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Selenium Priming Selectively Ameliorates Weed – Induced Phytotoxicity by Modulating Antioxidant Defense Components in Lentil (*Lens culinaris* Medik.) and Grass Pea (*Lathyrus sativus* L.)

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Author's contribution

The only author DT performed the whole research work. He wrote the first original draft of the paper. He read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Present investigation was performed to assess the phytotoxic effect of weed, *Ageratum conyzoides* L. on two pulses, lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) and the ameliorative role of selenium (Se) to counter it.
Place and Duration of Study: Leaf samples of *Ageratum* were collected from the lentil-growing fields of Kalyani, West Bengal, India during winter of 2011-12. The lab-based works were performed at Department of Botany, R.P.M. College, West Bengal, India throughout the period of 2012.
Study Design: The study was carried out using four replicates treatment^{-1} in a completely randomized block design.
Methodology: Ageratum leaf aqueous extract of 200 mg ml^{-1} (w/v) was prepared and used on the two crops in (a) nutrient media, and (b) media + Se concentrations of 10, 20 and 40 µM separately. Also, Se was added alone (no extract) in nutrient media. A control (no exogenous Se, no extract) was maintained. The experiment was performed in an environmentally controlled growing chamber and plants were grown till flowering. Different morpho-physiological parameters along with root-tip mitosis and flower bud meiosis were

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studied at appropriate stages with standard methodology and analysed statistically.
Results: Leaf extract induced phytotoxicity through significant perturbation in ascorbate and glutathione redox and activities of SOD, APX, DHAR, GR, CAT and GPX, resulting in over-accumulation of H_2O_2 and lipid peroxidation product as the obvious marking of oxidative damage with more severe effect on lentil. Se-priming significantly ameliorated this situation at 20 and 40 µM in grass pea and at 40 µM in lentil by modulating antioxidant defense machinery in favor of growth. Se alone at 20 µM promoted growth of both crops but exhibited toxic effect on lentil at 40 µM. Phytotoxic effect of extract was also manifested by aberrant mitotis and meiosis in both plants.
Conclusion: *Ageratum* leaf extract triggered oxidative stress. Se-priming at selective concentration ameliorated extract-induced phytotoxicity in lentil and grass pea by modulating anti-oxidant defense components.

Keywords: Ageratum conyzoides L.; leaf extract; phytotoxicity; antioxidant defense; mitosis and meiosis; lentil; grass pea.

1. INTRODUCTION

Selenium (Se) is vital in global public health systems as it is involved in intra- and extra-cellular glutathione peroxidases, iodothyronine deiodinase, thioredoxin reductase and in many seleno-proteins. In all these cases Se is present as amino acid selenocysteine which forms the part of catalytic active site of enzyme. Despite its importance in animal and human micro-nutrition is well-known, reports on Se in plant growth and development is still nascent. Accumulating evidences indicated that Se at proper concentration may promote antioxidative mechanisms of plants, delay senescence, act against oxidative stress by increasing the GSH–Px activity and decreasing lipid peroxidation in higher plants [1], protect plants from fungal infection and heavy-metal toxicity, enhance tolerance of plants to UV-induced oxidative stress as well as a promotive growth effect in aging seedlings [1-3]. Furthermore, Se-induced changes in the activities of superoxide dismutase, ascorbate peroxidase and other antioxidant defense enzymes have been reported in different plants such as wheat, rapeseed, soybeans and coffee cell suspension cultures [2-4]. Yet, Se is toxic to most organisms at elevated concentrations, largely because of its similarity to sulfur (S), which leads to nonspecific replacement of S by Se in proteins [5].

Growing evidences indicate that weed-induced phytotoxicity may be manifested at physiological, cellular and metabolic levels on recipient crops, and may involve perturbations in oxidative metabolism in the form of allelopathy [6,7]. In plants, the formation and scavenging of various reactive oxygen species (ROS) are usually in balance. However, when a plant experiences stresses, the balance is tilted in favor of oxidative state, subsequently, resulting in oxidative damage to cellular proteins, lipids and DNA [8]. To counter this condition, plants have developed a complex but well coordinated antioxidant defense system in which ascorbate and thiol-compound glutathione play major roles through ascorbate-glutathione cycle and outside it [9]. Superoxide dismutase (SOD) constitutes the first line of defense, dismuting superoxide radicals to H_2O_2 which is readily scavenged by ascorbate peroxidase (APX) within this cycle and catalases (CAT) outside this cycle [9]. However, during scavenging of H_2O_2, APX uses reduced ascorbate (AsA) as its co-factor and continuously generates dehydroascorbate (DHA) which is enzymatically reduced to AsA by dehydroascorbate reductase (DHAR), using reduced glutathione (GSH) as an electron donor [9,10]. The resulting GSSG or oxidized glutathione is then recycled to GSH by

glutathione reductase (GR) [9]. Besides SOD and CAT, glutathione peroxidase (GPX) outside this cycle scavenges H_2O_2 and lipid peroxides in plant cell [10,11]. Se-dependent stimulation of GPX activity was reported in algal taxa and in some grasses [1,12].

Lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) are two important cool-season food legumes with a cultivation period of more than 8000 years, and have been in constant use as nutritional and pharmacological foods [13-17]. Worldwide most lentil production occurs under Mediterranean winter conditions (Middle East, Mediterranean region, Australia, etc.), during dry season in north Indian sub-tropical savannah and under temperate summer climates in North America. The eastern half of Indo-Gangetic plain of South Asia including India, Nepal and Bangladesh share 32% lentil production [17]. On the other hand, grass pea has been cultivated both as food and animal feed in Indian Subcontinent, Australia, the Mediterranean Europe, North Africa, and parts of America [15]. In India, both crops are grown as winter pulse crop either as monocrop or often in mixed cropping with oil-seeds and cereals [13,15]. Besides sensitivity to diverse types of biotic and abiotic stresses [18,19], slow growth in early seedling stages make the crops poorly competitive to weeds [13,20] and weed-induced oxidative stress has primarily been observed in grass pea [6]. The early growth of both lentil and grass pea in rice fallow often coincides dense weeds [13], among which *Ageratum conyzoides* L. has been considered one of the worst invasive taxa [21]. Accumulating evidences also indicate that high weed diversity in crop growing areas may be related to increasing drought and heavy metal contamination and greater fitness of alien weeds than the cultivated crops in these adverse agro-climatic conditions [22,23]. Phytotoxicity such as allelotoxicity induced by obnoxious weeds is a major constrain for pulse food security, and thus, understanding of phytotoxicity is extremely important for integrated weed management in the fields of edible pulse crop like lentil [20]. Despite immense importance in sustainable production of pulse crops, not much work has been done in this direction. The objectives of the present work were, therefore, set to 1) assess the phytotoxic effect of weed, *Ageratum conyzoides* L. on two pulses, lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) and 2) the ameliorative role of selenium (Se) to counter it.

2. MATERIALS AND METHODS

2.1 Preparation of Leaf Extract

Leaves of *Ageratum conyzoides* L. plants were collected at early flowering stage from the lentil-growing fields of Kalyani (22°59'N/ 88°29'E, Gangetic plain, clay-alluvial), West Bengal, India during winter of 2011-12. Extract preparation was carried out following protocols, earlier adopted in legume crops [6]. Collected samples were carefully cleaned and fully dried in shade for 96 h, ground and stored at room temperature. The extracts were prepared by soaking 30 g crushed mass in sterilized distilled water for 24 h at room temperature (25ºC), filtered through Whatman No. 1 filter paper, made final volume to 100 ml (300 mg ml^{-1} w/v). The extract was considered as stock solution (kept at 5ºC until used). The solution was further diluted with distilled water to get test concentrations of 200 mg ml^{-1}. The protocol and concentration of extracts were standardized by preliminary experiments on survival of lentil and grass pea plants.

2.2 Plant Material, Culture Conditions and Treatment Protocols

Fresh and healthy seeds of lentil (*Lens culinaris* Medik. cv. IPL 406) and grass pea (*Lathyrus sativus* L. cv. BioL 212) were surface-sterilized with NaOCl (0.1 %, w/v) and continuously washed under running tap water followed by distilled water. Seeds were allowed to germinate in the dark in two separate sets on moistened filter paper at 25ºC. Germinated seedlings were randomly placed in polythene pots (5 plants $pots^{-1}$) containing 300 ml of Hoagland's No 2 nutrient media, and were allowed to grow for 7 d. The plants were, then, subjected to the following four treatment protocols in eight separate sets as: (a) untreated control (no extract, no exogenous Se), (b) 200 mg ml^{-1} leaf extract, (c) 10 µM sodium selenate (Se, Na_2SeO_4, MW 188.94 g/mol, Sigma-Aldrich, Bangalore, India), (d) 20 µM sodium selenate, (e) 40 µM sodium selenate, (f) 200 mg ml^{-1} extract + 10 µM Se, (g) 200 mg ml^{-1} extract + 20 µM Se, and lastly, (h) 200 mg ml^{-1} extract + 40 µM Se. Each treatment was replicated four times. Control and treated plants were allowed to grow till the onset of flowering (21 days). Nutrient solution was refreshed every alternate day to prevent depletion of nutrients, extracts as well as Se. The experiment was carried out in a completely randomized block design manner in an environmentally controlled growing chamber under a 14 h photoperiod, 25/20 (± 2ºC), relative humidity of 70 ± 2 % and a photon flux density of 180 µmol m^{-2} s^{-1}. Dry weight (g $plant^{-1}$) of shoot was recorded after oven drying at 60℃ for 48 h.

2.3 Measurement of Leaf Photosynthesis

Leaf photosynthetic rate was assayed following earlier methods [24] using a portable photosynthesis system (LI-6400XT, LI-COR, Inc, USA).

2.4 Determination of Leaf Glutathione and Ascorbate

Reduced (GSH) and oxidized (GSSG) glutathione contents were estimated following the method of Griffith [25]. Reduced (AsA) and oxidized (DHA) ascorbate contents were determined by the method of Law et al. [26]. Redox state of AsA was calculated by AsA/(AsA+DHA) and that of GSH by GSH/(GSH+GSSG).

2.5 Antioxidant Enzyme Assay

Fresh leaf tissue (250 mg) was homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 2% (w/ v) polyvinyl pyrrolidone (PVP) using a chilled mortar and pestle kept in an ice bath. The homogenate was centrifuged at 15,000 *g* at 4ºC for 30 min. Clear supernatant was used for enzyme assays. For measuring APX activity, the tissue was separately ground in homogenizing medium containing 2.0 mM ascorbate in addition to the other ingredients. All assays were done at 25ºC. Soluble protein content was determined according to Bradford [27] using BSA as a standard.

SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay following Beyer and Fridovich [28]. In this method 1 ml of solution containing 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 µM NBT, 0.025 % triton-X-100 was added into small glass tubes, followed by 20 µl of enzyme extract. Reaction was started by adding 10 µl of riboflavin solution (0.044 mg ml^{-1}) and placing the tubes in an aluminium foil-lined box having two 20-W fluorescent lamps for 7 min. After illumination, the absorbance of solution was measured at 560 nm. SOD activity was expressed as U (unit)

mg^{-1} protein. One unit of SOD was equal to that amount which causes a 50% decrease of SOD-inhibited NBT reduction.

APX (EC 1.11.1.11) activity was assayed following methods of Nakano and Asada [29]. Three milliliters of there action mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 ml enzyme extract. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε = 2.8 $mM^{-1}cm^{-1}$). APX activity was expressed as µmol ascorbate oxidized min^{-1} mg^{-1} protein.

DHAR (EC 1.8.5.1) activity was measured following the protocol of Nakano and Asada [29]. The complete reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.2 mM DHA and 0.1 mM EDTA in a final volume of 1 ml. Reaction was started by addition of suitable aliquots of enzyme extract and the increase in absorbance was recorded at 30 s intervals for 3 min at 265 nm. Enzyme activity was expressed as µmol ascorbate formed min^{-1} mg^{-1} protein.

GR (EC 1.6.4.2) activity was determined by monitoring the glutathione dependant oxidation of NADPH, as described by Carlberg and Mannervik [30]. In a cuvette, 0.75 ml 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA, 75 µl NADPH (2 mM), and 75 µl oxidized glutathione (20 mM) were mixed. Reaction was initiated by adding 0.1 ml enzyme extract to the cuvette and the decrease in absorbance at 340 nm was monitored for 2 min. GR specific activity was expressed as nmol NADPH oxidized min^{-1} mg^{-1} protein.

For CAT (EC 1.11.1.6) activity, extraction was performed in a 50 mM Tris–HCl buffer. The enzyme activity was assayed by measuring the reduction of H_2O_2 at 240 nm (ε = 39.4 $mM^{-1}cm^{-1}$) and 25ºC, as described by Chance and Maehly [31].

Total GPX (EC 1.11.1.9) activity was assayed, using cumene hydroperoxide (for Se and non-Se enzyme at 25℃ and pH 8.0) as substrates [11]. The rate of peroxide removal was measured with respect to the rate of NADPH oxidation at 340 nm (ε = 6.22 mM^{-1} cm^{-1}).

2.6 Estimation of Leaf H_2O_2 Content, Lipid Peroxidation and Membrane Electrolyte Leakage

Foliar H_2O_2 content was estimated following the methods of Wang et al. [32]. Fresh tissue of 0.1 g was powdered and blended with 3 ml acetone for 30 min at 4℃. Then the sample was filtered through eight layers of gauze cloth. After addition of 0.15 g active carbon, the sample was centrifuged twice at 3,000 *g* for 20 min at 4℃, then 0.2 ml 20% $TiCl_4$ in HCl and 0.2 ml ammonia was added to 1 ml of the supernatant. After reaction, the compound was centrifuged at 3,000 *g* for 10 min, the supernatant was discarded and the pellet was dissolved in 3 ml of 1 M H_2SO_4 and absorbance was measured at 410 nm. Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents following the method of Hodges et al. [33] and expressed as nmol MDA g^{-1} fresh weight. Electrolyte leakage (EL) was assayed by measuring the ions leaching from tissue into deionised water [34]. Fresh samples (100 mg) were cut into small pieces (about 5 mm segments) and placed in test tubes containing 10 ml deionised water. Tubes were kept in a water bath at 32℃ for 2h. After incubation, electrical conductivity (EC_1) of the bathing solution was recorded with an electrical conductivity meter (Systronics M-308, Kolkata, India). The samples were then autoclaved at 121℃ for 20 min to completely kill the tissues and release all electrolytes.

Samples were then cooled to 25℃ and final electric al conductivity (EC_2) was determined. The EL was expressed as a percentage by the formula, EL% = EC_1 / EC_2 × 100.

2.7 Cytogenetic Study

Root-tip mitosis and flower bud meiosis were studied following the procedures employed earlier [35-37]. For mitotic preparations, fresh and healthy roots were pretreated with 2 mM 8-hydroxyquinoline for 2 h at room temperature followed by fixation in 45% acetic acid for 15 minutes at 4℃. These were then hydrolyzed in a mix ture of 1N HCL and 45% acetic acid (2:1) at 60℃ for 10s. The root tips were stained a nd squashed in 1% aceto-orcein. The mitotic index (MI %) was calculated by dividing cells among the examined total cells. For meiosis, suitable sized flower buds were fixed between 9.00 A.M and10.00 A.M in propionic acid-alcohol (1:2) for 6 h, and were preserved in 70% alcohol for future studies. After washing the fixed buds in distilled water, anthers were smeared in 1% propiono-carmine solution to analyze meiosis in the microsporocytes. Photomicrographs were taken from well scattered plants

2.8 Statistical Analysis

The results presented are the mean values ± standard errors of at least four replicates. Multiple comparisons of means were performed by ANOVA (SPSS Inc. v. 10), and the means were separated by Duncan's multiple range test considering significant differences at 5% level.

3. RESULTS AND DISCUSSION

Ageratum leaf extract at 200 mg ml^{-1} caused severe growth inhibition in both lentil and grass pea seedlings as manifested by reduction in photosynthetic rate and shoot dry weight. Photosynthetic rate and shoot dry weight was decreased by about 3.0 and 2.5-fold in lentil and 1.8 and 2-fold in grass pea, respectively (Table 1). Reduction in dry weight due to weed effect on crop plants is one of the determining symptoms of phytotoxicity, and has been reported in different plants including grass pea [6,22,23].

Upon impositions of three different concentrations of Se, significant improvement of photosynthetic rate as well as shoot dry weight was observed in both crops at 20 µM (Table 1). At 40 µM Se, further enhancement of these two growth traits was observed in grass pea but in lentil, both the traits were reduced significantly in relation to control (Table 1). Marginal changes in dry weight were noticed in 10 µM. The results indicated marked variations between the two crops in response to Se treatment alone and revealed toxicity of Se on lentil crops at elevated level. Fairly wide scale differences were found among lentil genotypes for grain Se concentrations [38,39], and as lentil growing areas in South Asia are generally Se-deficient Se priming in soil is often recommended for Se-biofortification of pulse grains [38]. Present result clearly suggested that Se can selectively enhance the growth of lentil plants while in grass pea its application has been used in favor of growth even at high concentration.

Under 200 mg ml^{-1} extract + 10 µM Se treatment, photosynthetic rate and shoot dry weight of both crops decreased significantly in comparison to control (Table 1). It is noteworthy, that *Ageratum* leaf extract alone caused severe reduction in photosynthetic rate and dry weight. Similarly, exogenous application of 10 µM Se alone was neither beneficial nor detrimental on

plant growth, indicating inhibition of plant growth in 200 mg ml^{-1} extract + 10 µM Se may be caused by leaf extract, and Se at low concentrations showed no noticeable ameliorative effect. The failure of Se to counter growth inhibition in lentil was strongly substantiated by the marked reduction of photosynthetic rate and plant dry weight at 200 mg ml^{-1} extract + 20 µM Se, despite stimulation of photosynthesis and dry weight under 20 µM Se treatment alone (Table 1). In grass pea, by contrast, completely normal photosynthetic rate and shoot dry weight was observed at 200 mg ml^{-1} extract + 20 µM Se and further enhancement of photosynthesis and dry weight was noticed at 200 mg ml^{-1} extract + 40 µM Se (Table 1), indicating positive effect of Se priming in extract-treated grass pea seedlings at concentration > 10 µM. Interestingly, both 200 mg ml^{-1} extract and 40 µM Se were found inhibitory to lentil growth, but stimulation of plant growth was observed in seedlings subjected to 200 mg ml^{-1} extract + 40 µM Se (Table 1). The results suggested beneficial effect of Se at higher concentration on plant growth in presence of leaf extract in lentil. Improvement of photosynthetic apparatus and physiological capacity was also reported in Se-treated *Cucurbita pepo* under drought stress [40] and enhanced root growth in *Vicia faba* [41], biomass yield in lettuce [42] and plant growth in mustard [43] at low Se concentrations.

The inhibitory effects of *Ageratum* leaf extracts on leaf photosynthesis and shoot dry weight of lentil and grass pea in the present study were intimately associated with their intrinsic antioxidant defense perturbations. Compared to control, reduced ascorbate (AsA) and glutathione (GSH) contents were declined by nearly 2-fold and 3-fold with concomitant increase of their oxidized forms by about 2.5-fold and 3-fold, respectively, in lentil shoots (Table 2). This resulted in substantial reduction of their redox state (Table 2). In extract-treated grass pea, shoot AsA level and its redox state was quite normal but GSH content decreased by about 3-fold compared to control. Although there was no change in GSSG content, declining GSH level led to reduction of GSH redox in extract-treated grass pea shoots (Table 2). Similar situation was encountered in both plants grown in extract + 10 µM Se, where shoot dry weight decreased significantly in relation to respective controls of two crops (Table 2). At 20 µM Se alone, AsA content was quite normal while total GSH level and its redox increased significantly over control in both crops (Table 2). This was maintained in grass pea even at 40 µM Se. However, redox value of both AsA and GSH declined markedly in lentil exposed to this concentration and also, at extract + 20 µM Se (Table 2). Completely normal level of both AsA and GSH, however, were measured in lentil plants subjected to extract + 40 µM Se and in grass pea under extract + 20 µM Se and extract + 40 µM Se (Table 2).

The above results clearly pointed out severe alterations in redox status of AsA and GSH in extract treated seedlings of lentil and grass pea, indicating damaging potential of *Ageratum* leaf extract on cell antioxidant systems. The effect was more severe in comparison to earlier reports on grass pea seedlings, treated with *Lantana* leaf extract [6]. In order to ascertain the possible reasons behind the redox perturbations and its consequences on oxidative metabolism of both crops exposed to *Ageratum* leaf extract, activities of six prominent antioxidant defense enzymes were assayed under all the treatments.

Table 1. Rate of photosynthesis and shoot dry weight of 14-d-old lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) seedlings in nutrient media (control) and nutrient media treated with *Ageratum* leaf extract (200 mg ml^{-1}), and three Selenium concentrations (Se), and Se-primed extract-supplemented nutrient media in total eight separate set of experiments

Parameters	Control	Leaf extract	10 µM Se	20 µM Se	40 µM Se	10 µM Se + extract	20 µM Se + extract	40 µM Se + extract
Photosynthetic rate (µmol m^{-2} s^{-1}) -lentil	13.82 ± 0.12b	4.27 ± 0.08c	13.93 ± 0.16b	19.69 ± 0.23a	6.18 ± 0.10c	4.61 ± 0.08c	4.75 ± 0.12 c	14.09 ± 0.12b
Photosynthetic rate (µmol m^{-2} s^{-1}) –grass pea	16.57 ± 0.18b	9.23 ± 0.11c	16.61 ± 0.19b	20.59 ± 0.19b	31.27 ± 0.26a	9.76 ± 0.12c	16.71 ± 0.17b	19.89 ± 0.20b
Shoot dry weight (g plant^{-1})-lentil	0.18 ± 0.10b	0.072 ± 0.12d	0.20 ± 0.16a	0. 27 ± 0.13a	0.09 ± 0.14c	0.071 ± 0.20d	0.080 ± 0.22d	0.17 ± 0.11b
Shoot dry weight (g plant^{-1})-grass pea	0.23 ± 0.19b	0.12 ± 0.10c	0.24 ± 0.19b	0.31 ± 0.21a	0.38 ± 0.19a	0.13 ± 0.11c	0.23 ± 0.10b	0.30 ± 0.11a

Data are means ± SE of four independent observations. Means followed by same lower case letters are not significantly different at 5% level by Duncan's Multiple Range Test

Table 2. Shoot ascorbate (AsA) and glutathione (GSH) content (nmol g^{-1} DW) and redox states in 14-d-old lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) seedlings grown in nutrient media (control) and nutrient media treated with *Ageratum* leaf extract (200 mg ml^{-1}), and three Selenium concentrations (Se), and Se-primed extract-supplemented nutrient media in total eight separate set of experiments

Parameters [a]	Control	Leaf extract	10 µM Se	20 µM Se	40 µM Se	10 µM Se + extract	20 µM Se + extract	40 µM Se + extract
Reduced AsA -lentil	902.4 ± 10.6a	451.2 ± 8.8b	910.1 ± 10a	917.6 ± 11a	473.18 ± 10b	449.8 ± 9.8b	455 ± 12b	898.8 ± 10a
Reduced AsA-grass pea	1017 ± 10a	998.9 ±10.8a	1061 ± 10a	1189 ± 12.7a	1067 ± 19.6a	996 ± 12a	1011 ± 1 5a	1089 ± 10a
Dehydro ascorbate -lentil	103.8 ± 5.5c	260.2 ± 6.1a	104.0 ± 5.0c	105.8 ± 6.0c	228.8 ± 7.7b	230.5 ± 8 .1b	245 ± 9.0a	107.1 ± 8.5c
Dehydro ascorbate -grass pea	123.8 ± 4.8a	119.6 ± 5.1a	124.1 ± 6.1a	131 ± 7.3a	129 ± 9.1a	123.1 ± 5.5a	123.6 ± 5a	125 ± 4.8a
AsA redox (lentil)	0.893 ± 0.15a	0.634 ± 0.17b	0.897 ± 0.10a	0.897 ± 0.15a	0.675 ± 0.18b	0.661 ± 0.15b	0.647 ± 0.18b	0.902 ± 0.17a
AsA redox-grass pea	0.892 ± 0.10a	0.893 ± 0.10a	0.895 ± 0.15a	0.901 ± 0.15a	0.890 ± 0.21a	0.890 ± 0.19 a	0.891 ± 0.12a	0.895 ± 0.16a
GSH –lentil	196.6 ± 2.2b	65.6 ± 1.8d	195.8 ± 2.9b	249.6 ± 3.5a	81.8 ± 2.8c	66.2 ± 2.5d	68.3 ± 3.3d	200 .5 ± 2.8b
GSH-grasspea	213.2 ± 1.9a	70.8 ± 2.0b	216.0 ± 2.3a	283.8 ± 3.7a	285.1 ± 4.1a	68.9 ± 2.9b	211.2 ± 2.1a	244.8 ± 4.1a
GSSG-lentil	31.7 ± 1.8b	196.1 ± 3.1a	33.5 ± 2.0b	29 .7 ± 1.6b	146.3 ± 4.4a	176.5 ± 3.5a	169.2 ± 2.9a	30 .5 ± 2.2b
GSSG-grass pea	39.8 ± 2.5a	41.6 ± 1.9a	40.5 ± 1.5a	37.9 ± 2.2a	41.1 ± 2.5a	40.8 ± 1.8a	40.5 ± 2.8a	37. 9 ± 2.4a
GSH redox-lentil	0.863 ± 0.23a	0.251 ± 0.25c	0.855 ± 0.18a	0.895 ± 0.21a	0.361 ± 0.22b	0.272 ± 0.20c	0 .290 ± 0.18b	0.871 ± 0.22a
GSH redox-grass pea	0.843 ± 0.15a	0.631 ± 0.16b	0.843 ± 0.19a	0.882 ± 0.15a	0.874 ± 0.13a	0.630 ± 0.11 b	0.841 ± 0.18a	0.870 ± 0.18a

Data are means ± SE of four independent observations. Means followed by same lower case letters are not significantly different at 5% level by Duncan's Multiple Range Test

In extract-treated lentil, SOD activity was quite normal and CAT was high but levels of APX, GPX, DHAR and GR decreased by about 2-4-fold (Table 3). In extract-treated grass pea, on the other hand, SOD activity was nearly doubled to that of control but activities of APX, DHAR and CAT were normal and GPX and GR decreased (Table 3). The results suggested perturbations in antioxidant defense response due to extract treatment, and the two crops responded in different ways although both suffered growth inhibitions. Normal to elevated SOD activity indicated generation of superoxide radicals in response to extract treatment but as an obvious consequence of SOD activity, H_2O_2 is formed [9]. H_2O_2 is a diffusible ROS within cell and its dual roles as an inducer of stress and a signaling molecule to modulate antioxidant defense have been explored in many plants including legumes like lentil, grass pea and *Leucaena* [10,44,45]. With several isozymes, APX is the most prolific enzyme in plants engaged in H_2O_2-scavenging in chloroplast [10,29]. However, it exclusively requires AsA as its co-factor to reduce H_2O_2 [29]. Reduced APX activity in the treated lentil shoot was presumably due to low availability of AsA pool, caused by significant retardation in DHAR activity. It is noteworthy that regeneration of AsA from its oxidized form by the activity of DHAR plays pivotal role in maintaining of AsA redox within cell [9] and that greater importance of regeneration than biosynthesis of AsA has been revealed in ascorbate-deficient grass pea mutants [10] and also in other plants under stress [29]. This was further substantiated in the present grass pea seedlings, where AsA redox was quite normal in treated plants, strongly indicating effective regeneration of AsA by the normal activity of DHAR. By contrast, the declining level of GSH redox pool in both crops exposed to leaf extract was mainly due to reduced activity of GR within AsA-GSH cycle, impeding regeneration of GSH from GSSG. GSSG is continuously formed during consumption of GSH by the activity of DHAR, GPX and other cellular enzymatic systems [9], and its higher level in the present lentil shoot than GSH resulted in significant drop in GSH redox value, often considered as 'disulphide stress' in plants [46]. Low availability of GSH might pose severe detrimental effect on H_2O_2-scavenging machinery in which, besides APX and CAT, GPX plays important roles. Although GPX can use thioredoxin as its electron donor, in the present case low GSH pool in both treated crops presumably perturbed its activity, indicating disturbances in GSH-mediated oxidative metabolism in treated seedlings.

As an obvious consequence of the crippling of antioxidant defense under leaf extract treatment, the level of H_2O_2 increased about 2-fold in both crops over that of mother control plants (Fig. 1) despite high CAT activity in lentil and its normal level in grass pea (Table 3). Obviously, CAT alone could not cope with rising level of H_2O_2 in treated shoots. The failure of CAT-mediated scavenging of H_2O_2 in the backdrop of low APX and/or GPX was also found in grass pea seedlings treated with *Lantana camara* leaf extract [6]. The increase in H_2O_2 was accompanied with steep rise in malondealdehyde (MDA) content in shoots of present crops (Fig. 1). MDA is a product of membrane lipid peroxidation, and over-accumulation of this cytotoxic aldehyde along with H_2O_2 is an indication of oxidative damage caused by *Ageratum* leaf extract in both legumes. The severity of membrane damage was also evidenced by high percentage of electrolyte leakage in both crops (Fig. 1). The result agreed well with earlier findings on oxidative imbalance in grass pea seedlings caused by *Lantana* leaf extract [6]. In several plants, including beans and peas, abnormal accumulation of H_2O_2 and MDA was found as the marks of oxidative stress during salinity, drought and heavy metal toxicity [44,45,47,48].

The severity of oxidative damage resulted from treatment with *Ageratum* leaf extract was also observed in both crops exposed to leaf extract + 10 µM Se where barring SOD, activities of all other enzymes reduced considerably (Table 3). No stimulation of antioxidant defense response was observed in any crop when nutrient media was supplemented with 10

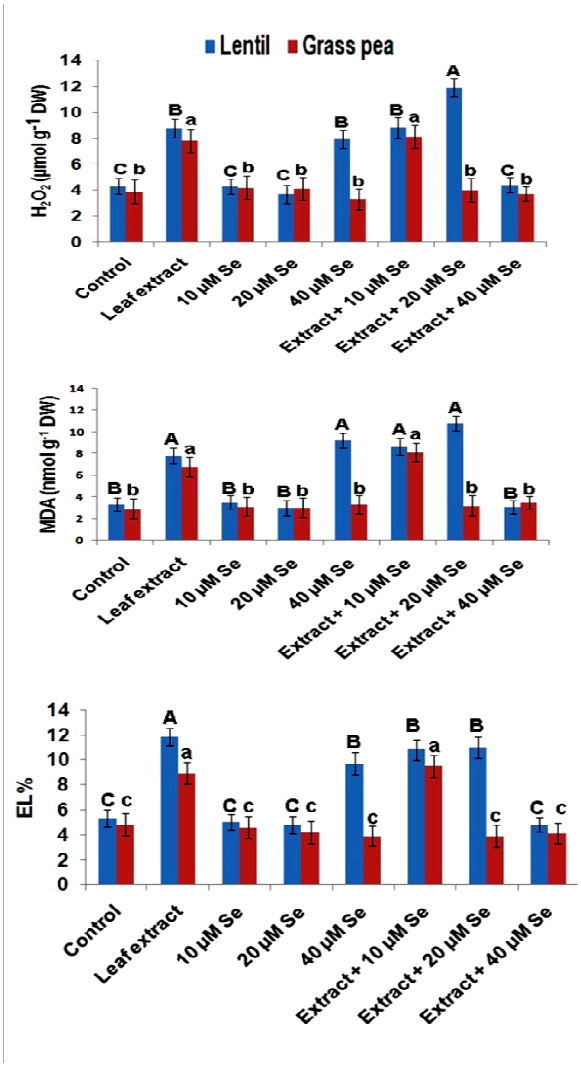

Fig. 1. Shoot H_2O_2, malondealdehyde (MDA) and electrolyte leakage (EL%) in 14-d-old lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) seedlings grown in nutrient media (control) and nutrient media treated with *Ageratum* leaf extract (200 mg ml^{-1}), and three Selenium concentrations (Se), and Se-primed extract-supplemented nutrient media. Data are means of at least four independent experiments with same alphabets above error bar represents non-significant differences among means at 5% level following Duncan's Multiple Range Test

µM Se alone (Table 3). This situation was significantly changed once the seedlings were subjected to 20 µM and 40 µM Se. Remarkable improvement of shoot dry weight in grass pea at these two concentrations and in lentil at 20 µM Se is presumably due to significant enhancement in activities of APX and GR within AsA-GSH cycle and GPX outside this cycle (Table 1, 3). This maintained redox balance of AsA and GSH in favor of reducing environment. The efficiency of antioxidant defense in presence of Se was also manifested by quite normal levels in SOD, DHAR and CAT activities in both crops (Table 3), collectively

preventing over-accumulation of H_2O_2 and concomitant oxidative damage to membrane. This was evidenced by even marginally lower level of MDA and electrolyte leakage in plants grown in Se-supplemented media than that in control plants. Completely opposite scenario, however, was observed in lentil plants grown in 40 µM Se (Fig. 1). Significantly higher SOD activity over control was accompanied with substantial loss in activities of APX, GPX and GR (Table 3). This led to lowering of AsA and GSH redox and impeded scavenging of H_2O_2 and peroxides, as suggested by over-accumulation of MDA content and EL% (Table 2, Fig. 1). Obviously, grass pea plants were better capable to utilize elevated Se level in favor of growth by modulating antioxidant defense while lentil although performed well in 20 µM Se could not tolerate high concentration of Se. The pro-oxidant role of Se at higher concentrations is certainly related to generation of excess ROS, and subsequently, tilting of redox balance in favor of oxidative stress in lentil at high doses, and agreed well with earlier reports on enhanced lipid peroxidation and cell membrane injury in lead-treated *Vicia faba* root [41], lettuce plants [42] and mustard seedlings [43] under high Se exposure. Decrease in rate of photosynthesis might be due to oxidative damage orchestrated by lipid peroxidation and subsequent leakage in chloroplast membrane and/or inhibition of chlorophyll synthesis as observed earlier in grass pea, lentil, beans, peas and other plants [10,42,48] and might be one of the prime reasons for dwindling shoot dry weight in the present materials.

Perhaps, one of the most intriguing questions in the present investigation was the stimulatory effect of 20 µM Se when applied solely with nutrient media on growth of lentil plant but its inhibitory effect and/or its failure to improve shoot dry weight when used in combination with leaf extract. Similarly, despite toxicity of 40 µM Se ameliorative action of this concentration was observed in the presence of extract on lentil. This apparent conflicting situation can be explained by comparing activities of SOD with other five antioxidant enzymes. SOD activity was quite normal at 20 µM Se (Table 3), but its enhancement by about 2-fold accompanied by significant reduction in other five enzymes at extract + 20 µM Se (Table 3) severely crippled recycling of AsA and GSH as well as effective scavenging of both H_2O_2 and lipid peroxides. Over-accumulation of H_2O_2 and MDA along with high EL% strongly suggested negative impact of leaf extract on plant oxidative metabolism and failure of Se to counter it at 20 µM. The inverse relationship between SOD and other five enzymes was also manifested by high SOD level but low activity of other five enzymes in lentil seedlings at 40 µM Se and the just opposite scenario at extract + 40 µM Se treatment (Table 3). In the latter case, effective scavenging of ROS was suggersted by quite normal levels of H_2O_2, MDA and EL% (Fig 1), preventing ROS-mediated cellular damage in lentil plants. Enhanced SOD level coupled with low GPX activity was ascribed to prime reasons to Se-toxicity of rye grass [49]. Present results revealed differential effect of Se on plant growth through modulation of defense response not only on GPX but also on other antioxidant enzymes activities and ROS-metabolism when applied alone. Presumably, Se priming in presence of extract synergistically acted in modulation of antioxidant defense and concomitant reduction of oxidative damage, as explained earlier in Se-treated rapeseed seedlings exposed to cadmium [2] and salinity [3] and sorghum leaves under high temperature stress [50]. Obviously, priming of Se influenced intrinsic cellular roles of leaf extract, modulating antioxidant defense especially, SOD in favor of plant growth in grass pea at both 20 and 40 µM and in lentil only at 40 µM. Increase in SOD activity was found associated with rising MDA content in Se-treated maize seedlings under water deficit stress [51]. Se application has tremendous effect on translocation of sulphur and phosphorus [42], and as Se as well as sulphur status is linked to GSH and other thiol metabolism in sulphur-deficient legumes like lentil and grass pea [52], further study is needed to ascertain the magnitude of relationship between leaf extract and Se in these two legumes.

Table 3. Shoot superoxide dismutase (SOD, U mg-1 protein), ascorbate peroxidase (APX, µmol ascorbate oxidized min^{-1} mg^{-1} protein), dehydroascorbate reductase (DHAR, µmol ascorbate formed min^{-1} mg^{-1} protein), glutathione reductase (GR, nmol NADPH oxidized min^{-1} mg^{-1} protein), catalase (CAT, nmol H_2O_2 min^{-1} mg^{-1} protein) and glutathione peroxidase (GPX, nmol min^{-1} mg^{-1} protein) activities in 14-d-old lentil (*Lens culinaris* Medik.) and grass pea (*Lathyrus sativus* L.) seedlings grown in nutrient media (control) and nutrient media treated with *Ageratum* leaf extract (200 mg ml^{-1}), and three Selenium concentrations (Se), and Se-primed extract-supplemented nutrient media in total eight separate set of experiments

Parameters [a]	Control	Leaf extract	10 µM Se	20 µM Se	40 µM Se	10 µM Se +extract	20 µM Se + extract	40 µM Se + extract
SOD -lentil	178.8 ± 10.8b	171.6 ± 8.9b	180.0 ± 10.5b	177.5 ± 10.1b	373.18 ± 13.9a	179.8 ± 9.3b	355 ± 12a	171.8 ± 10.6b
SOD –grass pea	191.3 ± 8.5b	382.9 ± 10.0a	186.4 ± 10.0b	183.8 ± 10.7b	191.7 ± 19.3b	189.9 ± 10.6b	190.6 ±10.7b	189 ± 10b
APX -lentil	153.4 ± 9.5a	76.2 ± 6.1c	154.0 ± 8.9a	175.8 ± 6.0a	88.8 ± 7.9b	100.5 ± 5.8b	45.8 ± 6.0d	197.1 ± 8.7a
APX–grass pea	161.3 ± 7.8b	159.0 ± 7.1b	164.0 ± 7.7b	187.5 ± 7.0b	222.8 ± 9.7a	113.1 ± 5.5c	223.6 ± 5. 8a	225 ± 5.3a
DHAR- lentil	0.81± 1.5a	0.27 ± 1.7c	0.83 ± 1.1a	0.89 ± 1.5a	0.38 ± 1.8b	0.36 ± 1.7b	0.24 ± 1.8c	0.97 ± 1.7a
DHAR -grass pea	0.89 ± 1.0a	0.89 ± 0.9a	0.89 ± 0.5a	0.90 ± 1.8a	0.90 ± 1.3a	0.77 ± 0.9a	0.89 ± 0.8a	0. 85 ± 0.6a
GR –lentil	26.8 ± 3.7c	9.2 ± 2.8d	25.5 ± 3.9c	49.7 ± 3.5b	11.8 ± 3.5d	16.2 ± 2.8d	8.3 ± 3.3d	60.5 ± 3. 8a
GR-grass pea	31.2 ± 3.9b	10.8 ± 4.4c	31.0 ± 2.8b	80.8 ± 3.7a	92.1 ± 4.4a	17.9 ± 1.9c	91.1 ± 2.3a	94.8 ± 3.1a
CAT-lentil	81.5 ± 3.8b	95.1 ± 3.3a	83.5 ± 4.0b	79.7 ± 3.6b	66.3 ± 4.3c	76.5 ± 3.5b	69.2 ± 4.0c	90.5 ± 4.2c
CAT-grass pea	89.8 ± 4.5a	91.6 ± 3.9a	90.5 ± 3.5a	77.9 ± 4.2b	91.1 ± 5.5a	80.8 ± 4.8b	80.5 ± 5.8b	97.9 ± 4.4a
GPX-lentil	63.3 ± 0.9b	16.1 ± 0.7d	65.5 ± 0.8b	79.5 ± 0.7a	36.9 ± 0.5c	27.2 ± 0.83c	19.0 ± 0.8d	87.3 ± 0.72a
GPX-grass pea	64.7 ± 0.8b	43.1 ± 0.6c	64.3 ± 0.9b	81.2 ± 0.8a	87.4 ± 0.89a	29.2 ± 0.71d	8.41 ± 0.88e	8.50 ± 0.80e

Data are means ± SE of four independent observations. Means followed by same lower case letters are not significantly different at 5% level by Duncan's Multiple Range Test

The onset of oxidative stress due to effect of *Ageratum* leaf extract was also felt in root-tip mitosis and flower bud meiosis of both crops. Compared to control, MI was reduced by 2-3-folds with more severe effect on lentil (mean 9.67 ± 0.45) than that in grass pea (18.38 ± 0.27) root tip. In contrast to usual distribution of 2n = 14 chromosomes in root tip mitosis and seven bivalents in meiosis I (Fig. 2a, b, g), chromotoxic effect of leaf extract was exhibited by chromosome breakage, tendency of sticky metaphase, multipolarity, unoriented chromosome/s, laggard and bridge formation in both mitosis and meiotic process of both crops (Fig. 2 c-f, j-l, n, o), suggesting induction of cytotoxic damage by leaf extract in legume crops. Similar aberrations were observed in both crop plants treated with extract + 10 µM Se (data not shown). By contrast, occurrence of aneuploid cells containing loss of one or two chromosomes from normal diploid complement (2n=2x=14; 2n=2x-1 or 2n=2x-2) was consistently observed in meiosis of lentil plants under extract + 20 µM Se treatment and in grass pea subjected to extract + 10 µM Se treatment (Fig. g-i). At 40 µM Se, binucleolate condition was observed in lentil meiocytes (Fig. 2 m) as a unique symptom of toxicity. Unequal 6-7 separation (Fig. 2 n) and anaphase bridge with disorganized scattered chromosomes was scored at anaphase I of grass pea exposed to extract + 10 µM Se treatment (Fig. 2 o). Disruption in both mitotic and meiotic process was also observed in grass pea plants subjected to *Lantana* leaf extract [6] and in other plants treated with weed extract [53].

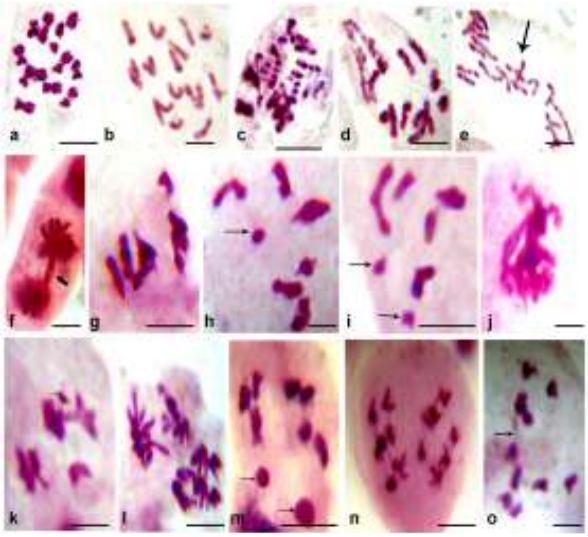

Fig. 2. Representative photographs of root-tip mitotis and flower-bud meiotic anomalies induced by *Ageratum* leaf extract, toxic Se concentrations and extract + Se treatment in lentil and grass pea; normal 2n=2x=14 chromosomes in lentil (a) and grass pea (b), chromosome breakage in lentil (c), tendency of chromosome stickiness at metaphase I in grass pea (d), unoriented chromosome at early anaphase I of lentil (e), sticky bridge at mitotic metaphase in lentil (f) under leaf extract treatment, normal 7 bivalents at meiosis metaphase I of lentil (g), aneploid cells with 2n=2x-1=13 chromosomes (6 bivalents + one univalent→) in lentil under extract + 20 µM Se treatment (h), aneuploid cells showing 2n = 2x- 2 = 12 chromosomes (5 bivalents + two univalents →) in grass pea under extract + 10 µM Se treatment(i), sticky metaphase I in lentil under leaf extract treatment (j), bridge with laggard (→) at anaphase I of lentil under leaf extract (k), multipolar orientation of meiotic chromosomes in grass pea exposed to leaf extract (l), binucleolate conditions (→) in diakinesis of lentil exposed to 40 µM Se (m), unequal 6-7 separation (n) and (o) anaphase bridge with disorganized scattered chromosomes at anaphase I of grass pea at extract + 10 µM Se treatment. Scale I SD = 10 µm

4. CONCLUSION

Ageratum leaf extract treatment at the concentration of 200 mg ml^{-1} induced phytotoxicity in both lentil and grass pea seedlings, with more severe effect on lentil. Severe perturbation in redox status of AsA and GSH as well as activities of SOD, APX, DHAR, GR, CAT and GPX was observed in presence of extract, resulting in oxidative damage. Exogenous application of Se triggered differential responses in two plants, exhibiting growth promoting effect on both crops when used solely at 20 µM but inhibited growth of lentil at 40 µM. Priming of Se in nutrient media following by addition of leaf extract induced oxidative stress and growth inhibition in both crops at 10µM but at 20 µM only in lentil plant. By contrast, leaf extract primed with Se at 20 and 40 µM showed significant stimulation of antioxidant defense components and amelioration of growth in grass pea. In lentil, alleviation of oxidative stress was only evidenced in plants exposed to media with 40 µM Se + extract. The impediment in normal cellular processes due to phytotoxicity of weed extract was also confirmed by disruptions in root-tip mitosis and flower bud meiosis, which was also found at toxic concentrations of Se. Based on responses of different morphological, biochemical and cytogenetic parameters, it can be concluded that *Ageratum* leaf extract has high potential to inflict oxidative damage in both lentil and grass pea but Se can selectively ameliorate this negative impact. Se concentration at 20 µM was found uniformly effective in both plants, while at 10 µM it has no beneficial effect and at 40 µM it was toxic to lentil. In presence of leaf extract, priming of 40 µM was highly beneficial for lentil while both 20 and 40 µM were ameliorative for growth of grass pea.

COMPETING INTERESTS

The author hereby declares that the present work has no competing interests.

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