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Effects of individual and combined heat and drought stress during seed filling on the oxidative metabolism and yield of chickpea (*Cicer arietinum*) genotypes differing in heat and drought tolerance

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Abstract. Drought and heat stress are two major constraints that limit chickpea (*Cicer arietinum* L.) yield, particularly during seed filling. The present study aimed (*i*) to assess the individual and combined effects of drought and heat stress on oxidative metabolism during seed filling, and (*ii*) to determine any genetic variation in oxidative metabolism among genotypes differing in drought and heat tolerance and sensitivity. The plants were raised in outdoor conditions with two different times of sowing, one in November (normal-sown, temperatures <32°C–20°C (day–night) during seed filling), and the other in February (late-sown, temperatures >32°C–20°C (day–night) during seed filling). Plants were regularly irrigated to prevent any water shortage until the water treatments were applied. At both sowing times, the drought treatment was applied during seed filling (at ~75% podding) by withholding water from half of the pots until the relative leaf water content (RLWC) of leaves on the top three branches reached 42–45%, whereas leaves in the fully irrigated control plants were maintained at RLWC 85–90%. Drought-stressed plants were then rewatered and maintained under fully irrigated conditions until maturity. Several biochemical parameters were measured on the leaves and seeds at the end of the stress treatments, and seed yield and aboveground biomass were measured at maturity. Individual and combined stresses damaged membranes, and decreased PSII function and leaf chlorophyll content, more so under the combined stress treatment. The levels of oxidative molecules (malondialdehyde (MDA) and H_2O_2) markedly increased compared with the control plants in all stress treatments, especially across genotypes in the combined heat + drought stress treatment (increases in leaves: MDA 5.4–8.4-fold and H_2O_2 5.1–7.1-fold; in seeds: MDA 1.9–3.3-fold and H_2O_2 3.8–7.9-fold). The enzymatic and non-enzymatic antioxidants related to oxidative metabolism increased under individual stress treatments but decreased in the combined heat + drought stress treatment. Leaves had higher oxidative damage than seeds, and this likely inhibited their photosynthetic efficiency. Yields were reduced more by drought stress than by heat stress, with the lowest yields in the combined heat + drought stress treatment. Heat- and drought-tolerant genotypes suffered less damage and had higher yields than the heat- and drought-sensitive genotypes under the individual and combined stress treatments, suggesting partial cross-tolerance in these genotypes. A drought-tolerant genotype ICC8950 produced more seed yield under the combined heat + drought stress than other genotypes, and this was associated with low oxidative damage in leaves and seeds.

Additional keywords: ascorbate peroxidase, catalase, chlorophyll content, electrolyte leakage, glutathione reductase, superoxide dismutase.

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Introduction

Drought and heat stress are two major environmental factors that can markedly affect plant productivity (Lipiec *et al*. 2013). These two stresses often coincide to severely limit crop growth and productivity (Prasad *et al*. 2008; Grigorova *et al*. 2012; Lipiec *et al*. 2013). In combination, drought stress and heat stress affect the yield of many crops more than the individual effects of each stress (Rollins *et al*. 2013). Prolonged exposure of plants to stress can significantly alter plant biochemistry and metabolism, thereby influencing an array of processes including growth, development, yield and quality (Prasad *et al*. 2008; Farooq *et al*. 2009). Although water and heat stress have been extensively studied independently, relatively little is known about their combined effects on crops (Queitsch *et al*. 2000).

Studies have examined the combined effects of water and heat stress on the growth and productivity of cereal crops such as sorghum (*Sorghum bicolor* L.; Machado and Paulsen 2001), wheat (*Triticum aestivum* L.; Bahar and Yildirim 2010) and maize (*Zea mays* L.; Kebede *et al*. 2012), and some legume crops such as cowpea (*Vigna unguiculata* L. Walp.; Hall 2004), groundnut (*Arachis hypogaea* L.; Hamidou *et al*. 2013) and chickpea (*Cicer arietinum* L.; Gan *et al*. 2004; Canci and Toker 2009; Awasthi *et al*. 2014). However, the physiological and biochemical responses remain largely unknown.

Seed filling is the final stage of growth for any grain crop and involves transport processes to import constituents and biochemical processes related to the synthesis of carbohydrates, proteins and lipids in seeds. Periods of water limitation as well as heat stress during seed development cause substantial yield losses in various crops, as reported for cereals (Barnabas *et al*. 2008) and legumes (Leport *et al*. 1998; Davies *et al*. 1999; Canci and Toker 2009). Legumes are highly sensitive to abiotic stresses during seed filling (Krishnamurthy *et al*. 2010; Devasirvatham *et al*. 2012; Hamidou *et al*. 2013; Farooq *et al*. 2016).

The production of reactive oxygen species (ROS) is an inevitable consequence of aerobic metabolism during stressful conditions (Bhattacharjee 2012; Sharma *et al*. 2012). Heat stress and drought stress, individually or in combination, lead to the overproduction of ROS (O^{2-}, $^1O_2$, H_2O_2, OH^-) in different organelles, providing a major threat to cellular metabolic processes. The overproduction of ROS leads to deleterious reactions that damage biological structures including thylakoidal membranes and the photosynthetic apparatus, resulting in DNA nicking, and amino acid and protein oxidation (Asada 1999; Vranova *et al*. 2002). ROS directly attack membrane lipids and increase lipid peroxidation (Mittler 2002). Several studies have reported increased malondialdehyde (MDA, a product of lipid peroxidation) and H_2O_2 content under drought in species including hyacinth bean (*Lablab purpureus*; D'Souza and Devaraj 2011), chickpea (Patel and Hemantaranjan 2012), maize (Voothuluru and Sharp 2013), sugarcane (*Saccharum officinarum* L.; Boaretto *et al*. 2014) and wheat (Du *et al*. 2012, 2013; Wang *et al*. 2016), and under heat stress in species including cotton (*Gossypium hirsutum* L.; Mohammed and Tarpley 2010), rice (*Oryza sativa* L.), maize (Kumar *et al*. 2012) and sorghum (Tan *et al*. 2011). Little is known about the combined effects of these two stresses on oxidative damage in plants.

Plants tend to combat ROS production by inducing an antioxidant system consisting of enzymatic and non-enzymatic components, which are maintained at steady-state levels under stressful conditions (Tuteja *et al*. 2012). Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and enzymes of the ascorbate-glutathione cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (GR) (Noctor and Foyer 1998; Foyer and Noctor 2003). Non-enzymatic antioxidants include phenolic compounds, ascorbate, reduced glutathione (GSH), carotenoids and tocopherols (Apel and Hirt 2004; Gill and Tuteja 2010). Increased activity of many antioxidant enzymes to combat oxidative stress has been observed during drought or heat stress in crops including lentil (*Lens culinaris* Medik.; Chakraborty and Pradhan 2011), pearl millet (*Pennisetum glaucum* L.; Kolupaev *et al*. 2011), soybean (*Glycine max* L.; Akitha Devi and Giridhar 2015), pea (*Pisum sativum* L.; Osman 2015), wheat (Du *et al*. 2012) and rice (Pandey and Shukla 2015).

Chickpea is a major food legume grown worldwide for its high nutritional value. It is usually grown under rainfed rather than irrigated conditions, where terminal drought is often accompanied by heat stress during seed filling, which can be detrimental for seed yield (Canci and Toker 2009; Krishnamurthy *et al*. 2011). Previously, we reported a marked reduction in sucrose metabolism in the leaves and seeds of chickpea exposed to heat and drought stress combined (Awasthi *et al*. 2014). Several reports are available on oxidative stress associated with drought or heat stress, but no study has investigated the combined effects of drought and heat stress in chickpea. The objectives of this study were (*i*) to assess the individual and combined effects of drought and heat stress on the biochemical processes related to oxidative metabolism during seed filling, and (*ii*) to identify genetic variation in oxidative metabolism in genotypes contrasting for drought- and heat-tolerance and sensitivity. It was hypothesised that (*i*) the response of chickpea genotypes would vary to drought, heat and their combination in terms of growth and yield as well as oxidative damage and redox status in leaf and seed tissues, and (*ii*) there may be some cross-protection mechanisms against heat and drought in differentially sensitive genotypes.

Material and methods

Genotypes

This study used six chickpea genotypes with matching phenology but contrasting for heat and drought tolerance: two heat-tolerant (ICC1356, ICC15614), two heat-sensitive (ICC4567, ICC5912), one drought-tolerant (ICC8950) and one drought-sensitive (ICC3776). The origin of the genotypes is presented in Table 1 and phenology in Table 2. Designation as heat-tolerant and heat-sensitive or drought-tolerant and drought-sensitive was based on seed yields of the chickpea mini-core collection under heat and drought stress in the field (Krishnamurthy *et al*. 2010, 2011; Devasirvatham *et al*. 2012).

Experimental details

The experiments were conducted at Panjab University, Chandigarh (30°44′N, 76°47′E), India. The six genotypes were grown under outdoor conditions with two different times

Table 1. Country of origin, seed colour and type, and 100-seed weight of the six chickpea genotypes used in this study

Genotypes were identified as heat-tolerant (HT), heat-sensitive (HS), drought-tolerant (DT) and drought-sensitive (DS) based on seed yields under heat and drought conditions in the field (Krishnamurthy *et al*. 2010, 2011)

Genotype	Country of origin	Seed coat colour and seed type	100-seed weight (g)
ICC1356 (HT)	India	Yellow brown (Desi)	14.9
ICC15614 (HT)	Tanzania	Yellow brown (Desi)	14.6
ICC4567 (HS)	India	Dark brown (Desi)	13.7
ICC5912 (HS)	India	Mosaic (Kabuli)	16.4
ICC8950 (DT)	India	Yellow brown (Desi)	13.2
ICC3776 (DS)	Iran	Black (Desi)	10.5

Table 2. Time to first flower, first pod and maturity (days after sowing) in six chickpea genotypes contrasting for heat tolerance (HT) and heat sensitivity (HT), drought tolerance (DT) and drought sensitivity (DS) in normal-sown well-watered (control), normal-sown drought-stressed (drought stress), late-sown well-watered (heat stress), and late-sown drought-stressed (heat + drought stress) treatments

Values are the means ± s.e.m. ($n = 3$). Within parameters, values followed by the same letter are not significantly different ($P > 0.05$)

Genotype	Control	Drought stress	Heat stress	Heat + drought stress
Time to first flower (l.s.d. genotype × treatment = 2.9)				
1356 (HT)	64 ± 2.5a	62.5 ± 2.1a	46 ± 2.2b	44.2 ± 2.0bc
15614 (HT)	61 ± 2.3a	64.2 ± 2.7a	44 ± 2.4bc	42.6 ± 2.6bc
4567 (HS)	63 ± 2.1a	62.8 ± 2.5a	46 ± 2.3b	48.4 ± 2.5b
5912 (HS)	62 ± 2.4a	64.3 ± 2.9a	42 ± 2.2bc	44.3 ± 2.4bc
8950 (DT)	61 ± 2.3a	63.1 ± 2.4a	41 ± 2.6bc	43.5 ± 2.3bc
3776 (DS)	60 ± 2.6ab	61.8 ± 2.5a	43 ± 2.2bc	42.6 ± 2.1bc
Time to first pod (l.s.d. genotype × treatment = 2.4)				
1356 (HT)	114 ± 2.2a	112.4 ± 2.2a	61 ± 2.3bc	59.4 ± 2.4c
15614 (HT)	111 ± 2.5ab	114.4 ± 2.4a	58 ± 2.5c	57.1 ± 2.1c
4567 (HS)	110 ± 2.3ab	108.4 ± 2.1ab	59 ± 2.2c	61.2 ± 2.3bc
5912 (HS)	112 ± 2.5a	115.3 ± 2.4a	60 ± 2.4c	62.5 ± 2.4bc
8950 (HS)	113 ± 2.2a	115.6 ± 2.3a	62 ± 2.4bc	64.3 ± 2.3b
3776 (DS)	110 ± 2.4ab	112.5 ± 2.2a	60 ± 2.6c	59.5 ± 2.1c
Time to maturity (l.s.d. genotype × treatment = 3.9)				
1356 (HT)	160 ± 2.3a	128 ± 2.5b	88 ± 2.4c	81 ± 2.5d
15614 (HT)	162 ± 2.5a	126 ± 2.2b	86 ± 2.6c	79 ± 2.3d
4567 (HS)	163 ± 2.6a	128 ± 2.4b	83 ± 2.5cd	74 ± 2.5de
5912 (HS)	160 ± 2.4a	126 ± 2.3b	85 ± 2.3c	75 ± 2.6de
8950 (DT)	164 ± 2.5a	127 ± 2.2b	83 ± 2.3cd	78 ± 2.4d
3776 (DS)	161 ± 2.2a	123 ± 2.4b	84 ± 2.5c	71 ± 2.7f

of sowing. The first was in November (normal sowing time) so that the average day–night temperatures during seed filling were <32°C−20°C, and the second was in February (late-sown) to ensure that plants were exposed to higher average day–night temperatures, i.e. >32°C−20°C, during seed filling. Daily maximum, minimum and mean air temperature, daily maximum, minimum and mean relative humidity, and photoperiod were recorded during both growing seasons (Fig. 1). Ranges of maximum and minimum (i.e. day and night) temperatures during seed filling were 27.0°C–30.5°C and 10.9°C–18.6°C, respectively, for the plants sown in November, and 32.1°C–37.0°C and 21.0°C–22.8°C for the plants sown in February. Day and night relative humidities were 25–45% and 83–85%, respectively, for the plants sown in November, and 20–25% and 48–70% for the plants sown in February (Fig. 1). The mean day–night vapour-pressure deficits during the seed-filling period were 2.0–0.3 kPa in the November-sown chickpeas and 4.5–1.1 kPa in the February-sown plants. The photoperiod range was 12.1–12.5 h during the normal-sown growing season and 13.0–13.3 h during the late-sown growing season (Fig. 1).

The field soil (sand 63.4%, silt 24.6%, clay 12%) was mixed with sand in a 3 : 1 ratio. Farmyard manure was then added to the soil–sand mixture in a 1 : 3 ratio, along with tricalcium phosphate fertiliser (at 10 mg kg^{-1}). Each earthen pot (300 mm diameter, 236 mm depth) was filled with 8 kg of this soil mixture. *Rhizobium ciceri* was used to inoculate the seeds before sowing. Five seeds were sown in each pot and thinned to three per pot at 20 days after sowing. The plants were grown inside a wire enclosure that prevented damage by birds and animals and could be covered with plastic (90% light transmission) to prevent infrequent rain events from affecting the treatments. The plants were regularly irrigated to prevent any water shortage until the water treatments were applied.

There were four treatments: control, normal-sown (November) and well watered; drought stress, normal-sown and drought-stressed during seed filling; heat stress, late-sown (February) and well watered; heat + drought stress, late-sown and drought-stressed during seed filling. There were 12 pots per genotype, with three pots in each of the four treatments, in a completely randomised block design. The pots were moved regularly within the enclosure to remove any positional effects.

Application of drought stress in normal- and late-sown conditions

The soil-water content at field capacity of the soil mixture, determined with a FieldScout TDR 300 soil moisture meter (Spectrum Technologies, Aurora, IL, USA), was 19.5% and it was maintained until the onset of seed filling by watering the plants twice daily at 09 : 00–10 : 00 and 16 : 00–17 : 00 Indian Standard Time. The drought treatment in both the normal- and late-sown plants was applied during seed filling (~75% podding) by withholding water from half of the pots. The relative leaf water content (RLWC), measured between 11:00–11 : 30, was taken as an indicator of the plant water deficit; water was withheld in the drought treatments until the RLWC of leaves from the top three branches reached 50% (i.e. 43–45%) of that on fully irrigated plants, with an RLWC of 85–90%. This occurred after 13 days in the normal-sown drought-stressed plants and after 7 days in the late-sown heat + drought-stressed plants. The gravimetric soil-water content (dry-weight basis) was measured by collecting soil from 70–120 mm depth in pots. In the drought-stressed (normal-sown) plants, the soil-water content decreased from 19.5% to 9%, and in the heat + drought-stressed (late-sown) plants, it decreased from 19.5% to 8% before rewatering to 100% field capacity. All plants were then watered twice daily until seed maturity.

Sample collection

For the measurement of stress injury and biochemical analyses related to oxidative metabolism and antioxidative defence, and to obtain homogeneity for comparison of the biochemistry among the genotypes grown at the two different sowing times, seeds and subtending leaves were randomly collected at 11 : 00 from three plants per genotype and treatment in three replications at the end of stress period just before re-watering, when RLWC was 42.5–45% in the drought-stressed plants and 85–90% in the well-watered plants. Leaves and seed samples for biochemical analysis were collected from the second and third branches from the top. In the late-sown plants, the samples were collected after the plants had experienced heat stress (day–night temperatures >32°C−20°C) for at least 7 days consecutively in the heat stress and combined heat + drought stress treatments. The samples were stored at −80°C in a deep freezer. To measure the aboveground biomass and yield components at maturity, three plants from one of the three pots per replicate per genotype per treatment were cut at soil level, with care taken to include the leaves that had fallen

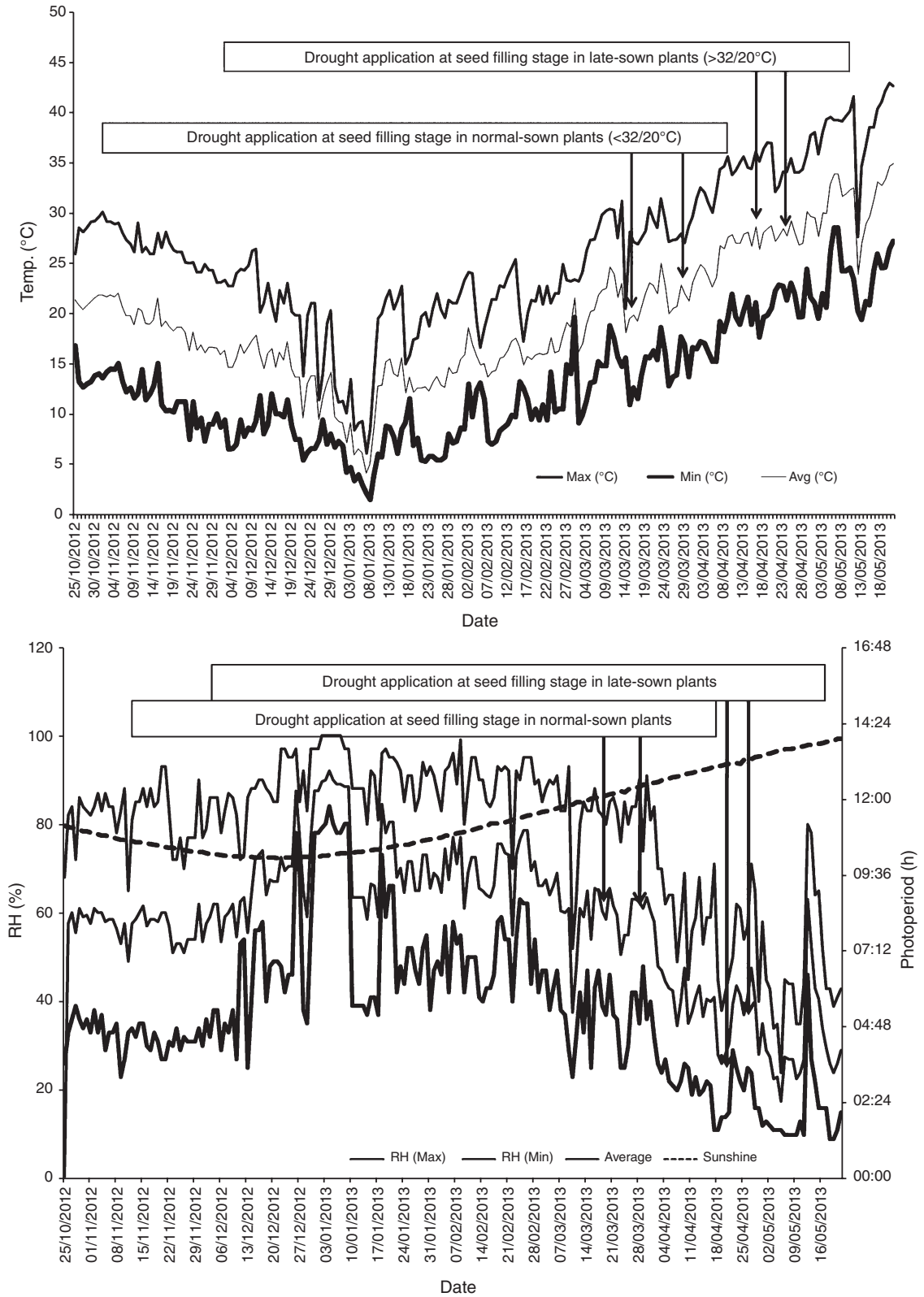

Fig. 1. Maximum, minimum and mean temperature (top panel), and maximum, minimum and mean relative humidity (RH, %) and photoperiod (h) (bottom panel), between 25 October 2012 and 21 May 2013. Arrows show the beginning (first arrow) and end (second arrow) of the drought treatment imposed on chickpeas sown in November (normal sowing), and the beginning (third arrow) and end (fourth arrow) of the drought treatment imposed on chickpeas sown in February (late sowing).

onto the pot and those that were collected near maturity and before they fell. The number of filled pods was counted, and seeds were removed from the pods and counted. The seeds and remaining plant parts (stems, leaves and pod shells, hereafter simply termed aboveground biomass) were oven-dried separately for 72 h at 45°C. Average values of all the traits measured on the three plants per pot were expressed on a per-plant basis.

Relative leaf water content

The RLWC was measured according to the method of Barrs and Weatherley (1962). Fresh leaves were excised and cut into smaller segments (10 mm), weighed (fresh weight, FW) and then floated on distilled water under low light (150–200 μmol m^{-2} s^{-1}) for 3 h to obtain the turgid weight (TW). Leaf samples were oven-dried at 80°C for 24 h and weighed (dry weight, DW). RLWC was calculated as:

$$\text{RLWC} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100$$

Electrolyte leakage

Stress injury to leaves was measured as electrolyte leakage (Premchandra *et al*. 1990). Fresh leaf samples (1.0 g) were washed three times with deionised water to remove electrolytes adhering to the surface. Samples were placed in closed vials containing 10 mL deionised water and incubated at 25°C on a rotary shaker for 24 h; the electrical conductivity of the solution was determined with a conductivity meter (CM 180; ELICO, Hyderabad, TG, India). Electrolyte leakage was expressed as electrical conductivity in μmhos g^{-1} DW.

PSII activity

The photochemical efficiency of the leaves was measured as chlorophyll fluorescence by using the dark-adapted test of the modulated chlorophyll fluorometer (OS1-FL; Opti-Sciences, Hudson, NH, USA) at 11 : 00 at the end of the stress period. With this system, chlorophyll fluorescence is excited by a 660-nm solid-state light source, with filters blocking radiation at wavelengths >690 nm. The average intensity of this modulated light was adjusted from 0 to 1 μE. Detection was in the range 700–750 nm using a PIN (p-type, intrinsic, n-type) photodiode (a silicone-based gated diode that features a p-i-n diode to detect incident light) with appropriate filtering to remove extraneous light. The leaves were dark adapted for 0.75 h before measurement. The clamped leaves were then exposed to 695 nm modulated light, and the variable fluorescence/maximum fluorescence (Fv/Fm) ratio (the maximum quantum yield of PSII photochemistry) was recorded. PSII activity was expressed as the Fv/Fm ratio. The same leaves were used to measure chlorophyll content.

Chlorophyll content

For the estimation of chlorophyll content, chlorophyll was extracted by grinding fresh leaves (1.0 g) in 80% acetone, followed by centrifugation at 5700*g* for 600 s. The absorbance of the supernatant was read at 645 nm and 663 nm, and total chlorophyll was calculated (Arnon 1949) against 80% acetone as a blank. The chlorophyll content was measured as:

$$\text{Chl a} = 12.9\ (\text{Abs}_{663}) - 2.69\ (\text{Abs}_{645}) \times V/1000 \times W \quad (1)$$

$$\text{Chl b} = 22.9\ (\text{Abs}_{645}) - 4.68\ (\text{Abs}_{663}) \times V/1000 \times W \quad (2)$$

$$\text{Total Chl} = \text{Chl a} + \text{Chl b} \quad (3)$$

where V is volume, W is tissue weight, Abs_{663} is absorbance at 663 nm and Abs_{645} is absorbance at 645 nm. The total chlorophyll content was expressed as μmol g^{-1} DW.

Oxidative molecules

Malondialdehyde

Lipid peroxidation of membranes was estimated from MDA content, a product of lipid peroxidation, using the method described by Heath and Packer (1968). Fresh leaf and seed tissue (500 mg) was homogenised in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 11 320*g* for 300 s, and a 1-mL aliquot of the supernatant was treated with 4 mL 0.5% thiobarbituric acid in 20% TCA; the mixture was heated at 95°C for 0.5 h and then quickly cooled in an ice bath. After centrifugation at 5700*g* for 600 s, the absorbance of the supernatant was read at 532 nm. The MDA content was calculated by its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} DW.

Hydrogen peroxide

The content of H_2O_2 was estimated by the method of Mukherjee and Chaudhari (1983). Fresh leaf and seed tissue (500 mg) was homogenised in 5 mL chilled acetone (80%) and filtered through Whatman filter paper, and 4 mL titanium reagent was added followed by 5 mL ammonia solution. The mixture was centrifuged at 5030*g*, and the supernatant discarded. The residue was dissolved with 1 M H_2SO_4 and the absorbance recorded at 410 nm. The extinction coefficient of H_2O_2 is 0.28 μmol^{-1} cm^{-1}. The content of H_2O_2 in samples was obtained from a standard curve using pure H_2O_2 and expressed as μmol g^{-1} DW.

Enzymatic and non-enzymatic antioxidants

Superoxide dismutase

Activity of SOD (E.C. 1.15.1.1) was measured following the method of Dhindsa *et al*. (1981). Fresh leaf and seed samples (500 mg) were homogenised in 50 mM chilled phosphate buffer (pH 7.0) and centrifuged at 5030*g* for 600 s at 4°C; the supernatant was treated as the enzyme extract. The reaction mixture (3 mL) contained 13 mM methionine, 25 mM nitro blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM sodium bicarbonate, 50 mM phosphate buffer (pH 7.8) and 0.1 mL enzyme extract. The reaction was started by adding 2 mM riboflavin to the mixture followed by exposure to a 15-W fluorescent light for 600 s. The absorbance was recorded at 560 nm and the total SOD activity of the samples assayed by measuring its ability to inhibit the photochemical reduction of NBT. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of the photochemical reduction of NBT. It was expressed in units of SOD activity mg^{-1} protein.

Catalase

Activity of CAT (E.C. 1.11.1.6) was estimated by the method of Teranishi *et al*. (1974) with some modifications. Fresh leaf and seed samples (500 mg) were homogenised in 50 mM chilled phosphate buffer (pH 7.0) and centrifuged at 5030*g* for 600 s at 4°C; the supernatant was treated as the enzyme extract. Enzyme extract (0.1 mL) was added to 3 mL phosphate buffer (50 mM, pH 7.0), and a reaction was initiated by adding 200 mM H_2O_2. The decrease in absorbance was recorded at 410 nm for 180 s. CAT activity was measured by using the extinction coefficient 40 mM^{-1} cm^{-1} and expressed as mmol H_2O_2 decomposed mg^{-1} protein.

Ascorbate peroxidase

Activity of APX (E.C. 1.11.1.11) activity was determined by following the oxidation of ascorbate as a decrease in absorbance at 290 nm (Nakano and Asada 1981). Fresh leaf and seed samples (500 mg) were homogenised in ice-cold 50 mM phosphate buffer and centrifuged at 5030*g* at 4°C; the supernatant (i.e. enzyme extract) was kept for assay. The reaction was carried out at 20°C in 3 mL reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbic acid (ASC), and 0.1 mL enzyme extract. The change in absorbance at A_{290} was recorded at 30-s intervals after adding H_2O_2. The rate constant was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and was expressed as mmol oxidised donor min^{-1} mg^{-1} protein.

Glutathione reductase

Activity of GR (E.C. 1.6.4.2) was measured using the method of Mavis and Stellwagen (1968). GR enzyme solution containing 0.30–0.60 units mL^{-1} of glutathione reagent in cold reagent, i.e. 1% bovine serum albumin (BSA), was prepared immediately before use. The reaction mixture contained 0.65 mL deionised water, 1.5 mL phosphate buffer (100 mM, pH 7.6), 0.1 mL glutathione oxidised (GSSG), 0.35 mL β-NADP, 0.20 mL BSA and 0.2 mL enzyme solution. The contents of the reaction mixture were immediately mixed by inversion and the decrease in absorbance was read at 340 nm for ~180 s. The enzyme activity was expressed as mmol oxidised donor min^{-1} mg^{-1} protein.

Ascorbic acid

Estimation of ASC was done according to the method of Mukherjee and Chaudhari (1983). Fresh leaf and seed tissue (500 mg) was homogenised in 6% TCA, and the homogenate was centrifuged at 3650*g*. A 2-mL volume of 2% DNPH (2,4,dinitrophenyl hydrazine) was added to 4 mL supernatant followed by one drop of 10% thiourea. The mixture was boiled for 15 min in a water bath and cooled to room temperature; 5 mL pre-cooled H_2SO_4 at 0°C was added and the absorbance read at 530 nm. The ASC content was calculated from a standard curve plotted with known content of ASC and expressed as nmol g^{-1} DW.

Glutathione

Reduced glutathione (GSH) was estimated according to the method of Griffith (1980). The GSH content was measured by using fresh leaf and seed tissue (500 mg) homogenised in 2.0 mL metaphosphoric acid and centrifuged at 14 540*g* for 600 s. Aliquots (0.9 mL) of the supernatant were neutralised by adding 0.6 mL 10% sodium citrate. A total volume of 1.0 mL solution, containing 700 µL NADPH (0.3 mM), 100 µL 5,5′-dithio-bis-(2-nitrobenzoic acid) DTNB (6 mM), 100 µL distilled water and 100 µL enzyme extract, was prepared and stabilised at 25°C for 180–240 s. Later, 10 µL GR was added, and the absorbance was recorded at 412 nm. GSH content was calculated from a standard graph as described by Griffith (1980) and expressed as nmol g^{-1} DW.

Assay for dry weight and protein content

The assays are expressed on a DW or protein content basis. The DW of the fresh leaf and seed samples was obtained by weighing out a similar quantity (500 mg) of fresh leaf and seed tissue from the same position on the plant as for the enzyme assays, oven-drying the leaf and seed samples at 45°C for 48 h, and weighing the dry samples.

The soluble protein content of the leaves and seeds at the time of sampling was estimated by the method of Lowry *et al*. (1951). The fresh leaf and seed samples (100 mg) were macerated in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 510*g* for 600 s to obtain the supernatant. TCA (5 mL, 15%) was added to the supernatant and kept at 4°C for 24 h. The mixture was then centrifuged at 510*g* for 600 s to separate the precipitates. The supernatant was discarded and the precipitate dissolved with 0.1 N NaOH (1 mL) and then kept for 18 h for complete dissolution. Copper sulfate reagent containing 2% Na_2CO_3 (in 0.1 N NaOH) and 0.5% $CuSO_4.5H_2O$ (in 1% sodium potassium tartarate) was added to 1 mL dissolved precipitate and allowed to stand for 600 s, and then 0.5 mL Folin-phenol Ciocalteu's reagent (1 N, 1 : 1 ratio) was added. This mixture was kept for 0.5 h for colour development and the absorbance was read at 570 nm. The total protein content (mg g^{-1} DW) was obtained by using a standard curve with BSA.

Statistical analyses

Data for the six genotypes by four treatments and three replicates were analysed by a 2-way analysis of variance, using AGRISTAT statistical software (developed by the Indian Council of Agricultural Research, New Delhi). Tukey's post-hoc test was determined using Statistical Analysis System software (SAS Institute, Cary, NC, USA). Correlations were fitted with 'Analyse it' software of Microsoft Excel, using average values per genotype. Mean values along with the standard error of the mean (s.e.m.) for genotypes and treatments and the least significant differences (l.s.d.) for the interactions are presented.

Results

Results of ANOVA, indicating the level of statistical significance, are presented for all measured traits in Table 3. For all traits, the effects of treatment, genotype and treatment × genotype were significant, with the exceptions of genotype for days to first flower and SOD in seeds.

Phenology, growth and seed yield

In the control treatment, time to first flower varied from 60 to 64 days after sowing (DAS), time to first podding varied from

Table 3. Analysis of variance showing the level of statistical significance of the traits measured in six chickpea genotypes given four treatments (normal-sown well-watered, normal-sown drought-stressed, late-sown well-watered, late-sown drought-stressed) and the significance of the interaction of genotype × treatment

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n.s., not significant ($P > 0.05$)

	Genotype	Treatment	Interaction
Days to first flower	n.s.	***	*
Days to first pod	***	***	*
Days to maturity	***	***	**
Aboveground biomass	***	***	***
Seed weight	***	***	***
Chlorophyll	***	***	***
PSII function	***	***	***
Tissue damage	*	***	***
Malondialdehyde, leaves	***	***	***
Malondialdehyde, seeds	***	***	***
Hydrogen peroxide, leaves	***	***	***
Hydrogen peroxide, seeds	***	***	***
Superoxide dismutase, leaves	*	***	***
Superoxide dismutase, seeds	n.s.	***	*
Catalase, leaves	**	***	***
Catalase, seeds	***	***	***
Ascorbate peroxidase, leaves	***	***	***
Ascorbate peroxidase, seeds	***	***	***
Glutathione reductase, leaves	***	***	***
Glutathione reductase, seeds	***	***	***
Ascorbic acid, leaves	***	***	***
Ascorbic acid, seeds	***	***	***
Glutathione, leaves	**	***	***
Glutathione, seeds	**	***	***

110 to 114 DAS, suggesting that many of the early flowers aborted and did not produce a pod, and time to maturity varied from 160 to 164 DAS (Table 2). In all genotypes, late sowing in February reduced the number of days to flowering, podding and maturity compared with the normal-sown plants (Table 2), and reduced flowering and podding duration. Drought stress (normal-sown) imposed during podding reduced days to maturity, and combined heat + drought stress reduced the days to maturity further, particularly in the sensitive genotypes (Table 2).

Aboveground biomass (stems, leaves and pod shells) produced by the six genotypes varied from 6 to 8 g plant^{-1} in the control treatment (Fig. 2*a*). The shortened growth period due to the high temperatures in the heat stress treatment reduced aboveground biomass more than the drought stress (normal-sown) treatment (24–62% cf. 14–52%). Compared with the control, this drought stress treatment reduced aboveground biomass by 25% in the heat-tolerant genotypes, 37% in the heat-sensitive genotypes, 14.3% in the drought-tolerant genotype and 51% in the drought-sensitive genotype (Fig. 2*a*). When drought was imposed on the late-sown plants (combined heat + drought stress treatment), the biomass was reduced by 53–60% in heat- and drought-tolerant genotypes and by 70–83% in heat and drought-sensitive genotypes, compared with the control treatment (Fig. 2*a*).

Seed weight in the control treatment was 5.3–6.0 g plant^{-1} and did not differ among genotypes (Fig. 2*b*). Seed weight per plant decreased significantly more in the drought stress (1.8–3.9 g plant^{-1}) than the heat stress (2.5–4.8 g plant^{-1}) treatment, and the combined heat + drought stress treatment was the most inhibitory (1.1–3.9 g plant^{-1}), especially for the sensitive genotypes (Fig. 2*b*). The drought stress treatment reduced yields by 45–51% in heat-tolerant genotypes, 57–64% in heat-sensitive genotypes, 30% in the drought-tolerant genotype and 66% in the drought-sensitive genotype. Delaying planting to expose plants to heat + drought stress reduced seed yields of heat-tolerant genotypes by 48–50%, heat-sensitive genotypes by 74–78%, the drought-tolerant genotype by 28% and the drought-sensitive genotype by 79% compared with the control, and by 30%, 51%, 19% and 56%, respectively, compared with the heat treatment (Fig. 2*b*).

Photosynthetic efficiency and chlorophyll content of leaves

The photosynthetic efficiency (Fv/Fm) of the leaves was not significantly different among the genotypes in the control plants (mean = 0.75), and consistent with the effects on seed yield, it decreased more in plants subjected to drought stress (54–74%) than to heat stress alone (9–46%) (Fig. 3*a*). The combined heat + drought stress treatment showed the greatest reduction in photosynthetic efficiency (68–83%), with the smallest reduction occurring in the drought-tolerant genotype (ICC8950) (Fig. 3*a*).

The chlorophyll content of the leaves was 13.3–15.2 μmol g^{-1} DW in the control plants and did not vary significantly with genotype (Fig. 3*b*). Plants in the drought stress treatment lost more chlorophyll (20–52%) than those in the heat stress treatment (10–41%), and plants under combined heat + drought stress lost the most chlorophyll (25.3–60.5%) (Fig. 3*b*). Nevertheless, the decreases in chlorophyll were less than the decreases in photosynthetic efficiency (Fig. 3*a*). Drought- and heat-tolerant genotypes maintained significantly higher chlorophyll (9.41–10.3 μmol g^{-1}DW) than sensitive genotypes (5.37–6.43 μmol g^{-1} DW) in the combined heat + drought stress treatment (Fig. 3*b*).

Leaf injury and oxidative damage of leaves and seeds

Leaf damage was assessed on the basis of membrane integrity (electrolyte leakage). Some electrolyte leakage (0.8–1.3 μmhos g^{-1} DW) was observed in the control plants, but leaf damage increased in all genotypes in the drought stress treatment (1.3–2.0-fold), heat stress treatment (1.2–1.8-fold) and the combined heat + drought stress treatment (1.5–2.5-fold) (Fig. 3*c*). Under the individual stress treatments, tolerant genotypes had significantly less tissue damage than sensitive genotypes (Fig. 3*c*). In the combined heat + drought stress treatment, the most leaf damage occurred in the drought-sensitive genotype (ICC3776, 2.5-fold), whereas the least damage was observed in a heat-tolerant genotype (ICC1356, 1.5-fold).

Consistent with leaf damage, the MDA content, an indicator of membrane damage due to lipid peroxidation, markedly increased in the leaves in all stress treatments, especially the combined heat + drought stress treatment (Fig. 4*a*). Drought stress (normal-sown) significantly increased leaf MDA content in all genotypes (3.7–8.3-fold), especially in the drought-sensitive genotype (ICC3776, 8.3-fold). Heat stress also increased the MDA

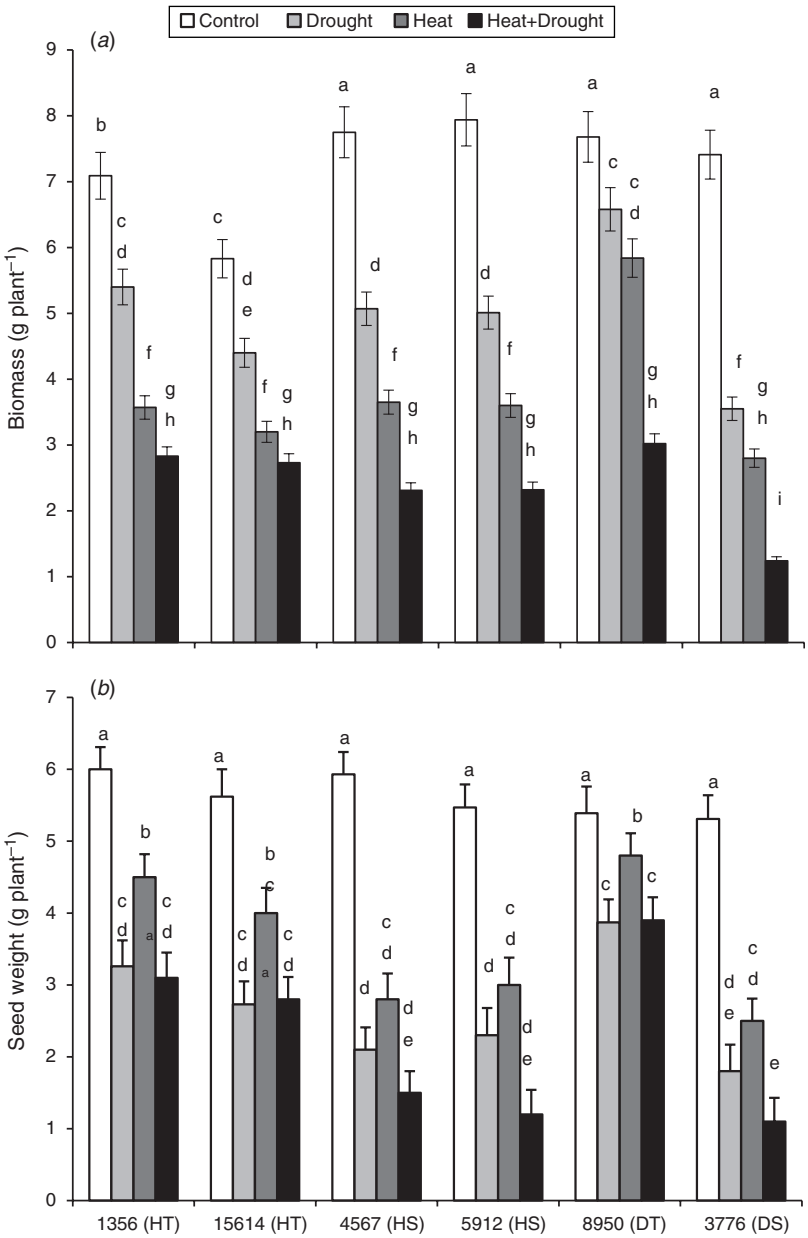

Fig. 2. (*a*) Aboveground biomass (stems, leaves and pod shells) and (*b*) seed weight per plant of six chickpea genotypes that were heat tolerant (HT), heat sensitive (HS), drought tolerant (DT) or drought sensitive (DS), in the normal-sown well-watered (Control), normal-sown drought-stressed (Drought), late-sown well-watered (Heat) and late-sown drought-stressed (Heat + Drought) treatments. Values of l.s.d. (genotype × treatment): biomass 0.52, seed weight 0.84. Values are means + s.e.m. ($n = 3$); within a parameter, means with the same letter are not significantly different.

content of the leaves in all genotypes, but to a lesser extent than drought stress (2.9–6.2-fold). Heat + drought stress increased leaf MDA content 4.8–8.4-fold compared with control plants, more than either alone except in the drought-sensitive genotype, ICC3776 (Fig. 4*a*). Irrespective of the stress treatment, the MDA content in the leaves was higher in the heat- and drought-sensitive genotypes than the heat- and drought-tolerant genotypes (Fig. 4*a*).

The MDA content of the seeds in the control plants was about half that in the leaves (Fig. 4*b*), but increased under the drought and heat stress treatments, more so under drought stress (1.5–2.0-fold) than heat stress (1.2–1.9-fold), and more so in the sensitive genotypes (Fig. 4*b*). The combined heat + drought stress treatment markedly increased seed MDA content (1.9–3.3-fold) relative to the control, with the greatest increase in the drought-sensitive genotype (ICC3776, 3.3-fold) (Fig. 4*b*) and

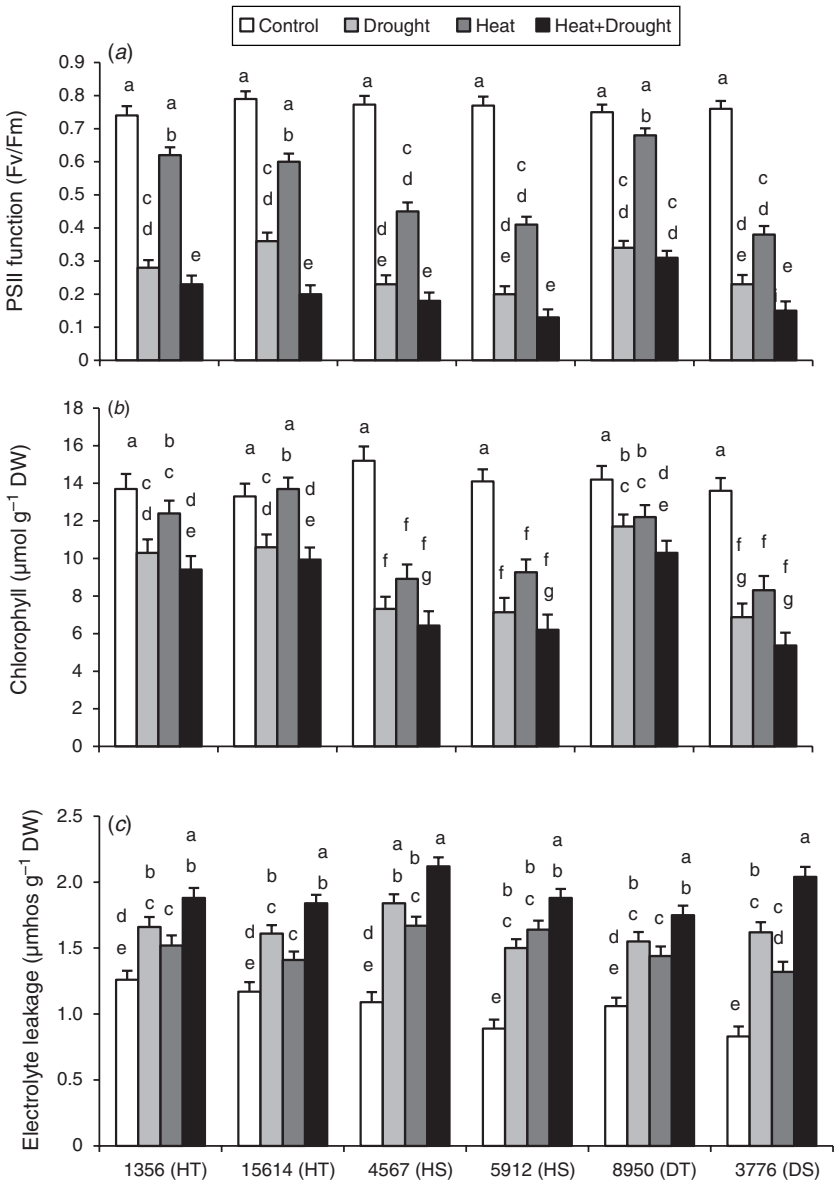

Fig. 3. (*a*) PSII function, (*b*) chlorophyll content, and (*c*) electrolyte leakage of six chickpea genotypes that were heat tolerant (HT) and heat sensitive (HS), drought tolerant (DT) or drought sensitive (DS), in the normal-sown well-watered (Control), normal-sown drought-stressed treatment (Drought), late-sown well-watered (Heat) and late-sown drought-stressed (Heat + Drought) treatments. Values of l.s.d. (genotype × treatment): PSII function 0.03, chlorophyll content 0.37, electrolyte leakage 0.29. Values are means + s.e.m. ($n = 3$); within a parameter, means with the same letter are not significantly different.

the two heat-sensitive genotypes (ICC5912 and ICC4567, 2.9-fold), and the smallest increase in a drought-tolerant genotype (ICC8950, 2.1-fold) and two heat-tolerant genotypes (ICC1356 and ICC15614, 1.9-fold).

Like MDA, the H_2O_2 content in the leaves increased significantly under all stress treatments: control, 1–2 µmol g^{-1} DW; heat stress, 2.2–4.5 µmol g^{-1} DW; drought stress, 4.5–9.2 µmol g^{-1} DW; heat + drought stress, 7.2–13.5 µmol g^{-1} DW (Fig. 4*c*). In this combined heat + drought stress treatment, the rise in leaf H_2O_2 content relative to the control treatment was greater in sensitive genotypes (6.5-fold) than in tolerant genotypes (5.7-fold); the greatest increase occurred in the drought-sensitive genotype (ICC3776, 7.1-fold) with the smallest increase in a drought-tolerant genotype (ICC8950, 5.4-fold).

The H_2O_2 content in the seeds of control plants, at 0.73 µmol g^{-1} DW in all genotypes, was about half that in the leaves (1.6 µmol g^{-1} DW). As in the leaves, H_2O_2 content increased in the heat stress treatment by 1.8–2.8-fold and in the drought stress treatment by 2.5–4.3-fold across genotypes,

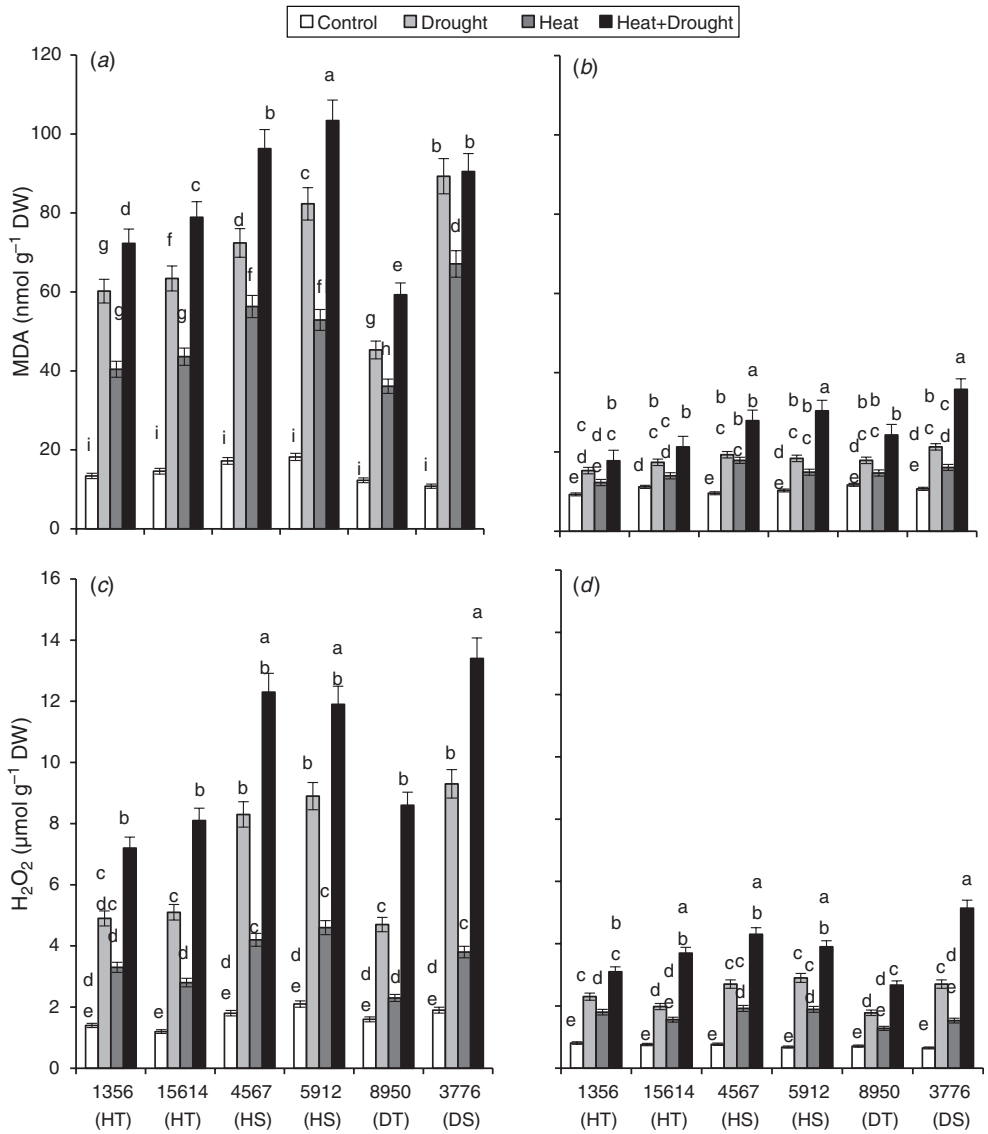

Fig. 4. (*a*, *b*) Malondialdehyde content (MDA) and (*c*, *d*) hydrogen peroxide (H_2O_2) content in the leaves (*a*, *c*) and seeds (*b*, *d*) of six chickpea genotypes that were heat tolerant (HT), heat sensitive (HS), drought tolerant (DT) or drought sensitive (DS), in the normal-sown well-watered (Control), normal-sown drought-stressed (Drought), late-sown well-watered (Heat) and late-sown drought-stressed (Heat + Drought) treatments. Values of l. s.d. (genotype × treatment): leaf MDA content 4.8, seed MDA content 3.4, leaf H_2O_2 content 0.50, seed H_2O_2 content 0.29. Values are means + s.e.m. ($n = 3$); within a parameter, means with the same letter are not significantly different.

with significantly higher increases in the heat- and drought-sensitive genotypes than heat- and drought-tolerant genotypes (Fig. 4*d*). Compared with the control, seed H_2O_2 content in the combined heat + drought stress treatment increased further (3.8–7.9-fold) across genotypes, with the largest increase in the drought-sensitive genotype (ICC3776, 7.9-fold) followed by the two heat-sensitive genotypes (ICC5912 and ICC4567 (5.6-fold) (Fig. 4*d*).

Activity of antioxidants in leaves and seeds

In the leaves of the control plants, the activity of SOD varied with genotype from 11 to 17 units mg^{-1} protein (Fig. 5*a*), CAT from 1.8 to 2.4 mmol H_2O_2 decomposed mg^{-1} protein (Fig. 5*c*), APX from 4.2 to 5.6 mmol oxidised donor min^{-1} mg^{-1} protein (Fig. 6*a*), GR from 4.3 to 5.2 mmol oxidised donor min^{-1} mg^{-1} protein (Fig. 6*c*) and GSH from 32 to 40 nmol g^{-1} DW (Fig. 7*c*). ASC content in the control treatment was 112–121 nmol g^{-1} DW in all genotypes (Fig. 7*a*). SOD, APX, GR, ASC and GSH were higher under drought stress than heat stress in most genotypes, whereas CAT had higher expression under heat stress than drought stress. The activity of most of the antioxidants under combined heat + drought stress was unexpectedly decreased compared with the heat stress treatment, except for CAT in the drought-tolerant genotype. For APX, GR and GSH, the activity

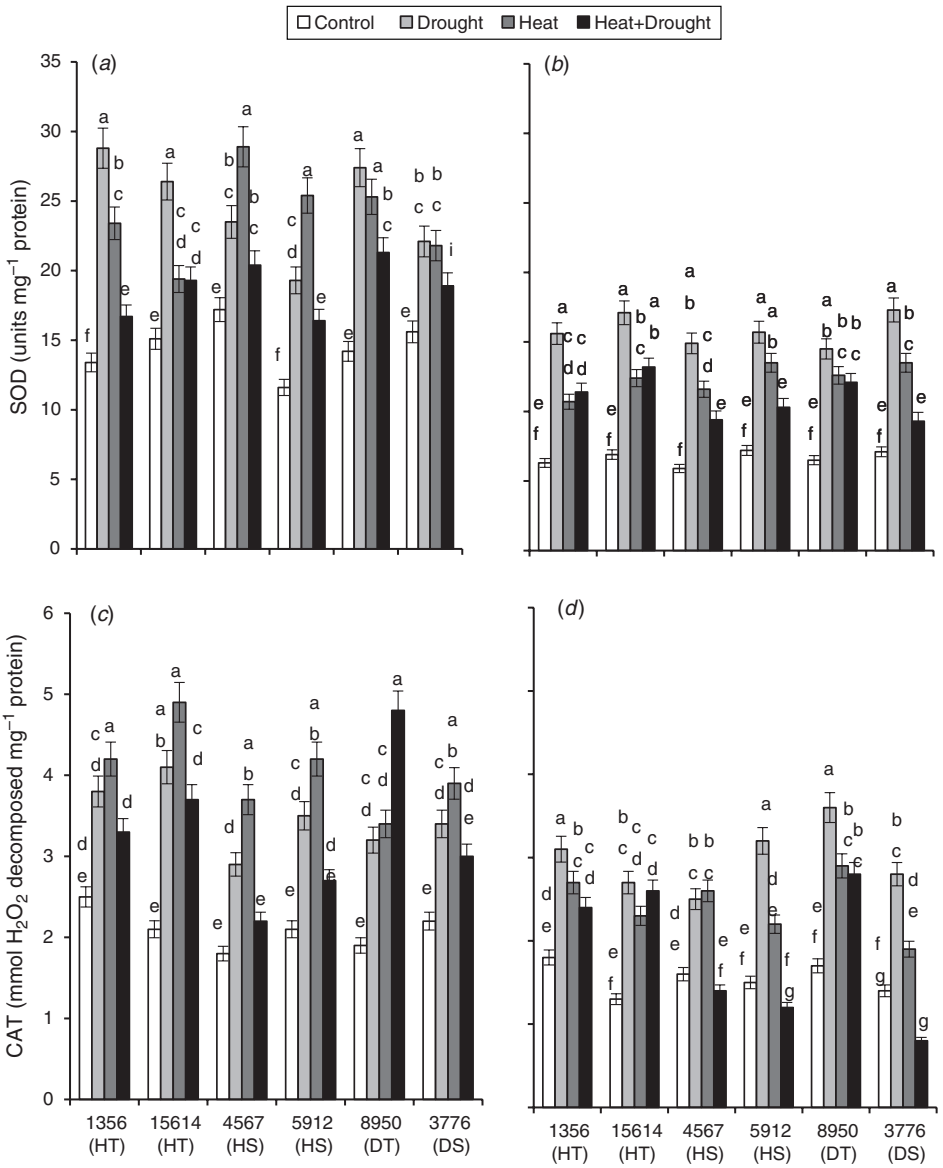

Fig. 5. (*a*, *b*) Superoxide dismutase (SOD) and (*c*, *d*) catalase (CAT) in the leaves (*a*, *c*) and seeds (*b*, *d*) of six chickpea genotypes that were heat tolerant (HT), heat sensitive (HS), drought tolerant (DT) or drought sensitive (DS), in the normal-sown well-watered (Control), normal-sown drought-stressed (Drought), late-sown well-watered (Heat) and late-sown drought-stressed (Heat + Drought) treatments. Values of l.s.d. (genotype × treatment): leaf SOD activity 5.5, seed SOD activity 2.5, leaf CAT activity 0.54, seed CAT activity 0.38. Values are means + s.e.m. ($n = 3$); within a parameter, means with the same letter are not significantly different.

of the heat- and drought-sensitive genotypes in the heat + drought stress treatment was lower than in the control (Figs 6*a*, *c*, 7*c*). Overall for enzymatic antioxidants, the combined heat + drought stress treatment increased leaf SOD by 18–50%, CAT by 22–152%, depending on genotype, compared with the control.

Content of ASC was found to increase under drought by 127–142% in the heat-tolerant genotypes and 133% in the drought-tolerant genotypes, whereas under heat stress, the increase was 70–106% in the heat-tolerant and 81% in the drought-tolerant genotype. In the combined heat + drought stress treatment, the tolerant genotypes (for heat and drought) showed a 54–64% increase relative to the control, and the sensitive genotypes showed ASC values similar to, or in the case of the drought-sensitive genotype significantly less than, the control and significantly less than individual stress treatments (Fig. 7*a*). The pattern in GSH was similar to ASC; thus, genotypes tolerant to drought and heat contained more GSH in the individual and combined stress treatments (Fig. 7*c*).

In the seeds of the control chickpea plants, the activities of the antioxidants SOD, CAT, APX and GR varied with genotype, but were ~50% of the values in the leaves (Figs 5*b*, *d*, 6*b*, *d*), whereas ASC activity was ~60% (Fig. 7*b*) and GSH ~80%

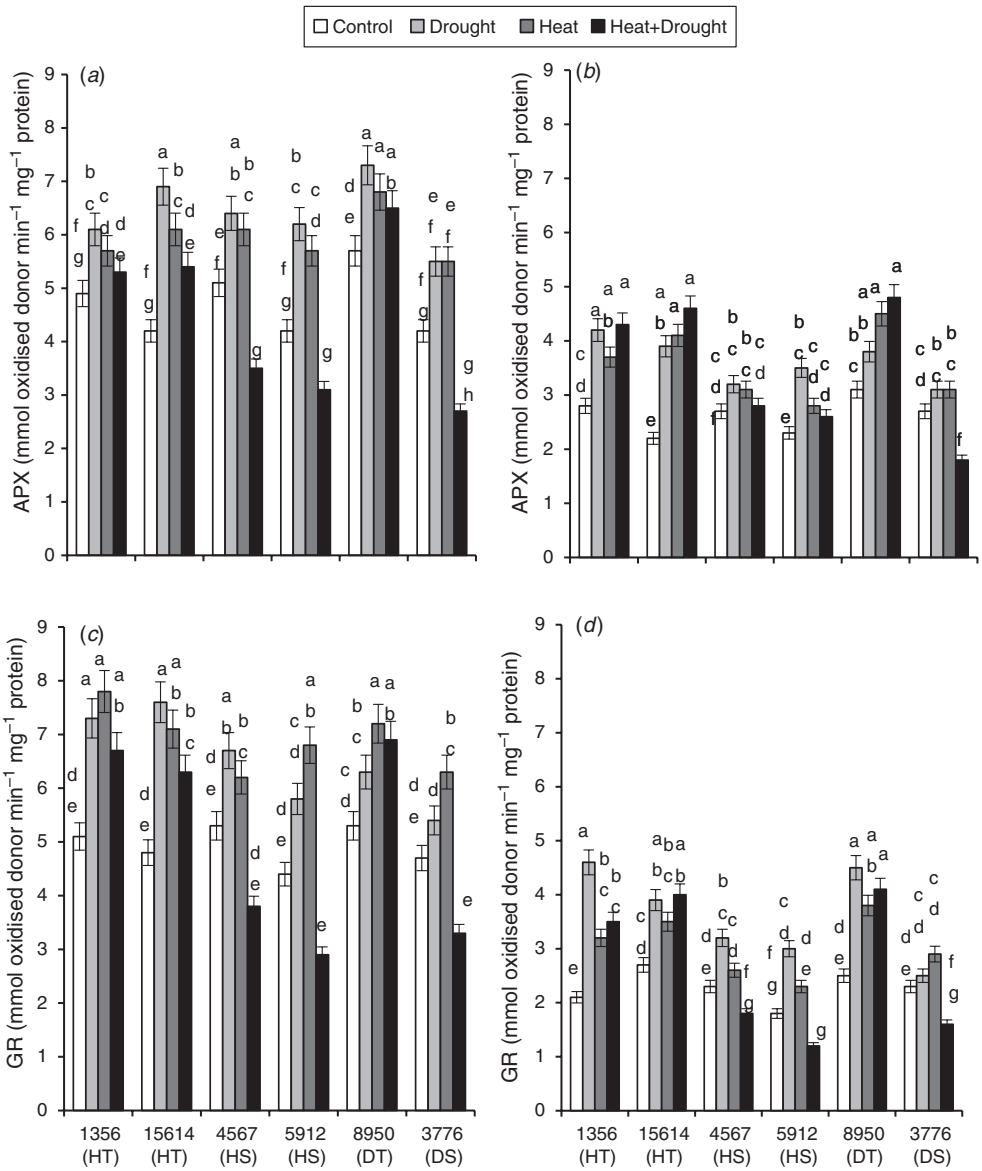

Fig. 6. (*a*, *b*) Ascorbate peroxidase (APX) and (*c*, *d*) glutathione reductase (GR) in the leaves (*a*, *c*) and seeds (*b*, *d*) of six chickpea genotypes that were heat tolerant (HT) and heat sensitive (HS), drought tolerant (DT) or drought sensitive (DS), in the normal-sown well-watered (Control), normal-sown drought-stressed treatment (Drought), late-sown treatment well-watered (Heat), and late-sown drought-stressed (Heat + Drought) treatments. Values of l.s.d. (genotype × treatment): leaf APX activity 0.56, seed APX activity 0.36, leaf GR activity 0.55, seed GR activity 0.59. Values are means + s.e.m. ($n = 3$); within a parameter, means with the same letter are not significantly different.

(Fig. 7*d*) of the values in the leaves. Individual treatments of drought stress and heat stress each increased antioxidant levels above the controls, with drought stress generally the antioxidant level to a greater extent than heat stress, which is similar in the observations in leaves (except for CAT, where heat stress tended to induce higher activity in the leaves; Fig. 5*c*, *d*). Combined heat + drought stress reduced the activity of all antioxidants in the seeds of the heat- and drought-sensitive genotypes compared with the heat stress treatment (except APX for heat-sensitive genotypes; Fig. 6*b*). However, the heat- and drought-tolerant genotypes had similar levels of antioxidant activity in the heat stress and heat + drought stress treatments (Figs 5*b*, *d*, 6*b*, *d*, 7*b*, *d*).

Seed content of ASC increased by 24–49% in drought-stressed plants and by 7–24% in heat-stressed plants, with a significantly greater increase in genotypes tolerant to drought and heat than in the sensitive genotypes. In the combined heat + drought stress treatment, the genotypes tolerant of drought and heat showed a 14–21% increase, whereas the sensitive genotypes showed a 15.6–25% decrease relative to the control.

Seed GSH showed an increase of 32–102% in drought-stressed plants and 30–82% in heat-stressed plants. The increase

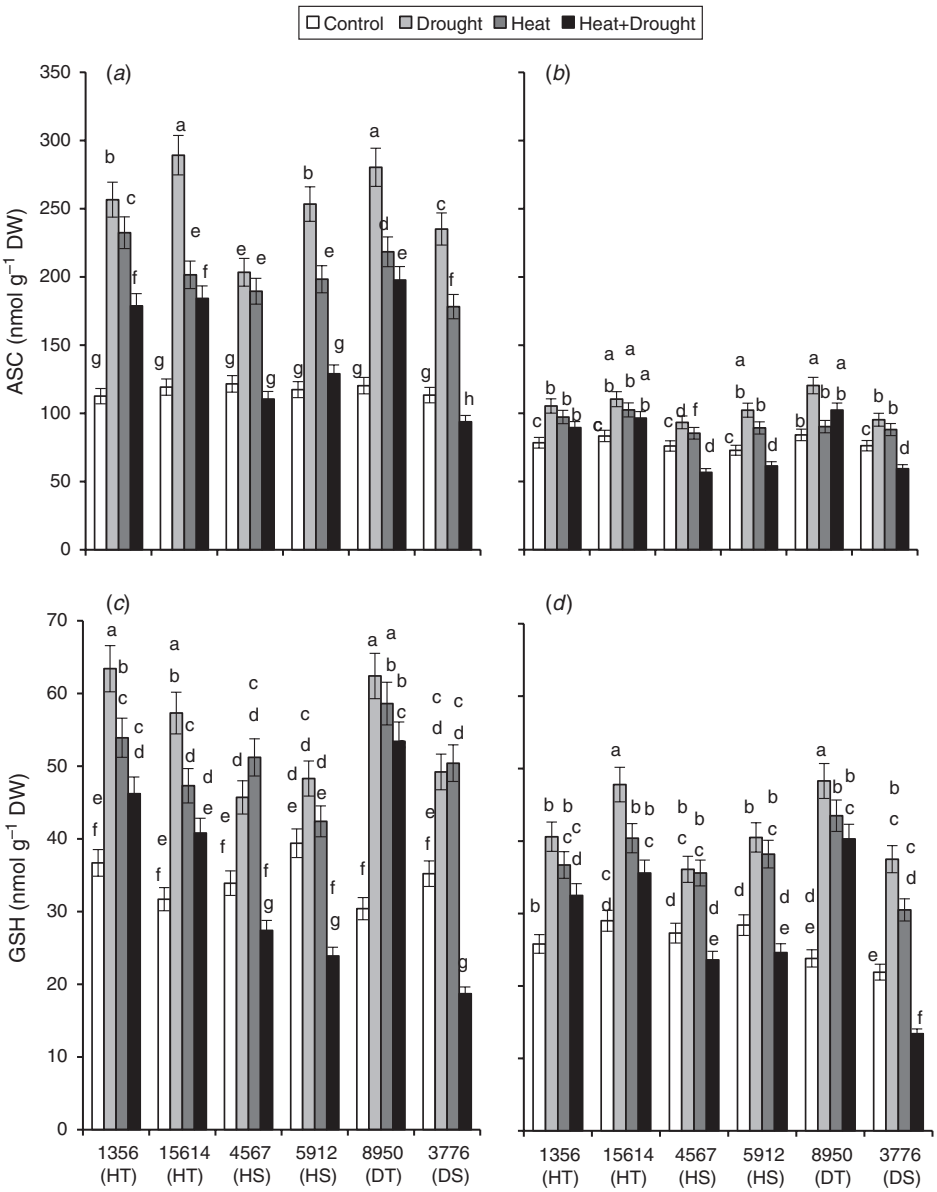

Fig. 7. (*a*, *b*) Ascorbic acid (ASC) and (*c*, *d*) reduced glutathione (GSH) contents in the leaves (*a*, *c*) and seeds (*b*, *d*) of six chickpea genotypes that were heat tolerant (HT) and heat sensitive (HS), drought tolerant (DT) or drought sensitive (DS), in the normal-sown well-watered (Control), normal-sown drought-stressed (Drought), late-sown well-watered (Heat) and late-sown drought-stressed (Heat + Drought) treatments. Values of l.s.d. (genotype × treatment): leaf ASC content 12.4, seed ASC content 8.9, leaf GSH content 5.6, seed GSH content 6.5. Values are means + s.e.m. ($n = 3$); within a parameter, means with the same letter are not significantly different.

was significantly more in the tolerant than the sensitive genotypes. Under heat + drought stress, GSH levels were lower than under individual stress treatments. GSH in genotypes tolerant to drought and heat showed a 22–69% increase, whereas GSH decreased by 13–38% in sensitive genotypes relative to the control.

Correlations

In the combined stress treatment, seed yield was positively correlated ($r = 0.82$) with aboveground biomass (stems, leaves and pod shells), and leaf chlorophyll content and photosynthetic efficiency (leaf PSII) were strongly and positively associated with biomass ($r = 0.87$, $r = 0.71$, respectively) and with seed yield ($r = 0.96$, $r = 0.94$, respectively) (Table 4). By contrast, leaf membrane damage was associated with a reduction in biomass ($r = –0.70$) and seed yield ($r = –0.76$), which suggests that membrane damage plays a vital role in inhibiting growth and seed yield. The correlations of biomass and seed weight were highly negative with contents of oxidative molecules MDA and H_2O_2 in leaves ($r = –0.62$, $r = –0.85$, respectively)

Table 4. Correlation coefficients (*r*) for leaf and seed traits with shoot biomass (leaves, stems and pod shells) and seed yield

EL, Electrolyte leakage; PSII, photosynthetic efficiency; Chl, chlorophyll; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; ASC, ascorbic acid; GSH, glutathione reduced

Trait	Biomass	Seed yield
Leaf EL	–0.696	–0.764
Leaf PSII	0.712	0.943
Leaf Chl	0.867	0.963
Biomass	–	0.825
Seed yield	0.825	–
Leaf MDA	–0.616	–0.953
Leaf H_2O_2	–0.849	–0.892
Leaf SOD	0.119	0.335
Leaf CAT	0.541	0.859
Leaf APX	0.858	0.992
Leaf GR	0.781	0.970
Leaf ASC	0.893	0.946
Leaf GSH	0.880	0.993
Seed MDA	–0.870	–0.788
Seed H_2O_2	–0.945	–0.893
Seed SOD	0.755	0.803
Seed CAT	0.893	0.964
Seed APX	0.912	0.951
Seed GR	0.747	0.951
Seed ASC	0.772	0.957
Seed GSH	0.955	0.920

and seeds ($r = –0.87$, $r = –0.89$, respectively). The enzymatic antioxidants CAT, APX and GR and non-enzymatic antioxidants ASC and GSH had strong positive correlations with biomass and seed yield in the combined stress treatment.

Discussion

Drought stress and heat stress and their combination during seed filling all had major impacts on the growth and yield of chickpea. Withholding water in normal-sown plants for 13 days so that the RLWC decreased to 50% of that in well-watered controls reduced seed yields by 30% in the drought-tolerant and 66% in the drought-sensitive genotype, but also reduced yields by 45–51% in the heat-tolerant and 57–64% in heat-sensitive genotypes. Delaying planting to expose the plants to heat stress (day–night air temperatures >32°C−20°C) for at least 7 days during seed filling reduced the seed yields of the heat-tolerant genotypes by 25–28%, the heat-sensitive genotypes by 45–58%, the drought-tolerant genotype by 28% and the drought-sensitive genotype by 53% compared with the controls. Thus, the effect of delayed planting to induce heat stress during seed filling was smaller than of the drought stress imposed during seed filling in chickpea sown at the normal time.

The genotypic variation for heat tolerance and sensitivity and drought tolerance and sensitivity identified in field studies in India was confirmed in this pot study. The genotypes selected for drought tolerance (high yields) and drought sensitivity (low yields) under drought conditions in the field (Krishnamurthy *et al*. 2010) were also drought-tolerant and drought-sensitive in this pot study. Likewise, the genotypes selected for heat tolerance (high yields) and heat sensitivity (low yields) when late-planted in the field (Krishnamurthy *et al*. 2011) were also heat tolerant and heat sensitive when late-planted in this study. The study has also shown that there is considerable crossover between heat and drought tolerance among chickpea genotypes, with drought-tolerant genotypes showing greater tolerance to heat than drought-sensitive lines, and heat tolerant genotypes showing greater tolerance to drought than the heat-sensitive genotypes. Compared with the well-watered plants, imposing drought stress, whether on normal-sown or late-sown chickpeas, reduced the seed yields of the heat-tolerant, heat-sensitive, drought-tolerant and drought-sensitive genotypes by a similar percentage (30%, 51%, 19% and 56%, respectively). We recognise that delaying planting to expose the plants to heat stress sped up the phenology, and that the reduced aboveground biomass may have been a consequence of this. However, seed yield was less affected than aboveground biomass by the delay in planting, so that the harvest index averaged across all genotypes was 34% in the drought stress treatment, but was 48% with delayed planting. This was higher than in the controls (44%) and similar to the combined heat + drought stress (47%), supporting the argument that the effects of delayed planting were not simply due to heat stress during seed filling, but also due to the faster phenological development. Nevertheless, the main purpose of this study was to determine the effects of drought and heat on growth and seed filling, and to determine whether the changes in aboveground biomass and seed yield can be explained by changes in the biochemistry induced by heat and drought treatments.

Seed filling is a critical growth stage in grain crops and involves various biochemical processes related to leaf assimilation and the synthesis of carbohydrates, proteins and lipids in the seeds. Seed yield and biomass were closely and positively associated with rates of photosynthetic efficiency and chlorophyll content of the leaves, suggesting that both aboveground biomass and seed yield were determined by the influence of the drought and heat treatments on photosynthetic capacity and assimilate transfer to the grain during the stress treatments.

Oxidative damage severely affects seed filling by hampering all metabolic processes, thereby damaging membrane properties, degrading proteins and deactivating enzymes, which further reduces yield (Farooq *et al*. 2009, 2016). In the present study, the chickpea plants grown under heat, drought and combined heat + drought stress showed a significant increase in oxidative stress, measured as an increase in MDA and H_2O_2 contents in the leaves and seeds. Both of these molecules increased markedly when plants were treated with heat or drought individually, more so under drought, and the combined heat + drought stress treatment increased these molecules further, suggesting an intensification of the damage. Oxidative molecules are formed by the leakage of electrons from electron-transport activities in chloroplasts, mitochondria and plasma membranes or as a by-product of various metabolic pathways localised in different cellular compartments during stress conditions, resulting in oxidative damage (Sharma *et al*. 2012; Suzuki *et al*. 2012). Cellular membranes are particularly susceptible to oxidative damage due to increased electrolyte leakage associated with lipid peroxidation, which was observed in the leaves in our study.

The oxidative damage was much higher in the leaves and seeds of sensitive than tolerant genotypes, which was correlated

with increased damage to the chlorophyll, photosynthetic efficiency and membranes, and a reduction in aboveground biomass and seed yield in all stress treatments. The increase in MDA content in our studies matches findings in drought-stressed plants (e.g. chickpea, Patel and Hemantaranjan 2012; soybean, Akitha Devi and Giridhar 2015; hyacinth bean, Rai *et al*. 2015), in heat-stressed plants (e.g. chickpea, Kumar *et al*. 2013*b*; mungbean (*Vigna radiata* L.), Mansoor and Naqvi 2013), and in combined heat + drought-stressed plants (e.g. birdsfoot trefoil (*Lotus japonicus*), Sainz *et al*. 2010; cotton, Sekmen *et al*. 2014). Likewise, the increased H_2O_2 levels observed in the present study are similar to observations in drought-stressed maize (Voothuluru and Sharp 2013), sugarcane (Boaretto *et al*. 2014) and field pea (Karataş *et al*. 2014), and heat-stressed wheat (Kumar *et al*. 2013*a*) and mungbean (Saleh *et al*. 2007; Mansoor and Naqvi 2013). The oxidative damage was much higher in the presence of both stresses in our studies, which is similar to observations recorded in birdsfoot trefoil (Sainz *et al*. 2010), cotton (Sekmen *et al*. 2014) and purslane (*Portulaca oleracea* L., Jin *et al*. 2015). In the present study, the content of MDA and H_2O_2 increased more in leaves than seeds under all stress treatments, suggesting higher oxidative damage in leaves than seeds.

Plants activate different enzymatic and non-enzymatic antioxidants to cope with oxidative damage (Hasanuzzaman *et al*. 2012; Suzuki *et al*. 2014). In our study, the chickpeas had increased activity of SOD (catalyses dismutation of superoxides), and CAT (dissociates hydrogen peroxide under individual heat or drought stress), which was in accordance with the rise in MDA and H_2O_2 in leaves and seeds. These findings concur with studies on drought-stressed plants of wheat (Devi *et al*. 2012), soybean (Akitha Devi and Giridhar 2015) and hyacinth bean (Rai *et al*. 2015), and heat-stressed plants of chickpea (Kaushal *et al*. 2011), wheat (Kumar *et al*. 2013*a*; Wang *et al*. 2015) and cucurbit species (Ara *et al*. 2013). In the combined heat + drought stress treatment, tolerant genotypes had higher CAT activity in leaves and seeds than sensitive genotypes. The combined heat + drought stress treatment severely inhibited CAT activity in seeds in the sensitive genotypes, which possibly increased H_2O_2 contents.

The enzymes APX (detoxifies H_2O_2 using ascorbate as a substrate) and GR (converts GSSG to GSH) are involved in the removal of H_2O_2 through the ascorbate–glutathione pathway (Mittler 2002; Suzuki and Mittler 2006). In the present study, increased activities of these enzymes, implied efficient detoxification of H_2O_2 in leaves and seeds in the individual stress treatments. These results are similar to findings in other plant species under drought stress, including chickpea (Patel and Hemantaranjan 2012), wheat (Devi *et al*. 2012) and barley (*Hordeum vulgare* L., Harb *et al*. 2015), and under heat stress, including wheat (Sairam *et al*. 2000; Wang *et al*. 2015, 2016) and mungbean (Kumar *et al*. 2011). In the combined heat + drought stress treatment, APX and GR activities in leaves and seeds were significantly suppressed compared with other treatments, less so in the tolerant genotypes than the sensitive genotypes. Thus, a combined heat + drought stress tolerance was associated with the ability to maintain higher activities of APX and GR in tolerant genotypes compared with the sensitive genotypes, as also reported in studies on cotton (Sekmen *et al*. 2014).

Non-enzymatic antioxidants such as ASC and GSH increased significantly in leaves and seeds of plants under the individual stress treatments. These two molecules are used in the ascorbate–glutathione cycle, which involves APX and GR enzymes (Kopczewski and Kuzniak 2013; Saed-Moucheshi *et al*. 2014), and their levels need to be maintained under stress to protect the cells from oxidative damage. Increased ASC and GSH contents in our studies are in accordance with previous studies in drought-stressed maize and rice (Nayyar and Gupta 2006; Chugh *et al*. 2011), wheat (Khanna-Chopra and Selote 2007; Du *et al*. 2013) and chickpea (Patel *et al*. 2011). GSH and ASC contents increased under heat stress, which matches observations in heat-stressed maize and rice (Kumar *et al*. 2012), mungbean (Kumar *et al*. 2011) and chickpea (Kumar *et al*. 2013*b*), indicating their significance in defence against heat stress. The combined heat + drought stress treatment decreased ASC and GSH contents in leaves and seeds, to a lesser extent in the tolerant than the sensitive genotypes, which may be a result of inhibited restoration due to a reduction in APX and GR activities. The endogenous contents of ASC and GSH correlated positively with the activities of APX and GR in the tolerant and sensitive genotypes, which further validates the fundamental role of the ascorbate–glutathione pathway in governing stress tolerance.

Stresses

Drought stress resulted in more oxidative damage (as indicated by MDA and H_2O_2 levels) than heat stress, possibly due to more dehydration of the tissues. Moreover, under drought stress, stomatal conductance (Awasthi *et al*. 2014) decreased, which likely resulted in an increase in leaf temperature and increased oxidative damage. This was apparent from the greater damage to membranes in drought-stressed than heat-stressed plants. At the same time, in most of the genotypes, various antioxidants such as CAT, APX, GR, ASC and GSH were significantly higher in the drought stress than the heat stress treatment. When both stresses were combined (heat + drought), oxidative damage (as MDA and H_2O_2 levels), along with membrane damage, increased markedly compared with the heat or drought stress treatments alone. This may be associated with a rapid dehydration of the leaf and seed tissues at high temperatures, as well as an increase in leaf temperature in the combined heat + drought treatment, which combined to induce a higher production of lipid peroxides and H_2O_2. All of the antioxidants decreased markedly in the combined heat + drought stress treatment compared with the single-stress treatments, as shown in a previous study with cotton (Sekmen *et al*. 2014), perhaps due to an overall reduction in cellular protein synthesis, which likely impaired the availability of various substrates such as ASC and GSH. It has been reported that ROS cause lipid peroxidation, and consequently membrane injury, protein degradation and enzyme inactivation (Sairam *et al*. 2005). Oxidative stress may also cause protein oxidation, with a loss of enzyme activity and the formation of protease-resistant cross-linked aggregates (Berlett and Stadtman 1997).

Organs

There were variations between the leaves and seeds with respect to oxidative damage, with leaves showing more damage than seeds, as shown by higher levels of MDA and H_2O_2. Accordingly,

the expression of various antioxidants was also found to be higher in leaves than in seeds. The greater oxidative damage in leaves might be related to the direct impact of the stress and more surface area exposed to high temperature and drought than in the seeds. Consequently, water relations are more affected in leaves than in seeds, possibly due to reduced vasculature in the latter. There were small differences in the expression pattern of the antioxidants in the leaves and seeds, depending on the stress environment and genotype. For example, CAT was expressed more in response to drought stress in seeds than in leaves, whereas in other antioxidants, no clear differences were discernible. The expression pattern of antioxidants was higher in tolerant genotypes than in sensitive genotypes in both leaves and seeds, suggesting a common defensive strategy in these two organs.

Genotypes

Chickpea genotypes responded differently to drought stress, heat stress and the combination of heat + drought stress at seed filling, the different responses being associated with variations in physiological and biochemical mechanisms. In the combined heat + drought stress treatment, tolerant genotypes, particularly the drought-tolerant genotype, produced significantly higher yields than the other genotypes, which correlated positively with pod and seed numbers (data not shown). Tolerant genotypes had larger seeds, which were linked to higher carbon assimilation and sucrose transport, than sensitive genotypes (Awasthi *et al*. 2014). Leaves of tolerant genotypes also had higher contents of chlorophyll, higher photosynthetic efficiency and generally less membrane damage, which corresponds with observations in other crops under similar situations of heat or drought stress applied individually (Kumar *et al*. 2012; Almeselmani *et al*. 2012; Mishra *et al*. 2012; Wang *et al*. 2016). Oxidative damage was less in the heat- and drought-tolerant genotypes, as reported for heat-tolerant genotypes of wheat (Almeselmani *et al*. 2006) and drought-tolerant genotypes of maize (Chugh *et al*. 2013), which correlated with less damage to membranes and chlorophyll. It was further observed that tolerant genotypes had significantly higher antioxidants, particularly APX and GR, as well as ascorbate and glutathione, in both leaves and seeds, which was associated with less oxidative damage. One drought-tolerant genotype in our study (ICC8950) had a higher degree of tolerance to the combined effects of heat and drought than the heat-tolerant genotypes, suggesting cross-tolerance of these two stresses, as indicated previously in chickpea (Canci and Toker 2009), and the likely presence of some common defence mechanisms. This can be attributed to the observed higher antioxidative ability, particularly of CAT, APX and GR in the leaves of this genotype, compared with others, under the combined heat + drought stress treatment. Future studies should explore the testing of more heat- and drought-tolerant chickpea genotypes against combined heat and drought stress to determine the adaptive mechanisms concerning antioxidants at the molecular level, which should be helpful for breeding for combined stress tolerance.

Conclusion

Our findings indicate that the intensity of oxidative stress varies in response to drought, heat and their combination. Drought alone and in combination with heat stress in late-sown plants resulted in more oxidative damage than in fully irrigated heat-stressed plants. With the combined heat and drought stress, we suggest that there was severe dehydration and a rise in leaf temperature due to reduced stomatal conductance, which exacerbated the oxidative damage. The expression of various antioxidants also varied according to the stress. Even though aboveground biomass and seed yield were reduced, the plants were able to defend themselves to some extent from the individual stresses, but these defences failed under the combination of heat + drought stress. We suggest that this was likely due to severe enzymatic inactivation arising from dehydration and the simultaneous elevation of the temperature, which caused a marked reduction in seed yield in all of the genotypes. A partial cross-tolerance existed for heat- and drought-tolerant genotypes, particularly for the drought-tolerant genotype, which was able to perform better under the combination of heat + drought stress. Studies are required to explore the underlying mechanisms associated with the combined heat + drought tolerance.

Conflicts of interest

The authors declare no conflicts of interest.

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