this study were taken as promoting Se tolerance[28]. Increase in glutathione values at the stress causing concentration of 100 µM suggests that their expression was increased to combat the oxidative stress encountered by these plants. This could be taken as the overwhelming effect of the stress generated on the plant at the high Se concentration which was also evident in the enhancement of cellular content of MDA. The GSH, NPT and MDA values of Toria and PS66 were found similar to that of *B. juncea* at the same concentrations.

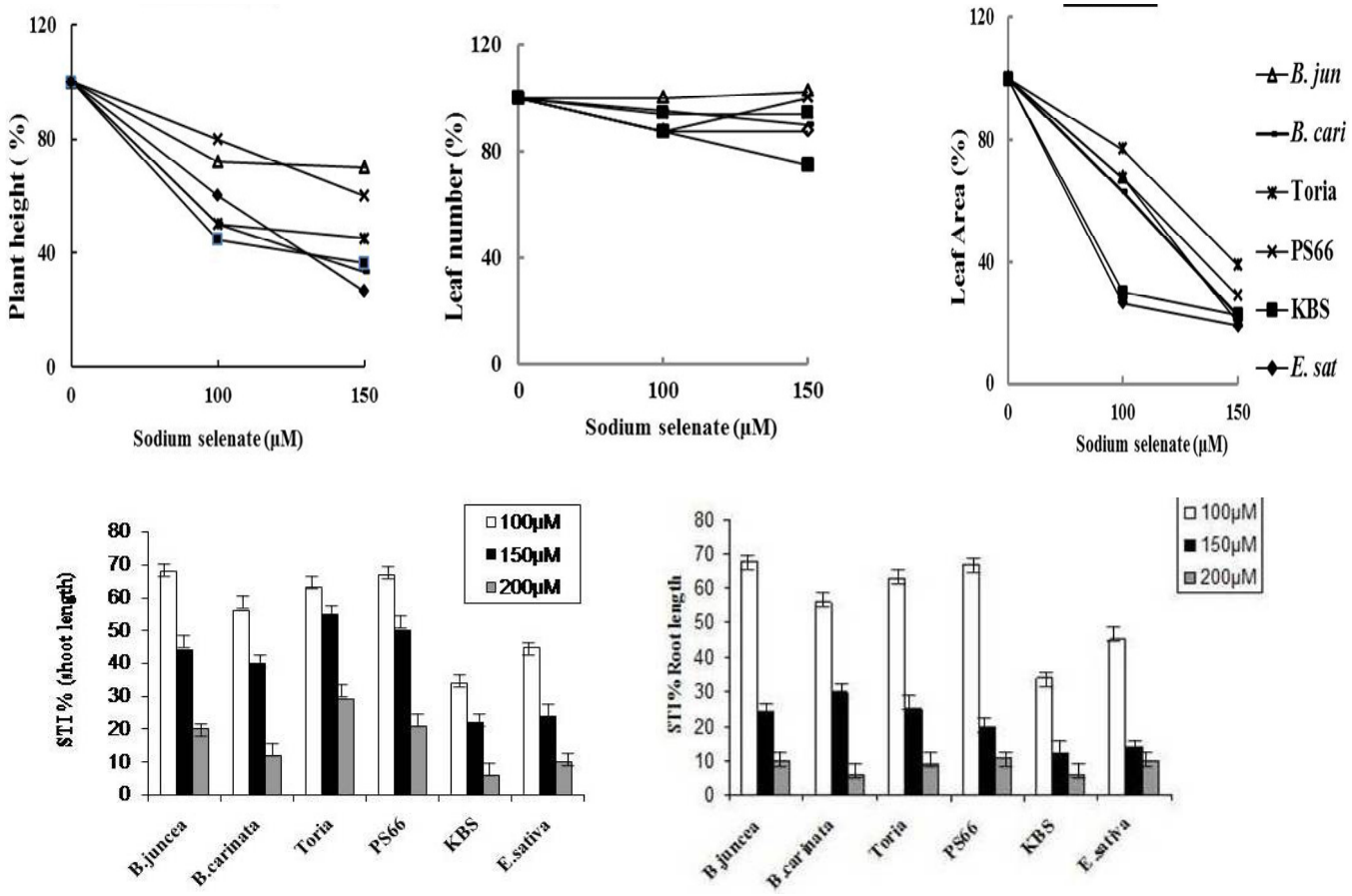
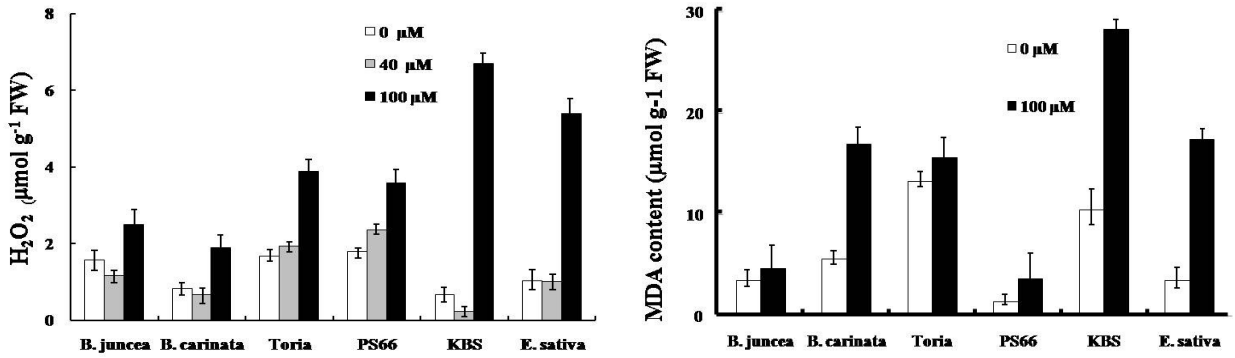


Figure-2
Growth parameters and selenium tolerance index of shoot length and root length of all the test species of *Brassica*. Bar = mean ± SD of three Independent experiments with triplicate samples.

Enzymatic antioxidant measurements and in-gel visualization: In keeping with the main objective of analyzing proteins for changes brought about by Se, the response of the *Brassica* species to 0, 40 and 100 µM Na_2SeO_4 treatment was judged by the expression of the antioxidative enzymes. As for *B. juncea*, the enzymes tested were –Catalase, Glutathione peroxidase and Peroxidase. The native PAGE assays were also performed for the same extracts. The spectrophotometric results have been depicted in figure-4 while the gels are shown in figure-5



A B

Figure-3

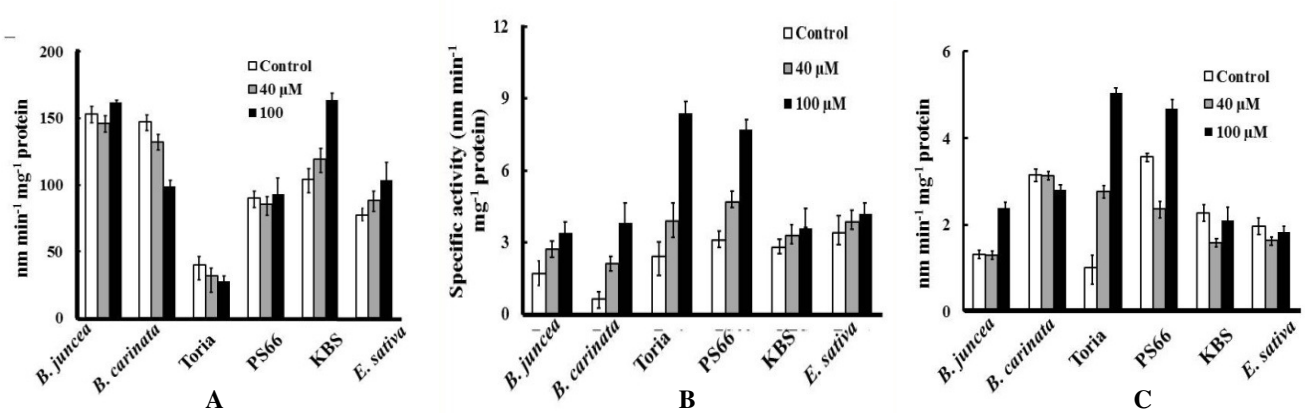
Oxidative stress indicators (A) H2O2 content (B) MDA content of all the test species at selenate concentrations of 0 µM; 40 µM; 100 µM. The values are mean ± SD of three independent experiments with triplicate samples

Table-1

Effect of sodium selenate on MDA content, Glutathione, NPT values of all the test species of *Brassica*

Species	MDA Contents		Glutathione		NPT Values	
	0	100	0	100	0	100
B. Juncea	3.48 ± 0.02	4.72 ± 0.12	2.34 ± 0.12	3.09 ± 0.18	7.78 ± 0.34	10.43 ± 0.43
B.Carinata	5.59 ± 0.12	16.9 ± 0.19	0.90 ± 0.18	0.45 ± 0.23	13.35 ± 0.76	7.28 ± 0.77
Toria	3.27 ± 0.17	4.17 ± 0.08	7.03 ± 0.21	9.79 ± 0.38	14.31 ± 0.12	17.41± 0.23
PS66	1.38 ± 0.24	3.71 ± 0.03	5.03 ± 0.15	7.08 ± 0.67*	24.59 ± 0.23	28.9 ± 0.34
KBS	10.4 ± 0.45*	28.2 ± 0.14	2.01 ± 0.23	0.90 ± 0.13	10.82 ± 0.89*	8.89 ± 0.12
Eruca Sative	3.48 ± 0.13	17.4 ± 0.23	0.90 ± 0.56	0.03 ± 0.05	11.56 ± 0.76	7.25 ± 0.34

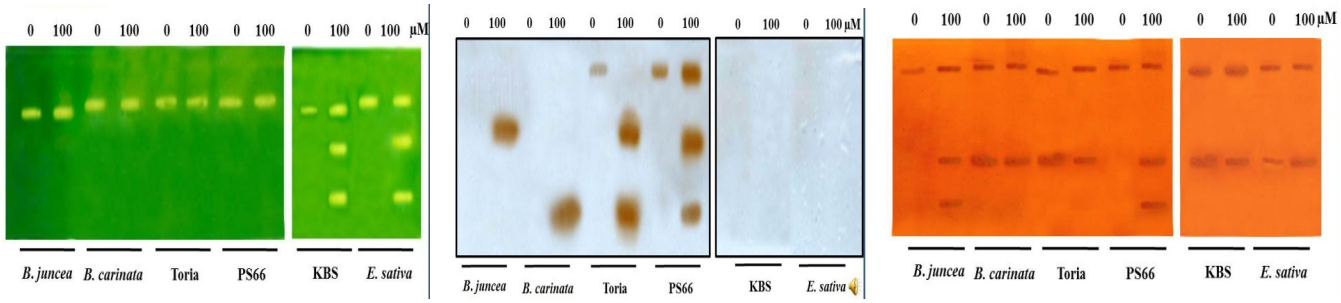
Untreated leaves (0 µM) and those treated with 100µM of Selenate were harvested and assayed. The values are mean± SD of three independent experiments (n=3). *Treatment significantly different from control (0 µM) ($p<0.05$; one-way ANOVA, LSD).



A B C

Figure-4

Spectrophotometric assay of antioxidative enzyme of all the test species at selenate concentrations of 0 µM; 40 µM; 100 µM where: A--Catalase; B--Glutathione peroxidase; C—Peroxidase. The values are mean±SD of three independent experiments with triplicate samples. Significant difference from control (without selenate were calculated at $p<0.05$ by two-way ANOVA followed by Turkey's HSD post hoc test



A **B** **C**

Figure-5

In gel assay of antioxidative enzymes of all the test species at selenate concentrations of 0 µM and 100 µM where: A--Catalase; B--Glutathione peroxidase; C—Peroxidase

The catalase activity of PS66 did not register wide variation over the three concentrations used, both spectrophotometrically and in the native gel. Toria was found to contain little basal activity of this enzyme at steady state and it further decreased at the two concentrations of Na_2SeO_4. KBS and *E. sativa* both showed increase in catalase activity on selenium exposure and this increase was found to manifest in the appearance of isozymes on the native gel. For the rest of the species no distinguishing changes were observed for catalase on the native gels.

The basal levels of glutathione peroxidase in control plants were found to be either very low or absent. The activity was found to be negligible for KBS and *E. sativa*. The GPX activity showed remarkable increase in the presence of selenium at both the concentrations for Toria and PS66. The appearance of two isozymes for both Toria and PS66, on the native gel, corroborated with the spectrophotometric readings. Among the antioxidative enzymes, the activity of glutathione peroxidase was found to be particularly remarkable under Se stress. The enzyme GPX shows very low basal activity in non-stressed plants. But a positive tie-up between the Se elevation and GPX activity has investigated in a number of studies[29, 30]. The much reported antioxidative consequences of Se (at low concentrations) on plants have also been linked with increase in GPX activity. Studies on GPX enzymes isolated from Aloe vera[31], barley seeds and *Chlamydomonas reinhardtii*[32] have reported the presence of an inorganic selenium atom associated with proteins catalyzing peroxidase activity earlier on. But direct proof for the existence of a UGA opal codon being decoded to incorporate selenocysteine in the plant kingdom came with studies[33]. These need not be solitary cases. It could be possible that plants over expressing GPX in the presence of Se incorporate, Se in the enzyme active site replacing sulfur. In this study, the results obtained with the more tolerant species were strongly suggestive of such a replacement. They are indicative of the singular ability of GPX in being induced by the presence of Se and bringing about Se tolerance in such plants as it is over expressed in PS66 and Toria. While for *B. carinata*, basal activity of the enzyme in control plants was detected in the spectrophotometric readings but its presence was found missing in the native gel for the same. A single band was observed for *B. carinata* as well as *B. juncea* at 100 µM. The findings for KBS and *E. sativa* GPX activity were absence from both spectral and native gel results.

Another distinct observation was that catalase did not participate in stress mitigation in the species where GPX was over expressed. No changes were observed in CAT in Toria and PS66. Also, the species that did over express CAT were unable to counter Se stress. The reasons could be that CAT is a highly compartmentalized enzyme found predominantly in the peroxisome, then in the cytosol and has little substrate likeness as it requires two molecules of H_2O_2 concurrently to affect catalysis[34]. GPX is present throughout the cell and has higher substrate affinity in the presence of glutathione as a reductant[35]. With the abundant elevation of glutathione seen in the Se-affected plants, the GPX could have carried out its role more efficiently than catalase. *B. carinata* was found to contain moderate catalase activity but the activity decreased with increase in selenium concentrations applied.

Peroxidase activity was found to exist in all the control plants at a moderate level. The enzyme activity did not show much variation at 40 µM Se exposure. It was found to increase significantly at 100 µM for Toria and PS66. The native gel results were distinguishable only for the increase in activity in Toria. *B. carinata*, KBS and *E. sativa* responded with little change in activity.

SDS- PAGE results of the extracts treated Na_2SeO_4: It was considered necessary that the changes observed in the enzyme activity be checked by observing the protein banding pattern through SDS-PAGE. Sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS–PAGE) was carried with the 0 µM and 100 µM treated extracts according to Laemmli's (1970). 100 µg proteins from the extracts of all the 6 species (*B. juncea*, *B. carinata*, Toria, PS66, KBS and *E. sativa*) were loaded on 10% SDS gels for PAGE.

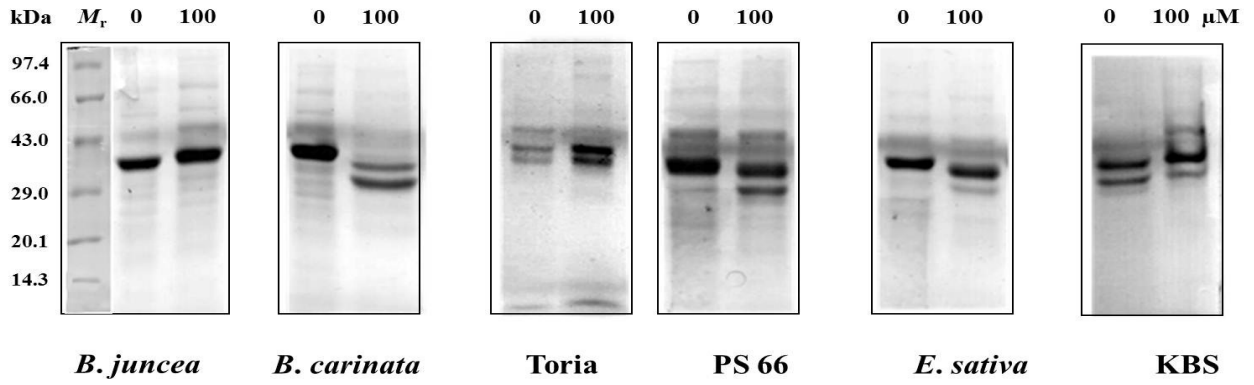


Figure-6
SDS PAGE analysis of all the test species at selenate concentrations of 0 µM and 100 µM

The results observed (Figure-6) were found to show some changes in all the species at 100 µM selenium treated extracts over control except *B. juncea*. In *B. carinata,* the 0 µM lane revealed a single prominent band of high intensity while in the 100 µM lane two bands could be clearly distinguished. In case of PS66, the intensity of the lower molecular weight proteins in the 100 µM lane was found greatly increased while the intensity of the moderate molecular weight proteins for control treatment was greater. In case of KBS; the first band, out of the three clearly visible in the 100 µM lane, was observed missing in case of control samples but was prominent in the treated extract. In contrast to this result, in case of Toria, the first of the three bands was found missing in the 100 µM extract while being clearly visible in the control sample. In *E. sativa,* in the 100 µM extract sample as compared to the 0 µM lane, appearance of another band, albeit a little faded, could be clearly made out. It was observed that different species responded with differing changes in their banding pattern; an observation that further brings out the differences among the species response to selenium.

Conclusion

To conclude *Brassica rapa* PS66 and Toria proved to be an efficient Se tolerance plant among all the test species as was an evident from the increased expression of glutathione peroxidase (GPX) in the treated plants. The future line of research will focus on purification and characterization of glutathione peroxidase from treated plants and to develop GPX mutant for the non accumulator plants. 2D analysis of the wild and Se treated plants will also add to our knowledge of the expression of stress proteins.

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