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REGULAR ARTICLE

Selenium supplementation to lentil (*Lens culinaris* Medik.) under combined heat and drought stress improves photosynthetic ability, antioxidant systems, reproductive function and yield traits

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Abstract

Purpose Increasing temperatures are generating heat and drought stress, especially for the cool-season crops such as lentil,; selenium can mitigate the adverse effects of various abiotic stresses but has never been tested in plants facing combined heat and drought stress.

Methods In this study, contrasting heat-sensitive and heat-tolerant lentil genotypes were grown in the absence (control) or presence of selenium (1, 2.5, 5.0 mg kg^{-1} dry soil). At the onset of flowering, plants were subjected to combined heat and drought stress by moving in a controlled environment [32/20 °C day/night (12/12 h), 50% soil field capacity, 500 µmol m^{-2} s^{-1} light intensity, 65–70% RH] for 20 days, up to completion of maturity stress.

Results The results revealed that lentil plants exposed to the combined stress + Se significantly increased endogenous leaf Se concentration, pod number (32–36% in sensitive, 19–24% in tolerant genotypes), and seed yield (21–35% in sensitive, 21–25% in tolerant genotypes), compared to the combined stress treatment alone. Se supplementation significantly improved leaf water status and osmolyte accumulation (such as proline, glycine betaine, and

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reducing sugars), which stabilized membranes and photosynthesis-related traits, enhanced the expression of various enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and non-enzymatic (ascorbate and reduced glutathione) antioxidants and flower function, and improved pollen function, pod set, pod number, and seed number.

Conclusions Our study showed the potential benefits of using selenium as a supplement in the low-Se soils to protect against combined heat and drought stress in lentil.

Keywords Legumes · Abiotic stress · Water deficit · High temperature · Selenium

Introduction

Selenium (Se), a group VIA metalloid, is present in the soil from 0.1–2.0 mg kg^{-1} depending on the geographical area (Dhillon & Dhillon 2003). Selenium is an considered an essential micronutrient for animals and humans, yet its essentiality in higher plants is ambiguous and unresolved. Se may be an essential micronutrient for plants such as Se-hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* (Galeas et al. 2007). Se is a structural component of some specific selenoproteins (such as glutathione peroxidase) and seleno-tRNAs (Lee et al. 1989). Though the evidence of Se as an essential micronutrient for Se-hyperaccumulators has only been hypothesized, yet definitely not proved. In non-accumulators, Se can exert favorable effects at low concentrations, including increased growth in ryegrass (*Lolium perenne*), lettuce (*Lactuca sativa*), potato (*Solanum tuberosum)*, and buckwheat (*Fagopyrum esculentum;* Hartikainen 2005). Several studies have suggested that Se helps to ameliorate various abiotic stress injuries induced in plants exposed to cold (Chu et al. 2010), drought (Hasanuzzaman and Fujita 2011), high temperature (Djanaguiraman et al. 2010; Iqbal et al. 2015), salinity (Hasanuzzaman et al. 2011; Kaur and Nayyar 2015) by enhancing antioxidants to mitigate oxidative damage. However, there is a lack of information on the effect of Se on plants facing combined stresses.

Lentil (*Lens culinaris* Medik.) is an important cool-season food legume in India, requiring low temperatures during vegetative growth and warm temperatures at maturity (Kumar et al. 2016). The optimum temperatures for growth are 18–30 °C (Sinsawat et al. 2004; Roy et al. 2012). Rising temperatures worldwide due to climate change are detrimental to the growth and yield performance of cool-season crops such as lentil (Prasad et al. 2017). In India, lentil sowings are often postponed due to the delayed harvest of the preceding crop especially in the northern part of India, thus exposing the plants to heat stress (> 30 °C) during later growth. Moreover, in the Indo-Gangetic region, lentil is often grown at comparatively higher temperatures. If sown late, the crop suffers from heat stress at the flowering and seed-filling stages, significantly reducing its yield potential (Tickoo et al. 2005). Across south-eastern Australia, a heat wave (35 °C for six days) in 2009 decreased lentil yields by 70% (Delahunty et al. 2015).

Heat stress is also accompanied by drought stress due to rapid water loss from the soil and plants (Wahid et al. 2007). Consequently, lentil may face the combined effects of heat and drought stress, especially during the reproductive and seed-filling stages, which seriously impacts pod and seed numbers (Sehgal et al. 2017). The susceptibility of lentil to hot and semiarid regions is supported by many studies (Oktem et al. 2008; Barghi et al. 2012; Allahmoradi et al. 2013). The effect of Se supplementation on plants facing combined heat and drought has not been investigated, and thus formed the basis of the current study. We hypothesized that Se supplementation at low doses in soil would impart protection to lentil plants subjected to combined heat and drought stress.

Materials and Methods

1. Plant materials and growth condition

The study involved four lentil genotypes—a heat-tolerant (HT; IG2507), a heat-sensitive (HS; IG2821), a drought-tolerant (DT; DPL 53) [seeds procured from Indian Institute of Pulses Research, India], and a drought-sensitive (DS; LL699) [seeds procured from Punjab Agricultural University, Ludhiana, India]. The phenology is shown in Supplementary Table S1. The contrasting genotypes were selected based on their response to high temperature (tested at 32/20 °C) and drought stress (tested at 50% soil field capacity) in our earlier studies (Sita et al. 2017; Sehgal et al. 2017). The seeds

were sown after inoculation with species-specific rhizobium in pots (7 kg capacity) filled with sandy loam soil (63.4% sand: 24.6% silt: 12% clay) mixed with sand (3:1 ratio). Farmyard manure (FYM; Cattle manure; contain about 3% nitrogen, 2% phosphorus, and 1% potassium; pH 6.5; total organic carbon: 67%; Ca: 36.1 mg kg^{-1} Mg: 13.2 mg kg^{-1} Zn: 11.2 mg kg^{-1}; Cu 2.1 mg kg^{-1} Fe: 34.5 mg kg^{-1} Mn: 83.4 mg kg^{-1}.

(3 (soil:1 (FYM)] and tricalcium phosphate (10 mg kg^{-1} of dry soil) were added at sowing and flowering. The plants were sown in November at Panjab University, Chandigarh, India, in a natural outdoor environment (see Supplementary Fig. S1 for temperature data) until the onset of flowering (101–104 days after sowing). The average temperature from sowing (1 November 2017) to the onset of flowering (9–13 February 2018) was 26/15 °C (day/night), with light intensity ranging from 1300–1550 µmol m^{-2} s^{-1} and mean relative humidity (RH) ranging from 62–65%. For the Se treatment, sodium selenate (1.0, 2.5, or 5.0 mg kg^{-1} dry soil) was thoroughly mixed in the soil prior to sowing; control plants received no Se. The soil used in the experiment contained 0.26 mg kg^{-1} Se. The soil was sandy loam with a pH of 7.1 and available N, P and K at 54, 43 and 158 kg ha^{-1} respectively. The pots were moved to a controlled environment of the growth chamber (500 µmol m^{-2} s^{-1} light intensity, 65–70% RH] at the onset of flowering (101–104 days after sowing). The plants were initially kept at 28/18 °C (day/night, 12/12 h, 1 day) before gradually increasing the temperature (2/1 °C; day/night, per day) to the required level (heat stress; 32/20 °C), to expose them to combined heat and drought stress [32/20 °C day/night (12/12 h). The irrigation was withheld two days prior to heat stress exposure to establish 50% soil field capacity,, which was maintainedfor 20 days, along with heat stress, up to completion of maturity. For drought stress treatment, the soil moisture was measured daily with a probe (Field Scout TDR 300 Probe, Spectrum Technologies, Inc., United States) at 15 cm depth as well as using the gravimetric method, periodically to maintain 50% field capacity. Control plants were placed at 25/15 °C (day/night, 12/12 h, with similar light and RH values) separately in another chamber and maintained under fully irrigated conditions (70% field capacity).

The treatments were as follows:

1. Control
2. Heat + drought stress alone, without Se
3. Heat + drought stress + Se (1.0 mg kg^{-1} dry soil; 1 ppm)
4. Heat + drought stress + Se (2.5 mg kg^{-1} dry soil; 2.5 ppm)
5. Heat + drought stress + Se (5.0 mg kg^{-1} dry soil; 5 ppm)

Se doses of 1, 2 and 5 mg/kg of dry soil indicate Se concentrations, not selenate.

The plants were assessed for various reproductive, physiological, and biochemical traits in the leaves at flowering (10 days after exposure to stress) and seed-filling (17 days after exposure to stress) stages. Phenology was recorded during different growth stages, while yield traits were examined at maturity.

The experiment was performed on four contrasting genotypes (One heat-tolerant, one heat sensitive, one drought sensitive, one drought tolerant) involving five treatments. Each treatment comprised of eight pots per genotype (two plants per pot) in triplicate (24 pots per treatment; 48 plants per treatment). Three pots in triplicate (nine plants per treatment; 18 plants per genotype) were maintained separately for yield trait measurements. The pots were kept following a factorial randomized block design (RBD) in the controlled environment.

Phenology and yield traits

Phenology observations (Supplementary Table S1) were recorded on five plants per genotype per replicate (15 plants per genotype), pooled, and averaged. Mature seeds were harvested for yield data; the seeds were oven-dried at 45 °C for three days, weighed, and average values per plant recorded.

Endogenous selenium concentration

The endogenous Se concentration in leaves, collected from the control and stressed plants, was measured using the spectrophotometric method of Revanasiddappa and Kumar (2002). The leaf samples were digested with 10 ml nitric oxide (10 ml) for 20 min, cooled for some time, subsequently, 0.5 ml perchloric acid was added, and the mixture was heated again for another 10 min, followed by cooling. To the cooled residue, 10 ml water and 5 ml HCl were added; the

mixture was boiled for 10 min to convert Se (VI) to Se (IV). The solution was neutralized with diluted NaOH, and further diluted to 50 ml by adding 5 ml EDTA (5%). The mixture (3 ml) was analyzed for Selenium by addition of 1% KI and 1 ml HCL (1 mol L^{-1}) followed by gentle shaking until the appearance of yellow color, indicating the production of iodine. To this mixture, 0.5 ml thionin (0.01%) was added accompanied by shaking for 2 min; the absorbance of the resulting solution was measured at 600 nm against distilled water. Selenium concentration was measured using a calibration graph. The recovery of Se in plant samples accoding to this method is 99.8%.

Stress injury

Membrane damage (as electrolyte leakage)

The damage to leaf tissues was assessed on the basis of membrane injruy (as electrolyte leakage) and cellular viability [as TTC (2, 3, 5-triphenyl tetrazolium chloride) reduction test]. Fresh leaves (young, second-to-third node from the top) growing below the flowers were collected and excised into segments to measure electrolyte leakage (Lutts et al. 1996) and cellular viability (Steponkus and Lanphear 1967), as detailed previously (Kaushal et al. 2013).

Oxidative molecules and antioxidants

The oxidative damage was measured in terms of malondialdehyde (MDA; Heath and Packer 1968) and hydrogen peroxide (H_2O_2 concentration; Mukherjee and Choudhuri 1983) from the fresh leaves, as explained in detail in our previous study (Sita et al.2017).

For assaying superoxide dismutase (SOD; E.C. 1.15.1.1) activity from fresh leaf tissue, the extraction was done in a pre-cooled phosphate buffer (50 mM; pH 7.0), followed by centrifugation (3360 g) at 4 °C for 5 min. The supernatant was tested for enzyme activity according to the method of Dhindsa and Matowe (1981), which was expressed as Units mg^{-1} protein. Catalase activity (CAT; E.C. 1.11.1.6) was assayed as per Teranishi et al. (1974). The enzyme extract prepared for assaying SOD activity was also used for CAT activity. To the reaction mixture [enzyme extract (0.1 mL) and phosphate buffer (pH 7.0; 50 mM)], H_2O_2 (200 mM) was added to start the reaction. The optical density (at 410 nm) was read for 3 min. The activity of ascorbate peroxidase (APX; E.C. 1.11.1.11) was assayed (Nakano and Asada, 1981) from the same enzyme extract, which was prepared for SOD assay. The enzyme activity was measured as the decline in absorbance at 290 nm by recording the oxidation of ascorbate. Glutathione reductase was assayed from the same enzyme extract following the method of Mavis and Stellwagen (1968), as explanined earlier (Awasthi et al. 2014).

Ascorbic acid was estimated according to the method of Mukherji and Chaudhari (1983). Plant tissue was homogenized in 6% TCA, and the homogenate was centrifuged at 3,649.15 g for 15 min. The supernatant was used as an extract for estimation as detailed previously (Awasthi et al. 2014). Reduced glutathione (GSH) was estimated following the method of Griffith (1980). Fresh leaf tissue was homogenized in 2 mL of metaphosphoric acid, centrifuged for 15 min at 14,539.59 g. The aliquots of the supernatant were neutralized by putting 0.6 mL of 10% sodium citrate to 0.9 mL of the extract and measured for GSH concentration, as explained earlier (Awasthi et al. 2014).

Leaf water status

Relative leaf water content (RLWC) was measured to assess leaf water status (Barrs and Weatherley 1962). Fresh leaves subtending flowers were collected, floated in a Petri dish containing distilled water for 2 h, followed by surface-drying with filter paper. The leaves were weighed again (turgid weight, TW), oven-dried at 110 °C for 24 h, and re-weighed (dry weight, DW). RLWC (%) was calculated as $(FW-DW)/(TW-DW)\times 100$. The stomatal conductance (g_s) of leaves below the flowers was measured with a portable leaf porometer (Decagon Devices, USA) (Kaushal et al. 2013).

Osmolytes

The proline concentration was assessed from oven-fresh leaf tissue was dried in hot air oven at 60 °C, which was extracted for proline measurement using 3% sulphosalicylic acid, and centrifuged at 2,150 g for 20 min. The supernatant was treated with an acidic ninhydrin reagent, and absorbance was read at 520 nm. Toluene was used as a blank (Bates et al.

1973). For measuring glycine betaine concentration, leaf tissue was oven-dried and crushed to a fine powder before adding 20 mL deionized water and shaking at 25 °C for 24 h. The extract was diluted (1:1) with 2 N H_2SO_4 to measure glycine betaine concentration using spectrophotometer at 365 nm (Grieve and Grattan 1983). The concentration was reducing sugars was estimated according to the method of Sumner and Howell (1935), as explained previously (Awasthi et al. 2014).

The soluble proteins were estimated from the oven-dried leaves were homogenized in 0.1 M phosphate buffer (pH 7.0), followed by centrifugation at 514 g for 15 min (Sita et al. 2017). The concentration of soluble proteins was quantified following the method of Lowry et al. (1951).

Photosynthetic ability

Chlorophyll fluorescence (Fv/Fm ratio) was measured on young leaves (2^{nd} and 3^{rd} nodes), below the flowers, using a chlorophyll fluorometer OS1-FL (Opti-Sciences, Tyngsboro, MA, USA) (Kaushal et al. 2013). Chlorophyll from fresh leaves (500 mg) was extracted using 80% acetone and centrifuged at 5,702 g. The absorbance of the supernatant was read in a spectrophotometer at 645 and 663 nm (Arnon 1949). For assaying the RuBisCo activity (EC: 4.1.1.39), fresh young leaf tissue was homogenized in a pre-chilled pestle and mortar kept in ice, using extraction buffer (50 mM BIS–TRIS-propane (BTP), pH 7.0) containing 2-mercaptobenzothiazole (MBT, 3 mM), polyvinylpyrrolidone (1.5%), benzamidine (1 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), dithiothreitol (DTT, 10 mM), adenosine 5'-triphosphate (0.5 mM), ethylenediamine tetraacetic acid (EDTA, 1 mM), and $MgCl_2$ (10 mM), $NaHCO_3$ (10 mM), as per the method of Wang et al. (1992). The activity was assayed according to the method of (Racker 1962), as explained previously (Awasthi et al.2014). Sucrose concentration was measured from the fresh leaves by extracting them two times in 80% ethanol for 1.5 h at 80 °C; the supernatant was pooled before being evaporated in an oven (air-circulating) at 40 °C and tested for sucrose concentration (Jones et al. 1977; Kaushal et al. 2013).

Reproductive function

Flowers were collected for analyzing the reproductive function from all treatments after 7 days of exposure to control and stress environments. Pollen grain germination was assessed in a growth medium containing potassium nitrate (990 mM; pH 6.5), calcium nitrate (1,269 mM), magnesium sulfate (812 mM), sucrose (10%), and boric acid (1,640 mM) (Brewbaker and Kwack 1963; Kaushal et al. 2013). Pollen grains were considered germinated when pollen tube length exceeded pollen grain diameter. Germination was measured on about 100 pollen grains per replicate.

Pollen viability was tested using 0.5% acetocarmine on about 200 pollen grains per genotype per treatment in five microscopic fields (Kaushal et al. 2013). Pollen grains were collected from flowers on the day of anthesis; the replicates were combined and examined for viability (Alexander 1969). Pollen viability was measured according to pollen grain size, shape (triangular or spherical), and color intensity. A dense color indicates higher pollen viability (Kaushal et al. 2013).

Stigma receptivity was examined using the esterase test, following Mattison et al. (1974). One day prior to flower opening, stigmas were harvested from flowers and placed at 37 °C for 15 min in a solution containing α-NAA and fast blue B prepared in phosphate buffer (0.1 M, pH 7). Stigmas develop colors of varying intensity depending on their receptivity, which was rated on a 1–5 scale (5-high receptivity, 1-low receptivity) (Kaushal et al. 2013).

Ovule viability was tested from the ovules were harvested from the ovary of flowers one day before anthesis, placed on a slide containing a few drops of TTC solution (0.5% TTC in 1% sucrose solution), The ovules were tested for viability based on the intensity of the red stain, particularly in the center. The color intensity indicates the respiring ability of the ovules and is rated on a 1–5 scale (5-highest intensity, 1-lowest intensity) (Kaushal et al. 2013).

Proximate analysis

Mature seeds of control and stressed plants were analyzed for various seed reserves. Soluble sugars and starch were extracted with 95% (v/v) ethanol and 30% (v/v) perchloric acid, respectively, and quantified

with the phenol/sulfuric acid method of Dubois et al. (1956) using glucose as a standard. The crude protein, ash (micro-Kjeldahl, N × 6.25), crude fat, crude fiber, and minerals were determined using standard AOAC procedures.

Statistical analysis

All the traits were analysed in 3 replicates. ANOVA (Genotypes x treatment x stages interaction) was conducted using Agristat software, and least significant values (LSD) values calculated ($P < 0.05$). Tukey's post-hoc test was used to compare the means.

Results

Among all the Se concnetraions, 2.5 mg kg^{-1} (2.5 ppm) Se yielded the best results for all traits in stressed lentil plants. Hence, the results presented below mostly pertain to that treatment.

Phenology

Flowering (appearance of buds) occurred at 103–104 days in tolerant and 101–102 days in sensitive genotypes (Supplementary Table S1). Podding (days to podding; DP) in control plants occurred at 121–122 and 120–121 days in the tolerant and sensitive genotypes, respectively (Supplementary Table S1). The combined heat and drought (H + D) stress treatment alone decreased DP to 117–118 days in tolerant and 113–114 days in sensitive genotypes. Podding in the combined stress + 2.5 ppm Se treatment occurred at 119 days in tolerant and 117–118 days in sensitive genotypes.

Maturity (days to maturity; DM) in control plants occurred at 141–142 days and 140 days in the tolerant and sensitive genotypes, respectively (Supplementary Table S1). The combined stress treatment alone decreased DM to 137–138 days in tolerant and 129–131 days in sensitive genotypes, increasing with Se (2.5 ppm) to 139–140 days in tolerant and 136–137 days in sensitive genotypes.

The flowering–podding duration in control plants was 17–19 and 19 days in the tolerant and sensitive genotypes, respectively (Supplementary Table S1). The combined stress treatment alone reduced this duration to 13–14 days in tolerant and 11–12 days in sensitive genotypes, impacting pod production. Se treatment (2.5 ppm) to stressed plants significantly increased the duration to 16 days in sensitive genotypes and 15–16 days in tolerant genotypes, compared to the combined stress treatment alone.

The podding–maturity duration in control plants ranged from 19–21 and 19–20 days in tolerant and sensitive genotypes, respectively (Supplementary Table S1). The combined stress treatment alone decreased the duration to 16–17 in tolerant and 12–13 days in sensitive genotypes. Se applied at 2.5 ppm concentration increased this duration to 19 days in sensitive genotypes, nearly reaching control plants, but did not significantly affect the tolerant genotypes, compared to the combined stress treatment alone.

Endogenous selenium

The soil used in the experiment contained 0.26 mg kg^{-1} Se. In control plants, the leaves of tolerant genotypes at the flowering (FL) and seed-filling (SF) stages had 13.4–18.4 and 18.4–19.4 μg g^{-1} Se and those of sensitive genotypes accumulated 16.3–18.4 and 17.5–20.4 μg g^{-1} Se, respectively (Fig. 1). The combined stress alone decreased leaf Se concentration by 32–37% and 42–53% (FL stage) and 46–53% and 53–56% (SF stage) in tolerant and sensitive genotypes, respectively, relative to the controls. The combined stress + Se supplementation (1, 2.5, and 5 mg kg^{-1} DW of soil) resulted in a manifold increase in leaf Se accumulation in all genotypes in a concentration-dependent manner.

Stress injury

Leaf injury from the combined stress was detected as membrane damage (using electrolyte leakage; EL; expressed as a percentage; Fig. 2A) and ranged 10.4–11.4% (FL stage) and 10.9–13.4% (SF stage). The combined stress treatment alone increased membrane damage to 23.7–24.1% and 17.8–19.3% (FL stage) and 25.2–27.4% and 20.4–21.3% (SF stage) in the sensitive and tolerant genotypes, respectively. With Se treatment (2.5 ppm), EL was significantly reduced, relative to the combined stress treatment alone, more so in sensitive genotypes than tolerant genotypes.

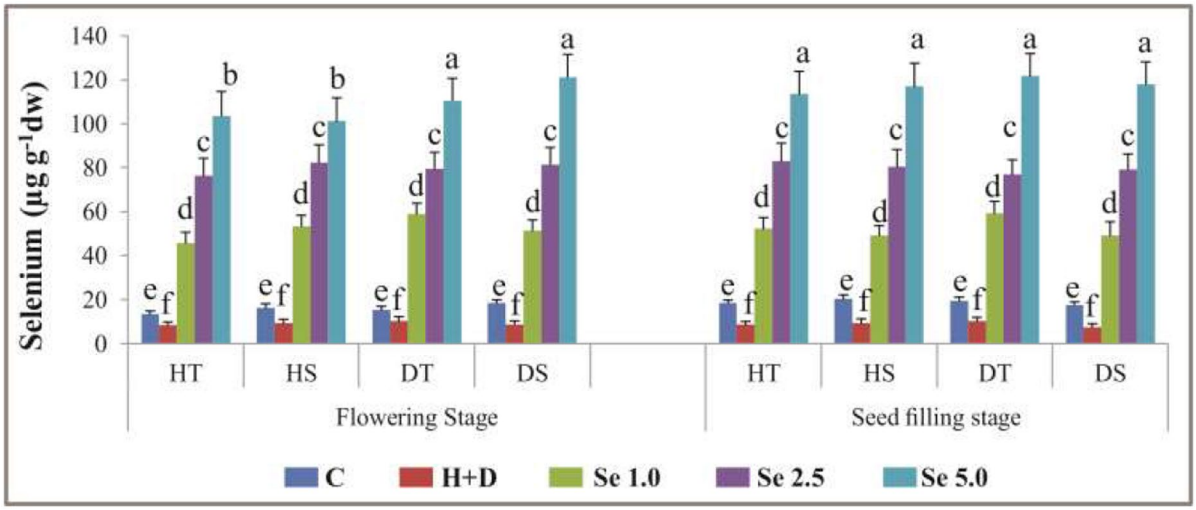

Fig. 1 Endogenous selenium in leaves of heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT), and drought-sensitive (DS) lentil genotypes in control (C), heat + drought stressed (H + D), and Se (1, 2.5, and 5 ppm) treatments at the flowering and seed-filling stages. Small vertical bars represent standard errors (n = 3). Different small letters on the bars indicate significant differences between treatments ($P < 0.05$). Tukey's post-hoc test was used to compare the means. Different small letters on the bars indicate significant differences between treatments ($p < 0.05$)

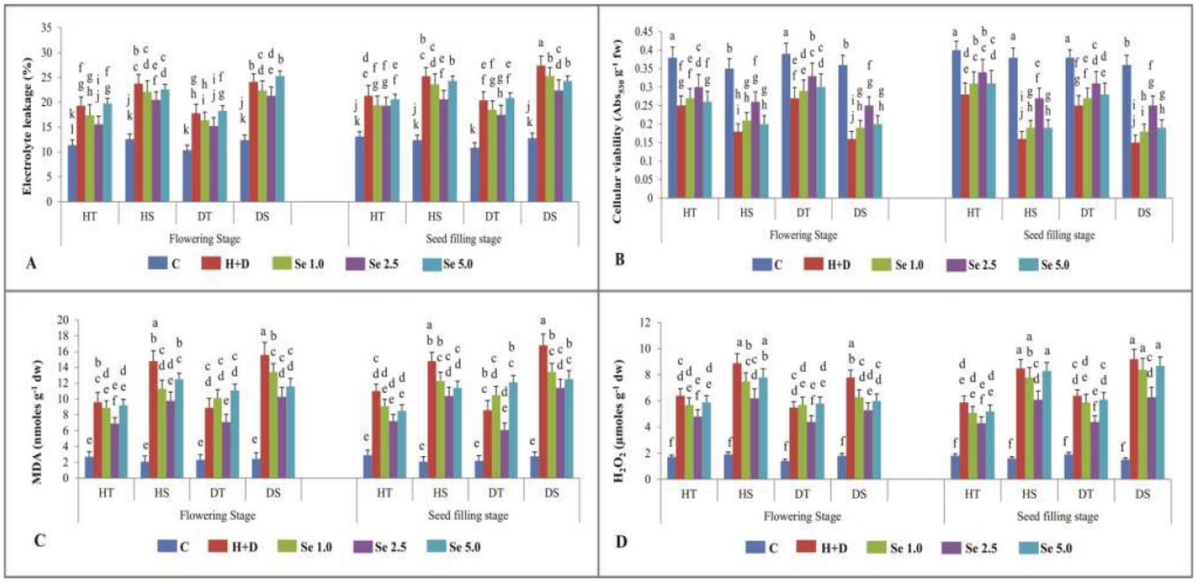

Fig. 2 Membrane damage (A), cellular viability (B), malondialdehyde (C), and hydrogen peroxide (D) concentration in leaves of heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT), and drought-sensitive (DS) lentil genotypes in control (C), heat + drought stressed (H + D), and Se (1, 2.5, and 5 ppm) treatments at the flowering and seed-filling stages. Small vertical bars represent standard errors (n = 3). Different small letters on the bars indicate significant differences between treatments ($P < 0.05$). Tukey's post-hoc test was used to compare the means

Another trait used to assess tissue damage was cellular viability (CV; Fig. 2B), which reflects the oxidizing ability of cells. Compared to the control, the combined stress treatment alone decreased CV by 48–55% and 30–34% (FL stage) and 58–59% and 31–34% (SF stage) in sensitive and tolerant genotypes, respectively. Se (2.5 ppm) supplementation remarkably improved CV, compared to the combined stress treatment alone, which was to a significantly higher extent in sensitive genotypes than tolerant genotypes.

Oxidative stress

Oxidative stress, assessed in terms of malondialdehyde (MDA; Fig. 2C) and hydrogen peroxide (H_2O_2; Fig. 2D) concentrations, is another indicator of tissue damage under stressful situations (Fig. 2C). MDA and Hydrogen peroxide concentration increased markedly in stressed plants, particularly in sensitive genotypes, relative to the controls. The combined stress + 2.5 ppm Se decreased the concentrations of both these molecules appreciably, compared to the combined stress treatment alone.

Enzymatic antioxidants

Superoxide dismutase (SOD; Fig. 3A) Catalase (CAT) activity (Fig. 3B) activity, in stressed plants decreased substantially in both categories of genotypes relative to the controls. Se at 2.5 ppm applied to the stressed plants significantly enhanced SOD and CATactivity, to a larger level in sensitive genotypes than tolerant genotypes, in comparison to the combined stress treatment alone.

Ascorbate peroxidase (APX;3C) and and Glutathione reductase (GR; (Fig. 3D) activity decreased by 32–34% and 30–40% (FL stage) and 37–38% and 35–38% (SF stage) in sensitive genotypes but increased APX and GR activity by 33–36% and 17–21% (FL stage) and 34–36% and 10% (SF stage) in tolerant genotypes, respectively relative to the controls. Se at 2.5 ppm resulted in significant improvement in APX and GR in both sensitive and tolerant genotypes, respectively, compared to the combined stress treatment alone.

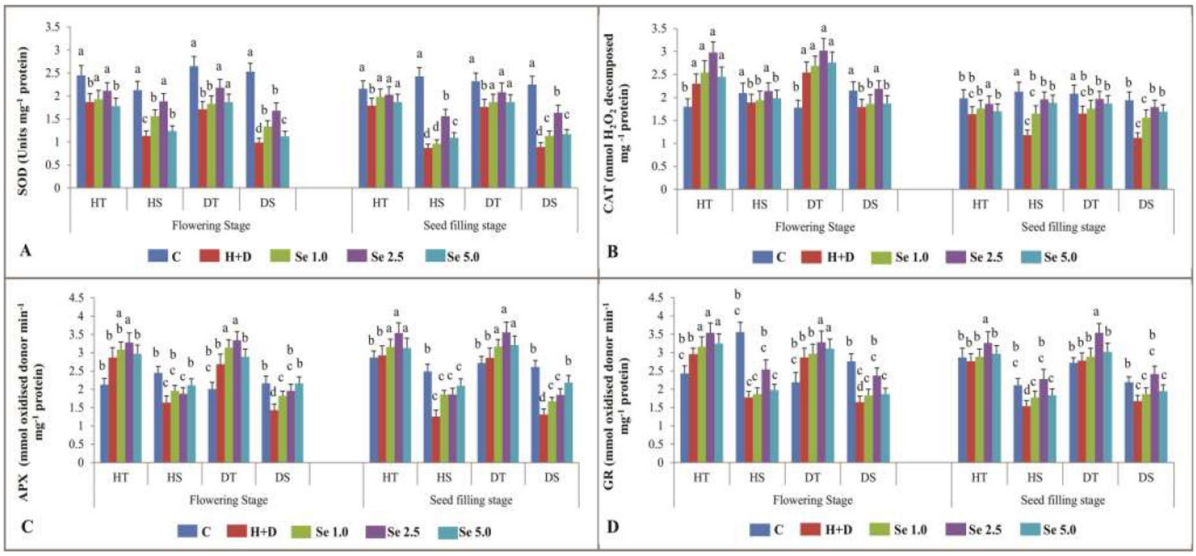

Fig. 3 Superoxide dismutase (SOD;A), catalase (CAT; B), ascorbate peroxidase (APX; C), and gluathione reductase (GR; D) activity in leaves of heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT), and drought-sensitive (DS) lentil genotypes in control (C), heat + drought stressed (H + D), and Se (1, 2.5, and 5 ppm) treatments at the flowering and seed-filling stages. Small vertical bars represent standard errors (n = 3 Different small letters on the bars indicate significant differences between treatments ($P < 0.05$). Tukey's post-hoc test was used to compare the means

Non-enzymatic antioxidants

As a result of stress, the ascorbic acid (Asc; Supplementary Fig. S2A) decreased in sensitive genotypes (12–26%, FL stage; 18–19%, SF stage) but increased significantly in tolerant genotypes (27–37%. FL stage; 17–21%, SF stage), relative to the controls. Se applied at 2.5 ppm concentration to stressed plants significantly increased Asc concentration, more so in sensitive genotypes (23–25%, FL stage; 23–32%, SF stage) than tolerant genotypes (11–19%, FL stage; 14–19%, SF stage), compared to the combined stress alone. Likewise, reduced glutathione (GSH; Supplementary Fig. S2B concentration with 2.5 ppm Se increased more in sensitive genotypes (55–61%, FL stage and 67–74%, SF stage) than tolerant genotypes (23–26%, FL stage) and 21–28%, SF stage), compared to the combined stress treatment alone.

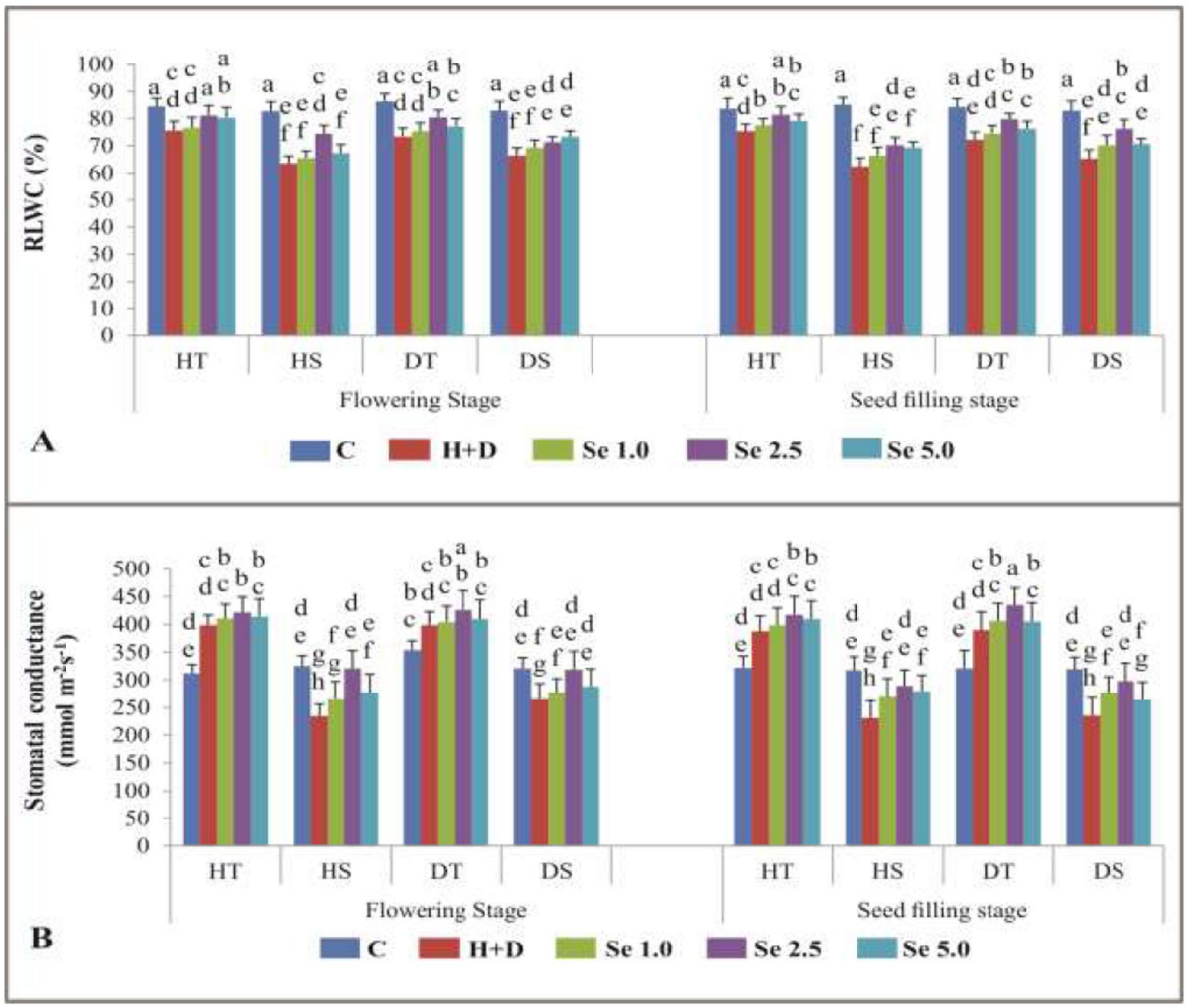

Fig. 4 Relative leaf water content (RLWC; A) and stomatal conductance (B) in leaves of heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT), and drought-sensitive (DS) lentil genotypes in control (C), heat + drought stressed (H + D), and Se (1, 2.5, and 5 ppm) treatments at the flowering and seed-filling stages. Small vertical bars represent standard errors (n = 3). Different small letters on the bars indicate significant differences between treatments ($P < 0.05$). Tukey's post-hoc test was used to compare the means

Leaf water status

Leaf water status (relative leaf water content; RLWC) was expressed in percentage (Fig. 4A), which ranged from 82.9–86.4% (FL stage) and 82.9–85.3% (SF stage) in control plants. The combined stress treatment alone decreased RLWC, more so in sensitive genotypes (63.5–66.5%, FL stage; 62.4–75.3%, SF stage) than tolerant genotypes (73.5–75.6%, FL stage; 72.3–75.3%, SF stage). Se application at 2.5 ppm to stressed plants significantly enhanced RLWC to 71.3–74.5% and 80.6–81.3% (FL stage) and 70.3–76.4% and 79.8–81.4% (SF stage) in sensitive and tolerant genotypes, respectively, compared to the combined stress treatment alone.

Stomatal conductance (g_S) values (Fig. 4B) declined markedly due to stress in sensitive genotypes but increased significantly in tolerant genotypes, relative to the controls. Se (2.5 ppm) increased g_S by 20–37% and 5–7% (FL stage) and about 25% and 8–11% (SF stage) in sensitive and tolerant genotypes, respectively, compared to the combined stress treatment alone.

Osmolytes

Proline concentration (Supplementary Fig. S3A) in stressed plants increased remarkably, particularly in tolerant genotypes (87–92%, FL stage; 72–78%, SF stage) than sensitive genotypes (about 38%, FL stage; 20–27%, SF stage), relative to the controls. Se applied to the stressed plants further increased proline concentrations in both categories of genotypes, compared to the combined stress alone.

Glycine betaine (GB; Supplementary Fig. S3B) markedly increased in stressed plants, to a larger extent in tolerant than sensitive genotypes, relative to the controls. Se applied at 2.5 ppm increased GB by 33–38% and 26–38% (FL stage) and 31–45% and 29–41% (SF stage) in sensitive and tolerant genotypes, compared to the combined stress alone.

Reducing sugars (glucose and fructose) (Supplementary Fig. S3C) increased noticeably due to stress; the tolerant genotypes showed more increase than sensitive genotypes, relative to the controls. In the presence of Se, the stressed plants showed further increase in reducing sugars, to a higher level in sensitive genotypes than tolerant genotypes, compared to the combined stress alone.

Photosynthetic ability

Photosynthetic function was based on traits such as chlorophyll (Chl) concentration (Supplementary Fig. S4A), photosystem II (PSII; Supplementary Fig. S4B) function, activity of carbon fixing enzyme (RuBisCo; Supplementary Fig. S4C), and sucrose concentration (Supplementary Fig. S4D). The Chl concentration in stressed plants, compared to control plants, decreased noticeably, more so in sensitive genotypes than tolerant genotypes, at both the stages, relative to the controls. Treatment of stressed plants with 2.5 ppm Se significantly increased Chl concentrations, which was significantly more in sensitive genotypes (17–26%, FL stage; 39–42%, SF stage) than tolerant genotypes (13–21%, FL stage; 17–27%, SF stage), compared to the combined stress alone.

PSII function (Supplementary Fig. S4B) was severely inhibited, much more in sensitive genotypes than tolerant genotypes at both the stages. In the presence of Se (2.5 ppm), PSII function showed significant enhancement (19–27% and 12–16% (FL stage) and 10–17% and 12–14% (SF stage) in sensitive and tolerant genotypes, respectively, compared to the combined stress treatment alone.

The combined stress treatment drastically reduced RuBisCo activity (Supplementary Fig. S4C;); the sensitive genotypes showed more inhibition (49–54%, FL stage; 49–51%, SF stage) than tolerant genotypes (22–26%, FL stage; 15–24%, SF stage), relative to the controls. Se-treated (2.5 ppm) plants recovered RuBisCo activity significantly; the impact was more in sensitive genotypes at both the stages than tolerant genotypes, compared with the combined stress alone.

Sucrose concentration (Supplementary Fig. S4D) substantially decreased more in stressed plants of sensitive genotypes (46–50%, FL stage; 48–50%, SF stage) than tolerant genotypes (17–26%, FL stage; 26–29%, SF stage), relative to the controls. When Se was supplemented to the stressed plants, a significant improvement was noticed in sucrose concentration, compared to the combined stress alone; the sensitive genotypes were more responsive than tolerant genotypes.

Reproductive function

Reproductive function was based on pollen germination and viability traits, stigmatic function, and ovular

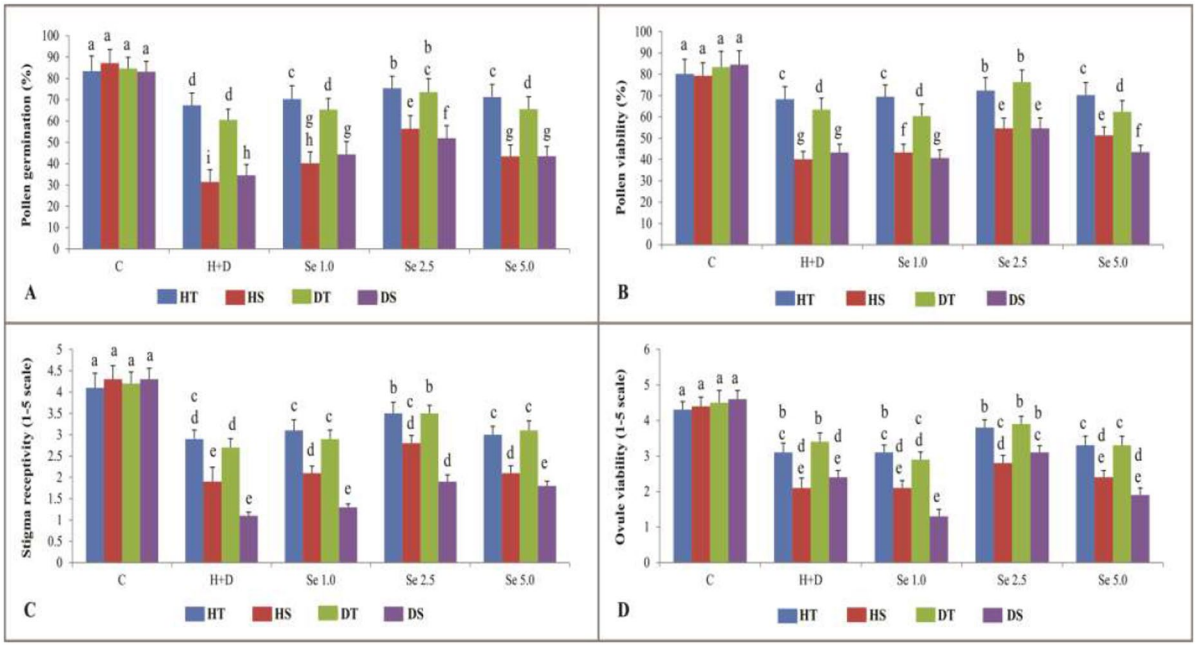

Fig. 5 Pollen germination (A), pollen viability (B), stigma receptivity (C), and ovule viability (D) in leaves of heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT), and drought-sensitive (DS) lentil genotypes in control (C), heat + drought stressed (H + D), and Se (1, 2.5, and 5 ppm) treatments at the flowering and seed-filling stages. Small vertical bars represent standard errors (n = 3). Different small letters on the bars indicate significant differences between treatments ($P < 0.05$). Tukey's post-hoc test was used to compare the means

function. Pollen germination (Fig. 5A) and pollen viability (Fig. 5B) were severely inhibited in stressed plants, compared to control plants, which was remarkably improved in plants growing with Se (2.5 ppm).

Stigmatic (Fig. 5C) and ovular function (Fig. 5D) was assessed on 1–5 scale (visual scoring; Fig. 5D); both these traits were substantially reduced as a result of stress, noticeably in sensitive genotypes, compared to tolerant genotypes. When the plants were grown in the presence of Se (2.5 ppm) stigmatic and ovule function improved by 47–72% and 33–39% in sensitive genotypes and 20–39% and 14–22% in tolerant genotypes, respectively, compared to the combined stress treatment alone.

Seed composition

The combined stress alone decreased seed starch (Supplementary Fig. S5A) concentrations by 37–51% in sensitive genotypes and 16–17% in tolerant genotypes, relative to the controls. In Se treated plants (2.5 pap), a significant improvement was noticed in seed starch concentration (22–44% in sensitive genotypes and 11–13% in tolerant genotypes, compared to the combined stress alone.

At the same time, seed protein concentration (Supplementary Fig. S5B) decreased by 36–42% in sensitive genotypes and 18–25% in tolerant genotypes relative to the controls. Se (2.5 ppm) improved seed protein concentrations, more so in sensitive genotypes (20–28%) than tolerant genotypes (12–14%), compared to the combined stress alone.

The combined stress treatment alone decreased seed fat (Supplementary Fig. S5C) concentration by 54–57% in sensitive genotypes and 22–28% in tolerant genotypes, relative to the controls. With Se supplementation (2.5 ppm), seed fat showed 47–52% increase in sensitive and about 14% in tolerant genotypes, in contrast to the stressed plants alone.

Growth and yield

Stress resulted in 52–57% and 39–40% reduction in biomass (Fig. 6A) over control in sensitive and

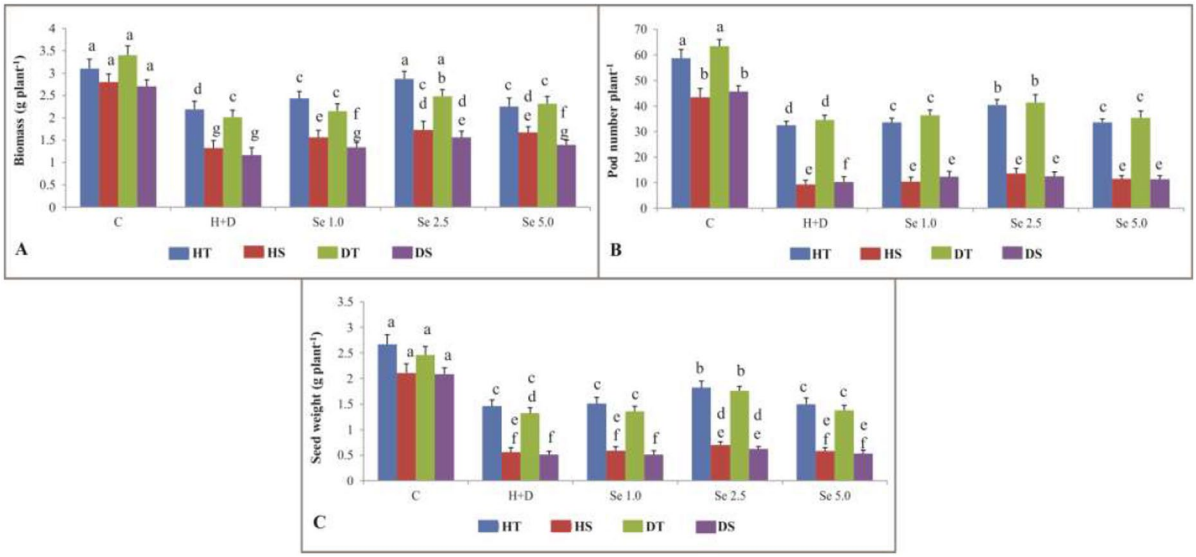

Fig. 6 Biomass (A), pod number(B), and seed weight per plant in heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT), and drought-sensitive (DS) lentil genotypes in control (C), heat + drought stressed (H + D), and Se (1, 2.5, and 5 ppm) treatments at the flowering and seed-filling stages. Small vertical bars represent standard errors (n = 3). Different small letters on the bars indicate significant differences between treatments ($P < 0.05$). Tukey's post-hoc test was used to compare the means

tolerant genotypes, respectively. With Se treatment to the stressed plants, the biomass recovered by 31–34% in sensitive and 23–25% in tolerant genotypes, compared to stress treatment alone,

Stress reduced the pod number per plant (Fig. 6B) by 76–80% in sensitive and 44–45% in tolerant genotypes, which was increased noticeably with Se (2.5 ppm) treatment to stressed plants; 32–36% in sensitive genotypes and 19–24% in tolerant genotypes, in comparison to the combined stress treatment alone.

Seed yield (Fig. 6C) in stressed plants declined by 73–75% in sensitive genotypes and 45–46% in tolerant genotypes, relative to the controls. A marked improvement in seed yield was noticed in stressed plants growing in the presence of Se (2.5 ppm). The sensitive and tolerant genotypes showed 21–35% and 21–25% recovery in seed yield with 2.5 ppm Se.

Discussion

The combined stress markedly reduced the growth (as biomass) and yield traits (pods and seeds), which was associated with marked reduction in Se accumulation in leaves, compared to the stressed lentil plants supplemented with Se. The combined stresses may disrupt Se uptake and its subsequent accumulation possibly as a result of reduced root hydraulic conductivity and stomatal conductance (Morales et al., 2003; Sehgal et al. 2017). Se supplementation through soil drenching to stressed lentil plants markedly enhanced the endogenous leaf Se concentrations, in contrast to stressed plants growing without Se. It was noticed that the control plants (- Se) also showed high endogenous Se concentration in leaves, which probably occurred due to volatile transfer of Se to these plants from + Se plants since both sets of plants were maintained in the same growth chamber, unlike in studies by Lyons et al. (2009) on *Brassica*, where plants were grown in the absence or presence of Se were kept in separate growth rooms. However, the endogenous levels in Se-supplemetned lentil plants were remarkably more than the control plants; the rates of Se volatilization in lentil remains to be investigated.

Our study indicated that the Se treatment, particularly at 2.5 ppm, was beneficial on stressed lentil plants, resulting in significant improvements in pod number and seed yield per plant due to increased vegetative and reproductive growth. The enhanced pod

numbers with Se treatment to stressed plants occurred as a result of maintenance of reproductive function, ensuring better pod set. Lentil has been found to be responsive to Se application, as shown in previous studies demonstrating increase in yield (Thavarajah et al. 2015) and Se-enrichment of seeds (Rahman et al. 2014).

Stress resulted in a notable reduction in leaf water status occurring likely due to disruption in root hydraulic conductivity (Morales et al., 2003) and inhibited stomatal conductance (Sehgal et al. 2017) coupled with soil moisture depletion. At the same time, reduction in osmolytes (such as proline, glycine betaine, and sugars) in stressed plants also decreased the turgor. Besides their involvement in turgor, these molecules have several functions, including serving as partial antioxidants in stressed plants (Burg and Ferraris 2008); their declining levels because of stress could be extremely detrimental in stress defense. Studies have reported increase in osmolytes in tomato (Qaseem et al. 2019) but decrease in chickpea (Awasthi et al. 2014) plants subjected to combined heat and drought stress, which was linked to an increase in stress injury at various tissue and cellular levels. Thus, the reduction in leaf turgor could disrupt leaf and flower function, as observed in the current study and other studies in wheat (Jiang et al. 2010; Qaseem et al. 2019), tomato (Zhou et al. 2019), lentil (Sehgal et al. 2019) and cotton (Carmo-Silva et al. 2012) plants exposed to combined heat and drought stress. Se supplementation resulted in marked increase in leaf turgor, possibly due to improved stomatal conductance and facilitated osmolyte accumulation, which stabilized leaf and flower function. Improved leaf water status with Se treatment has also been reported in drought-stressed wheat (Rady et al. 2020) plants.

Stress resulted in increase in membrane injury and reduction in cellular viability in lentil plants, which might have occurred due to reduction in leaf turgor and manifold increase in oxidative damage (i.e., MDA and H_2O_2 concentrations). MDA accumulation is a sign of lipid peroxidation, a commonly used stress indicator for membrane damage (Taulavuori et al., 2001). MDA levels increase in various crops subjected to combined stresses, including lentil (Sehgal et al. 2017), chickpea (Awasthi et al. 2017) causing tissue damage. Moreover, the direct impact of high temperature on leaf tissue, resulting in dehydration due to increased transpiration and reduced water availability, might also damage membranes (Hussain et al. 2019). Cellular viability, a measure of respiratory ability, is a useful trait in thermotolerance (Porter et al. 1994); its decline in stressed lentil plants indicated the inhibition of respiratory pathways, more so in sensitive than tolerant genotypes, which contributed to a greater degree of growth inhibition. The enhanced leaf water status in Se-treated plants significantly decreased damage to membranes and tissue viability, and are similar to the findings in heat-stressed tobacco cell cultures (Malerba and Cerana 2018) and drought-stressed strawberry plants (Zahedi et al. 2020). At the same time, Se remarkably reduced oxidative stress (in terms of MDA and H_2O_2 concentration), which could be related to the augmented expression of various enzymatic and non-enzymatic antioxidants by Se in our study and have similarity to findings in heat-stressed wheat (Iqbal et al. 2015) and sorghum (Djanaguiraman et al. 2018) and drought-stressed and wheat (Rady et al. 2020).

The photosynthetic ability of stressed lentil plants declined markedly due to a significant reduction in stomatal conductance coupled with the loss of chlorophyll, chlorophyll fluorescence and RuBisCo activity, as observed in wheat (Balla et al. 2006) and, tomato (Nankishore and Farell 2016) plants exposed to combined heat and drought stress. Severe reductions in chlorophyll under the combined stress could be related to the disorganization of chloroplasts, as observed in lentil (Sita et al. 2017), and/or increased oxidative damage (Rossi et al., 2017a, b), which disrupted the photochemical and biochemical reactions of photosynthesis. Consequently, sucrose concentration declined markedly in stressed leaves, impacting vegetative and reproductive growth (Aluko et al. 2021). Se supplementation improved Chl and chlorophyll fluorescence, which could be attributed to maintaining better leaf water status and minimizing oxidative damage that prevented chlorosis to stabilize RuBisCo activity and sucrose production. Recent studies have also shown stimulatory effects of Se treatment on photosynthetic efficiency in plants of heat-stressed cucumber (Shalaby et al. 2021), and drought-stressed wheat (Rady et al. 2020).

Stress resulted in a marked inhibition in reproductive function (poor pollen germination, viability, failure of pollen to germinate, impaired stigmatic and ovular activity) in lentil plants, decreasing the number

of pods and seeds. Previous studies have shown that combined heat and drought stress is highly detrimental to reproductive function in plants, relative to their individual impacts, as reported in lentil (Sehgal et al. 2017), wheat (Fábián et al.2019) and rice (Da Costa et al. 2021), which has been attributed to increases in reactive oxygen species, reactive nitrogen species and inhibited NO (nitric oxide) production (Fábián et al. 2019). The reproductive function was primarily disrupted due to the decrease in leaf water status inhibiting sucrose production and its translocation to flowers, as reported previously in lentil (Sehgal et al. 2017) and other crops, such as chickpea (Awasthi et al. 2014), rice (Jagadish et al. 2010), and maize (Hussain et al. 2019), treated with combined heat and drought stress. Moreover, the combined stress might have directly damaged flowers and their components due to heat and dehydration (as in tomato; Zhou et al. 2016), impairing developmental and functional aspects in lentil. Se-treated stressed lentil plants showed improved leaf function (in terms of water status and photosynthetic ability), which alleviated the damage to reproductive function, as reflected in the enhanced pollen germination, viability, stigmatic and ovular function resulting in less damage to pods and seeds. Consequently, the yield traits (pods and seeds) in lentil were markedly improved with Se enrichment in combined stressed environment. Previous studies have reported improved yield-related traits in heat-stressed cucumber (Shalaby et al. 2021), wheat (Iqbal et al. 2015) and drought-stressed wheat (Teimouri et al. 2014; Rady et al. 2020) with Se treatment. The present study is the first study demonstrating the beneficial effects of Se supplementation to combined heat and drought stressed lentil plants.

Conclusion

The present study revealed that Se addition to the soil of combined heat and drought stressed plants was markedly effective in alleviating the damage to leaves and flowers. Se acted through improving the leaf water status, associated to enhanced stomatal conductance and osmolytes' accumulation. At the same time, Se reduced the oxidative damage to leaf tissue, by remarkably enhancing the expression of antioxidants. Consequently, the photosynthetic ability in terms of chlorophyll retention, RuBisCo activity and PS II function was effectively maintained under stress environment ensuing sustained sucrose production. Thus, Se could improve the leaf functional status under combined stress environment, which contributed to maintenance of flower function thus promoting the production of pods and seeds.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate The authors declare consent to participate.

Consent for publication The authors declare consent for publication.

Conflict of interest The authors declare no competing interests.

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