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Selenium – an antioxidative protectant in soybean during senescence

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Abstract

Selenium (Se) is regarded as an antioxidant in animals and plants, even though considered as non-essential element in plants. To test its ability to counteract senescence related oxidative stress in soybean a pot culture experiment was conducted. The soybean plant was sprayed with sodium selenate (50 ppm) at 78 days after sowing (DAS). Soybean leaves were harvested at 80 and 90 DAS for analysis of oxidant production and antioxidative enzymes activity. Se positively promoted growth and acted as antioxidant by inhibiting lipid peroxidation and per cent injury of cell membrane. The antioxidative effect was associated with an increase in superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzymes activity. Significant increase in antioxidant enzyme activity was positively related to Se content. The decrease in antioxidative enzymes at 90 DAS was much faster in control plants than Se-sprayed plants. The reduction in SOD and GSH-Px may be associated with senescence-induced oxidative burst.

Introduction

Selenium (Se) is an essential micronutrient needed in antioxidation and hormone balance in human and animal cells (Berry et al., 1991; Pallud et al., 1997 Rotruck et al., 1973).

Being a powerful natural antioxidant, Se has immunostimulating, cardio protective and anti-carcinogenic activity in man and animals (Block et al., 1992; Golubkina et al., 2002). Rotruck et al. (1973) identified Se to be an essential component of the enzyme glutathione peroxidase (GSH-Px). Subsequently it was identified as essential in human and animal cells of Type I, II and III iodothyronine deiodinases (Berry et al., 1991; Pallud et al., 1997; Ramauge et al., 1996), thioredoxin reductase (Tamura and Stadtman, 1996) and for a number of selenoproteins (Behne et al., 1997; Gladyshev et al., 1998).

Even though higher plants are thought not to require Se and to have a low tolerance to it, there are increasing indications that Se may also have beneficial biological functions in higher plants (Stadtman, 1990). Recently, Hartikainen et al. (2000) demonstrated that, depending on the dosage, Se exerts dual effects on ryegrass. At low concentrations, it acts as an antioxidant and can stimulate the plant growth, whereas at higher concentrations it acts as a pro-oxidant thereby reducing the yield.

Senescence, an integral part of plant development, may coincide with the production of free

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oxygen radicals and can be regulated by a variety of environmental and autonomous factors (Kar and Feirabend, 1984). Se can increase the tolerance of plants to UV-induced oxidative stress as well as delay senescence and promote the growth of ageing seedlings (Hartikainen and Xue, 1999; Xue and Hartikainen, 2000). In higher plants, the results obtained by Xue et al. (1993a, b) with rape and soybean indicated that Se may inhibit lipid peroxidation in higher plants through GSH-Px and non-enzymatic reactions. On the basis of the antioxidative role of Se, it is theoretically possible that Se can delay plant senescence.

Monocarpic senescence of soybean has been studied extensively (Nooden et al., 1979, Nooden, 1984, 1988; Nooden and Thompson, 1985). But little information is available on the impact of Se on delaying senescence in soybean. Application of Se upto 50 ppm in soybean increased the yield of soybean by preventing chlorophyll degradation and maintaining longer leaf area duration (Djanaguiraman et al., 2004). Hence the objective of this experiment was to investigate the effect of Se on antioxidative systems to counteract senescence related oxidative stress in soybean.

Materials and methods

A pot culture experiment was conducted in Glasshouse at the Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore (11°N; 77°E; 426.7 m MSL) during 2002–2003. Soybean seed cv. CO_2 with field duration of 95 days was grown under the following conditions: photosynthetic photo flux density of 300–400 μM m^{-2} s^{-1} at the top of the pot, 16 h of light, 8 h dark and a relative humidity of 60%. The temperature in the glasshouse varied between 18 °C (by night) and 30 °C (by day). The plants were cultivated in earthern pots (75 cm diameter) containing coarse textured soil (pH 6.3 in 1:2.5 soil: water suspension and 2.8% of organic Carbon) taken from eastern farm of the university. The soil is low in available nitrogen (196 kg ha^{-1}), medium in available phosphorus (6 kg ha^{-1}) and high in available potassium (386 kg ha^{-1}). Prior to the experiment, the soil was air dried, sieved ($<$ 3–5 mm sieve size) and homogenized. Ten kilogram soil was weighed for the pot culture experiment with six replications in two sets. Seeds (4 per pot) were sown onto the upper layer of soil and covered with 100 g of soil. Nitrogen, phosphorus and potash were added to each pot at the rate of 22, 250 and 33 mg kg^{-1} of soil, respectively. No micronutrients were added.

The total Se content of the top of 0–300 mm soil was 0.11 μg g^{-1}, and water soluble Se was 0.02 μg g^{-1}. The plants were irrigated with deionized water from sowing to maturity. Se as sodium selenate at 50 ppm was sprayed on 78 days after sowrings (DAS), when the plants were at pod filling stage and the leaves were starting to senesce, using hand-operated high volume sprayer. The plants were completely drenched with the spray solution. Control plants were sprayed with deionized water. The soybean leaves were harvested twice, the first harvest was at 80 DAS and the second one was at 90 DAS. The leaves were washed and immediately frozen in liquid nitrogen and stored at −70 °C for enzyme analysis.

Plant analysis

Growth attributes such as plant height, number of leaves, leaf area and total dry matter production were measured from six replications ($n = 12$) from each treatment at harvest. Leaf area was measured using leaf area meter (LICOR 3100) and dry weight was taken after drying the samples in hot air oven at 60 °C till constant weight was reached. Estimation of Se content (μg g^{-1}DW) was done in individual plants ($n = 10$). Third leaf from the top was washed with distilled water and oven dried for 3 days at 80 °C. Se content in the leaf was quantified with atomic absorption spectrophotometer (Varion) at 196.1 nm (Kumpulainen et al., 1983).

With respect to antioxidants, attention was focused on Se-induced changes in GSH-Px, superoxide dismutase (SOD), catalase (CAT) and proline. Hydrogen peroxide, superoxide anion and lipid peroxidation were analysed for oxidant production. Apart from the antioxidant and oxidant production, the contents of chlorophyll, PS II photochemistry (Fv/Fm ratio) and cell membrane stability were also quantified as an indication of senescence processes. Antioxidant enzyme assays, oxidant assays, cell membrane stability, chlorophyll content and fluorescence were done on the third leaf from top of the plant.

Lipid peroxidation, reactive oxygen species (ROS) assay and cell membrane stability

The lipid peroxidation was determined by malondialdehyde (MDA) content produced by thiobarbituric acid (TBA) reaction at low pH as described by Behra *et al.* (1999). The pink chromogen was measured at 532, and at 600 nm for the correction of blank. Lipid peroxidation was expressed as MDA content in μM g^{-1} DW. The H_2O_2 level was colorimetrically measured as described by Okuda et al. (1991). H_2O_2 was extracted by homogenizing 0.5 g of tissue with 4 ml of perchloric acid (200 n*M*). The homogenate was centrifuged at $12{,}000 \times g$ for 10 min. Oxidation of ferrous ions to ferric ions by H_2O_2 in acidic pH was monitored and stable complex of ferric ions with Xylenol orange dye at 560 nm was measured. H_2O_2 was expressed in n*M* g^{-1} DW. Superoxide anion was estimated according to Chaitanya and Naithani (1994) and expressed as change in optical density (ΔOD) at 540 nm min^{-1} g^{-1} DW. To determine leakage of electrolytes, which is a measure of membrane integrity, leaf samples (10 leaf discs of 1.5 cm each) were washed thoroughly with deionized water. After 5 h, leaching of electrolytes was determined by measuring electrical conductivity and the per cent injury was assessed according to Chowdhury and Choudhuri (1985).

Antioxidant enzymes and proline

For SOD and catalase enzymes activity estimations, frozen tissue was homogenized in ice-cold 0.1 *M* Tris–HCl buffer at pH 7.8 containing 1 m*M* EDTA, 1 m*M* dithiothreitol and 5 ml of 4% polyvinyl pyrrolidone per gram fresh weight. The homogenate was filtered through a nylon mesh and centrifuged at $20{,}000 \times g$ at 4 °C. The supernatant was used for measuring enzyme activity.

Superoxide dismutase (EC 1.15.1.1) was determined by nitroblue tetrazolium (NBT) method of Beyer and Fridovich, (1987) by measuring the photoreduction of NBT at 560 nm. One unit of SOD activity equalled to the amount required to inhibit photoreduction of NBT by 50%. *CAT (EC 1.11.3.6)* was estimated according to Samantary (2002). One milliliter of the supernatant was added to the reaction mixture containing 1 ml of 0.1 *M* H_2O_2 and 3 ml of 0.1 *M* sodium phosphate buffer. The reaction was discontinued by adding 10 ml of 2% H_2SO_4 after 1 min of incubation at 20 °C. The reaction mixture was then titrated against 0.01 *M* $KMnO_4$ to determine the quantity of H_2O_2 used by the enzyme. Enzyme activity was expressed as μM H_2O_2 reduced min^{-1} mg^{-1} protein. *GSH-Px (EC 1.11.1.9)* activity was measured by a modification of the method of Flohe and Gunzler (1984) by using H_2O_2 as substrate. The enzyme was extracted by a pre-chilled $KNaHPO_4$ buffer at pH 7.0, with a homogenizer. The supernatant obtained by centrifugation (10 min at 3000 rpm) was used as an enzyme crude extract. For the enzyme reaction, 0.2 mL of the supernatant was placed in a testtube and mixed with 0.4 mL reduced glutathione (GSH) (0.1 m*M*) and 0.2 mL $KNaHPO_4$ (0.067 *M*). The above reagents without supernatant extract were used for the non-enzyme reaction. After pre-heating the mixtures in water bath at 25 °C for 5 min, 0.2 mL H_2O_2 (1.3 m*M*) was added to initiate the reaction. The reaction lasted for 10 min and was terminated by adding 1 mL 1% trichloro acetic acid, and the mixture was put in an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 3000 rpm, 0.48 mL of the supernatant was placed in a cuvette, and 2.2 mL of 0.32 *M* Na_2HPO_4 and 0.32 mL of 1.0 m*M* 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma) were added for colour development. The absorbance at wavelength 412 nm was measured with a Beckman spectrophotometer (USA) within 5 min. *Proline* was extracted in 3% sulfosalicylic acid and estimated by using acid ninhydrin reagent and measuring the absorbency of the toluene chromophore at 520 nm (Bates et al., 1973) and expressed as μg g^{-1} DW.

Chlorophyll contents and fluorescence

Chlorophyll was extracted in 80% acetone and estimated according to Arnon (1949). Chlorophyll fluorescence measurements were made with Plant Efficiency Analyser (PEA) (Hansatech, UK) following the method advocated by Lu and Zhang (1998). Measurements were made on intact leaves (third leaf from the top) which were

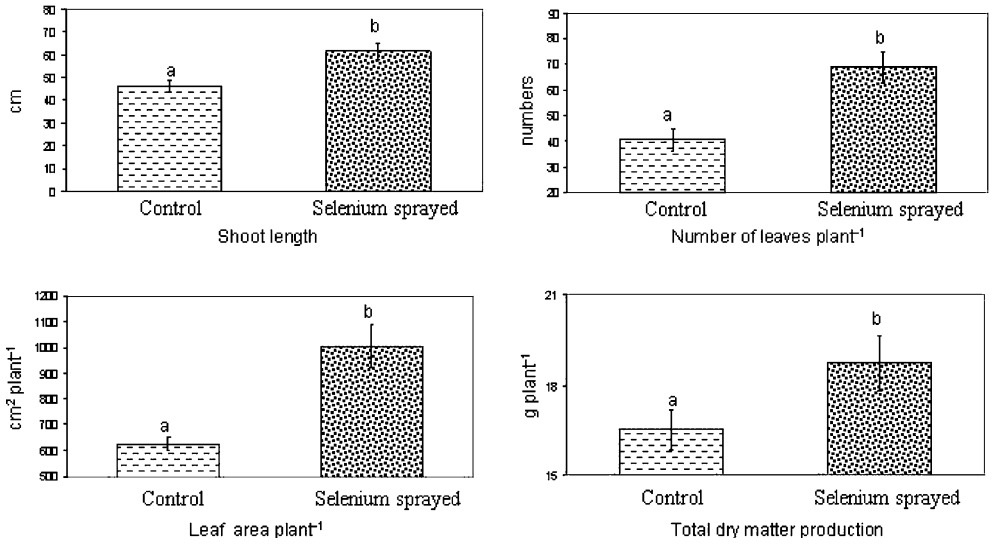

Figure 1. Effect of Selenium spary on growth and growth attributes in soybean at harvest.

dark adapted for 30 min prior to measurement. The minimal fluorescence level (Fo) with all PS II reaction centres open was assessed by measuring the modulated light which was sufficiently low (μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The maximal fluorescence level (Fm) with all PS II reaction centres closed were determined by a 0.8 s saturating pulse at 8000 μmol m^{-2} s^{-1} in dark adapted leaves (Lu et al., 2001). Using both light and dark fluorescence parameters, maximal efficiency of PS II photochemistry in the dark adapted state, was calculated as Fv/Fm = (Fm−Fo)/Fm (Van Kooten and Snell, 1990).

Statistical analysis

Statistical analysis was performed using SAS programme for PCs (SAS User's Guide version 6, 4th edn., SAS Institute, Cary, NC, 1990). The mean of three independent samples were taken to represent the result of each replicate. Three replicates were taken for all antioxidant enzyme assays, oxidants and for senescence indicators. The mean values ± SE are given in all the tables and figures. The data were analysed statistically using a general linear model for analysis of variance. Significance between control and treatment was determined using Duncan's multiple range tests.

Results

Plant growth and Se accumulation

Se application significantly promoted the shoot growth in terms of shoot length, number of leaves per plant, total leaf area per plant and total dry matter production (Figure 1). The shoot length, number of leaves and leaf area were increased by 24.8, 29 and 37.8%, respectively in Se treatment. The highest dry matter production of 18.73 g $plant^{-1}$ was recorded in Se treatment with an increase of 13.0% over control.

Leaf and grains varied significantly in their ability to accumulate Se (Figure 2). There was 2.7 and 2.6 fold increase in Se content in leaves at 80 and 90 DAS, respectively, and 1.4 fold in seeds by Se foliar spray. Both control and Se treatment showed an increasing trend in accumulating Se in leaf from 80 to 90 DAS.

ROS production and cell membrane stability

Se subdued MDA formation, H_2O_2 and superoxide anion production as compared to control plants. In the control plants the H_2O_2 content increased by 6.9 n*M* g^{-1}DW from 80 to 90 DAS as against 2.5 nm g^{-1} DW increase seen in Se treated plants (Figure 3). In the case of superoxide anion

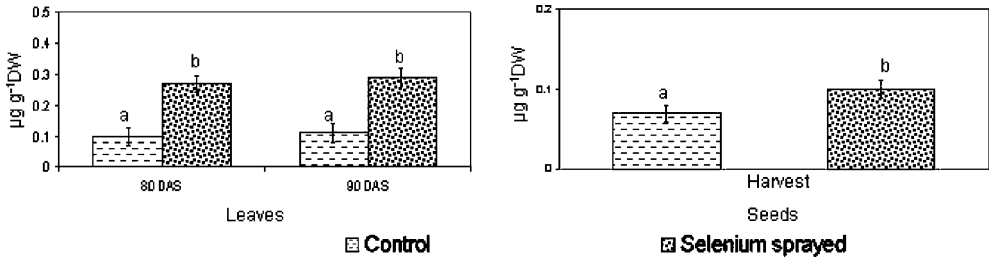

Figure 2. Effect of Selenium spray on selenium content in leaves (80 and 90 DAS) seeds (harvest) of soybean.

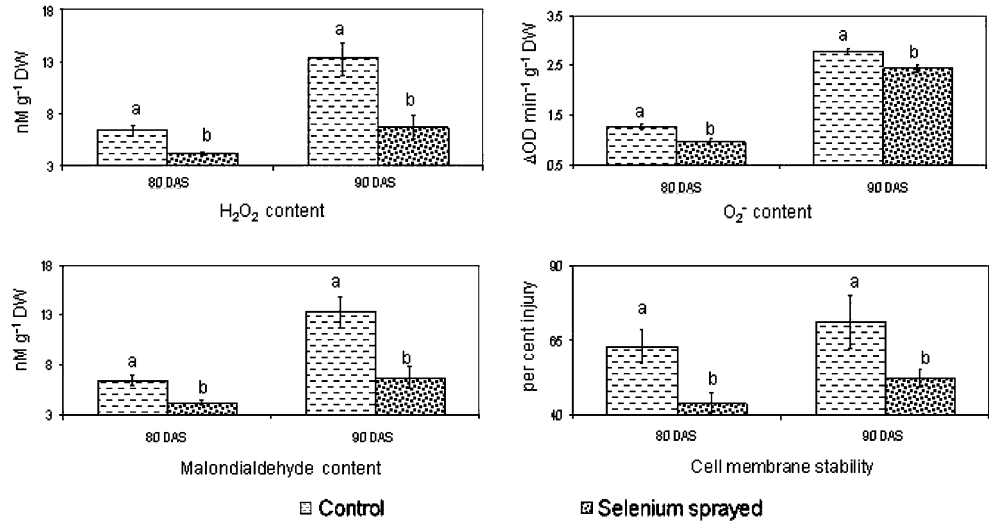

Figure 3. Effect of Selenium spray on $H_2O_2^-$, MDA production and cell membrane stability in soybean at 80 and 90 DAS.

production a significant increase was observed at both 80 and 90 DAS and the difference between control and Se treated was only 0.31 ΔOD min^{-1} g^{-1} DW and 0.34 ΔOD min^{-1} g^{-1} DW at 80 and 90 DAS, respectively (Figure 3). MDA content in plants was significantly higher under control at 80 and 90 DAS, the increase being 17.0 and 37.8% morethat plants treated with Se during the same period. (Figure 3). Se foliar spray significantly decreased the membrane injury at both 80 and 90 DAS. Both control and Se treatment recorded an increase of 9% from 80 to 90 DAS (Figure 3). However, Se treatment had a value of 52.1% injury as against 71.1% injury in control at 90 DAS. It is noteworthy that in Se treatment at 80 DAS the per cent injury was kept at a lower level than its corresponding control plants. This could be due to reduced H_2O_2 and O_2^- production in Se treated plants.

Antioxidant enzymes and proline

Significant increase in the activity of SOD was seen at 80 and 90 DAS by Se treatment over control plant. Increase in SOD activity by 2.5 enzyme unit mg^{-1} protein was observed from 80 to 90 DAS by Se treatment, as against a much lesser increase in contriol (Figure 4). GSH-Px activity increased from 4.7 to 7.3 μM min^{-1} mg^{-1} protein during 80 to 90 DAS in control plants. On contrary, in the plants sprayed with Se the increase was from 14.5 to 22.3 μM min^{-1} mg^{-1} protein during 80 to 90 DAS. In the 90 days old leaves, Se effectively increased the proline content

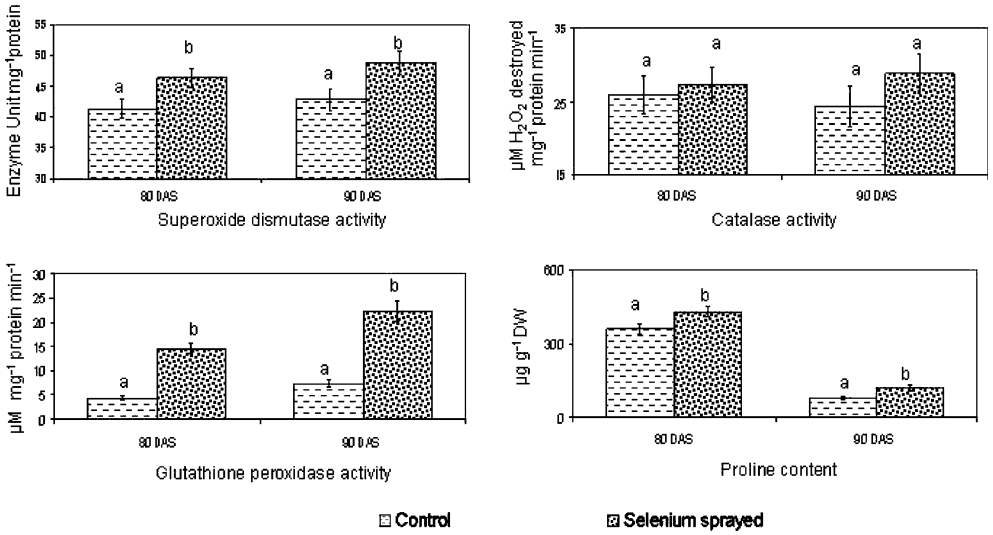

Figure 4. Effect of Selenium spray on superoxide dismutase, catalase, glulathione peroxides activity and proline content is soybean at 80 and 90 DAS.

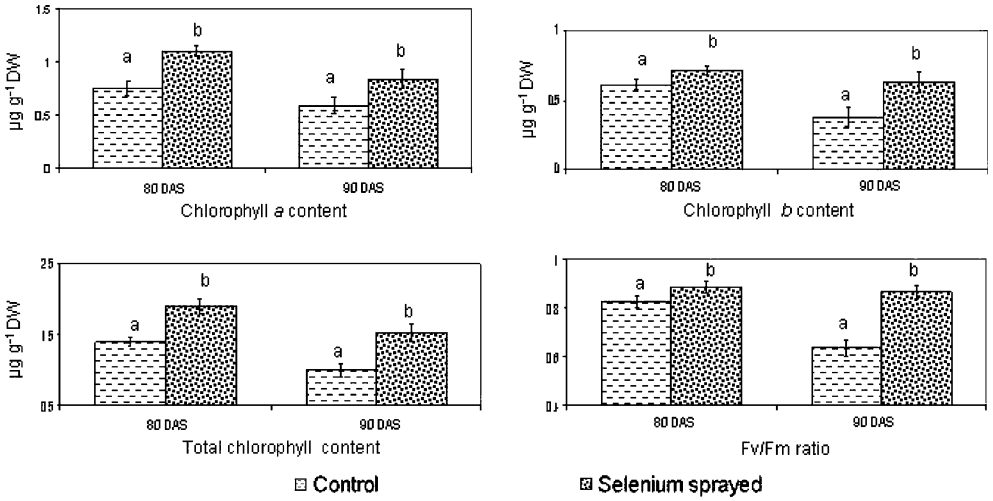

Figure 5. Effect of Selenium spray on chlorophyll pragmentation and fluorescence in soybean at 80 and 90 DAS.

by 50% over its unsprayed control plants. However, at 80 days the increase was only 19.4% (Figure 4).

Chlorophyll contents and fluorescence

In both Se sprayed and control plants, chlorophyll *a*, *b* and total chlorophyll contents decreased between 80 to 90 DAS. However, the magnitude of reduction was low in Se treated plants (Figure 5). In control plants, at 90 DAS the chlorophyll *a*, *b* and total chlorophyll contents showed 27.1, 60.7 and 39.5% decrease as compared to values at 80 DAS. Whereas, in Se sprayed plants a decrease of 30.2, 14.2 and 24.3% was recorded during the same period. The net result was that chlorophyll *b* was less degraded or may be converted to chlorophyll *a* in the Se treated plants.

Chlorophyll fluorescence measurements were taken to assess, whether Se spray could prevent photoinhibition during senescence. Reduction in the variable to maximum fluorescence (Fv/Fm) was monitored in leaves of both control and Se

treated plants at 80 and 90 DAS. The control plants showed a significant reduction in Fv/Fm ratio of 29.6% as against the Se treated plants which showed only 2.6% reduction from 80 to 90 DAS (Figure 5).

Discussion

ROS such as hydrogen peroxide (H_2O_2), the superoxide anion radical (O_2^-) and the hydroxyl radical (OH^-) are toxic by−products of cellular oxygen metabolism (Berlett and Stadtman, 1997). The intracellular concentration of ROS has been implicated as a determinant of apoptosis, aging, cancer, and cell cycle arrest (Epp et al., 1983; Stadtman, 1992). The direct reduction of H_2O_2 by GSH-Px and catalase as well as the scavenging of superoxide radicals by superoxide dismutase contribute to homeostasis of the intracellular redox state. Two selenoproteins, GSH-Px and thioredoxin reductase induced by Se serve to protect cells from oxidative stress. Whereas GSH-Px catalyses the reduction of peroxides that might damage cells and tissues in humans and mouse (Epp et al., 1983; Jeong et al., 2002), thioredoxin reductase provides reducing equivalents for various biochemical processes and antioxidant defenses (Prinz et al., 1997). The growth stimulating effect of Se may be related to its antioxidative function as demonstrated by diminished lipid peroxidation, H_2O_2 and superoxide radical production, and higher contents of chlorophyll *a, b* and total chlorophyll than control. 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.

The results revealed that in the senescing plants (80 and 90 DAS) cultivated without Se spray, the production of oxidants (H_2O_2 and O_2^-) was enhanced. These two are important ROS capable of causing oxidative damage (Shanker et al., 2004). The combined action of SOD and GSH-Px is critical in mitigating the effects of oxidative stress, since the former merely acts on the superoxide anion converting it to another reactive intermediate (H_2O_2) and the latter acts on H_2O_2 converting it to water and oxygen (Mates, 2000). In the present study, it was observed that catalase did not participate in active H_2O_2 reduction irrespective of date of sampling, although SOD was active in scavenging the superoxide produced at 80 and 90 DAS, as evidenced by significant difference between the treatments. The reason for this could be that, CAT is present only in the peroxisome and has low substrate affinity, as it requires simultaneous access of two molecules of H_2O_2 (Shanker et al., 2004).

Even though SOD is not a seleno enzyme, Se might have altered the transcript levels of SOD enzymes thus altering SOD gene expression. The reduction in SOD at senescence after Se spray is in accordance with the finding of Hartikainen et al. (2000) in ryegrass. The reduction in SOD activity at 90 DAS in control plants might be due to enhanced superoxide and hydrogen peroxide radical production, since they destroy the SOD enzyme, if superoxide and hydrogen peroxide radicals were not scavenged properly (Dhindsa et al., 1981). Whereas in Se treated plants the decrease might be due to spontaneous disproportion of superoxide radicals to hydrogen peroxides and singlet oxygen by Se itself.

It is possible that Se promotes scavenging of produced H_2O_2 through increased GSH-Px activity and, consequently, reduces the need for their scavenger, SOD. It is clear from the study that, Se enhanced the proline content by 50% at 90 DAS. The mechanism of proline accumulation under Se treatment is not known till date, but the role of proline under oxidative stress like senescence, includes stabilization of proteins, regulation of cytosolic pH and regulation of NAD/NADH ratio by singlet-oxygen quencher and scavenger of OH^- radicals (Matysik et al., 2002).

GSH-Px was more efficient in destroying H_2O_2 than was catalase under both 80 and 90 DAS, since Se could not increase the catalase activity in a significant manner. The reason for this could be that GSH-Px, which is present throughout the cell and has higher substrate affinity in the presence of glutathione as a reductant (Noctor and Foyer, 1998). Lipid peroxidation was reduced concomitantly with an increase in GSH-Px activity. The results revealed that during the start of senescence phenomenon (80 DAS) in the plants cultivated with Se, antioxidative capacity was strengthened by increased GSH-Px, which is in accordance with increased Se concentration (Xue et al., 2001). On the other hand, GSH-Px was not able to increase further

during late senescence period (90 DAS), which was in agreement with the slight increase in Se concentration (Figure 2). It can be attributed to Se loss as volatile compounds (Hu et al., 2002; Terry et al., 1992; Terry and Zayed, 1994), and GSH might have been required for regeneration of the GSH-Px and glutaredoxin system or GSH itself might be involved in detoxification of electrophiles (Lu, 1999). The positive correlation between the Se concentration and GSH-Px activity in tea and *Chlamydomonas reinhardtii* (Yokota et al., 1988), soybean and rape (Xue et al., 1993a), and lettuce and ryegrass (Hartikainen et al., 2000; Xue et al., 2001) suggests the presence of Se-dependent GSH-Px activity. The loss of protective effect of selenoprotein against H_2O_2 induced cytotoxicity in cells treated with an inhibitor of GSH synthesis indicates that selenoprotein is a GSH dependent antioxidant *in vivo* (Jeong et al., 2002).

The hypothesis of Hartikainen et al. (2000), that the increase in GSH-Px, which is a scavenger of H_2O_2 and lipid hydroperoxides, resulted in reduced formation of superoxide anion radicals (O_2^-) is confirmed in the present study by recording a lower value of superoxide anion content in Se treated plants coupled with increased GSH-Px and reduced SOD activity. A relative increase in leaf number was seen which may have been due to a significant Se-induced delay in total leaf abscission as against production of new leaves. According to Panigrahi and Biswal (1979), the content of total chlorophyll decline after the leaf reaches full expansion. The decline in chlorophyll content might be partially due to lipid peroxidation of chloroplast membranes (Heath and Packer, 1968) or due to the formation of hydroperoxides of fatty acids (Pieser and Yang, 1978). The increased chlorophyll content in Se treated plants over control might be attributed to efficient scavenging of ROS by SOD and GSH-Px or otherwise they would have destroyed the chlorophyll pigments (Thomas et al., 2001). Dark adapted value of Fv/Fm reflects the potential quantum efficiency of PS II, and is used as a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson, 2000). Se slightly improved the PS II photochemistry at 80 DAS over control, whereas at 90 DAS, it increased the ratio by two per cent. It might be probably due to alleviation of oxidative stress developed in chloroplast during senescence by enhanced expression of GSH-Px and by enhanced Cu/Zn SOD transcript levels (Seppanen et al., 2003). Down regulation of PS II photochemistry in control leaves can be attributed to the increase in the proportion of closed PS II centres and the decrease in the efficiency of excitation energy capture, which may lead to increased photo inhibition (Lu et al., 2001). Due to closed PS II centres, ROS may accumulate which may damage PS II reaction centre complexes (Grove et al., 1986), this might have happened in control plants. On the contrary, Se treated plants had more GSH-Px and SOD, which were involved in active scavenging of ROS, thus maintaing near optimum PS II activity (Foyer and Noctor, 2000; Seppanen et al., 2003). Hence, the present study clearly indicates the antioxidative role of Se by enhanced antioxidant enzymes production which, in turn postpones the senescence phenomenon in soybean.

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