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Individual and combined effects of transient drought and heat stress on carbon assimilation and seed filling in chickpea

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Abstract. High temperatures and decreased rainfall are detrimental to yield in chickpea (*Cicer arietinum* L.), particularly during grain filling. This study aimed to (i) assess the individual and combined effects of drought and heat stress on biochemical seed-filling processes, (ii) determine genotypic differences in heat and drought tolerance, and (iii) determine any cross-tolerance. Plants were grown outdoors in the normal growing season when temperatures during seed filling were <32−20°C or were planted late (temperatures >32−20°C; heat stress). Half of the pots were kept adequately watered throughout, but water was withheld from the others from the initiation of seed filling until the relative leaf water content reached 50% of the irrigated plants (drought stress); all plants were rewatered thereafter until seed maturit. Water was withheld for 13 days (normal sowing) and 7 days (late sowing), so soil moisture decreased by 54–57%. Tests on leaves and seeds were performed after the stress. Individual and combined stress damaged membranes, and decreased cellular oxidising ability, stomatal conductance, PSII function and leaf chlorophyll content; damage was greater under combined stress. Leaf Rubisco activity increased with heat stress, decreased with drought stress and decreased severely with combined stress. Sucrose and starch concentrations decreased in all seeds through reductions in biosynthetic enzymes; reductions were greater under combined stress. These effects were more severe in heat- and drought-sensitive genotypes compared with drought-tolerant genotypes. Drought stress had a greater effect than heat stress on yield and the biochemical seed-filling mechanisms. Drought- and heat-tolerant genotypes showed partial cross-tolerance.

Additional keywords: *Cicer arietinum*, high temperature, seed yield, water stress.

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Introduction

Drought and high temperature are important environmental factors restricting crop yields in many regions of the world. Although the two stresses often occur simultaneously (Shah and Paulsen 2003), relatively little is known about how their combination affects the legumes. Of further concern is global climate change, which seemingly will increase global temperature, alter the distribution of precipitation and intensify drought in semiarid and arid areas (Wigley and Raper 2001; Chaves *et al*. 2003; Turner and Meyer 2011), leading to less crop productivity in many regions of the world (Bai *et al*. 2004; Müller *et al*. 2009). The combined effects of heat and drought stress have been investigated in wheat (*Triticum aestivum* L.) (Wardlaw 2002), canola (*Brassica napus* L.) (Faraji *et al*. 2008), maize (*Zea mays* L.) (Cairns *et al*. 2013) and groundnut (*Arachis hypogea* L.) (Hamidou *et al*. 2013), but only to a limited extent in chickpea (*Cicer arietinum* L.) (Canci and Toker 2009). A combination of drought and heat stress affected the growth and productivity of these crops more than each individual stress (Canci and Toker 2009; Hamidou *et al*. 2013). Furthermore, combined drought and heat stress alters plant metabolism in a novel manner compared with single stresses due to differential activity and expression of metabolic pathways (Rizhsky *et al*. 2004; Wang and Huang 2004). In the perennial grass *Leymus chinensis* (Trin.) Tzvel, high temperature combined with severe soil drought reduced the function of PSII, weakened nitrogen

anabolism, strengthened protein catabolism and provoked lipid peroxidation (Xu and Zhou 2006). In general, the physiological and biochemical responses to heat stress, drought stress and the combination of the two are not well understood and need to be investigated further in each crop species.

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 high temperatures during seed development cause large yield losses in various crops, as reported for cereals (Barnabás *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 the phase of pod and seed set (Leport *et al*. 2006; Wang *et al*. 2006*a*; Krishnamurthy *et al*. 2010; Devasirvatham *et al*. 2012; Hamidou *et al*. 2013). Low leaf photosynthetic rates during seed filling (Singh *et al*. 1987) in heat-stressed chickpea plants are a major cause of reduced seed size with water shortage (Leport *et al*. 1998). The accumulation of various seed components (mainly starch and proteins) may be inhibited by heat or drought stress (Behboudian *et al*. 2001) due to inactivation of enzymatic processes involving starch (Wilhelm *et al*. 1999; Ahmadi and Baker 2001) and protein synthesis (Triboï *et al*. 2003).

Sucrose metabolism in leaves and seeds is critical for seed filling and a fundamental role has also been implicated for hexose–sucrose balance in regulating vital aspects of seed development (Weschke *et al.* 2000). Sucrose synthesised by the leaves or seeds is imported by the seeds and can be cleaved by invertase (Sturm and Tang 1999). The activity of vacuolar and cell wall-bound acid invertases predominates during kernel development, as observed in maize (Weschke *et al.* 2000). Drought stress during kernel development in maize decreases the activity of both vacuolar and cell wall-bound acid invertase (Zinselmeier *et al.* 1999; Andersen *et al*. 2002), with parallel reductions in ovary growth and hexoses. Moreover, metabolic pools downstream of sucrose in the starch formation pathway are depleted to impair seed filling (Zinselmeier *et al.* 1999). Hence, heat and drought stresses may compromise the metabolic and assimilate transfer processes necessary to accomplish seed filling.

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 extremely detrimental for seed yield (Canci and Toker 2009). Previous studies reported the inhibitory effects of drought stress on seed growth of chickpea genotypes (Davies *et al*. 1999; Yadav *et al*. 2006), as well as variations in seed yield among genotypes subjected to drought stress (Leport *et al*. 2006) and heat stress (Summerfield *et al*. 1984; Yang *et al*. 2004). Biochemically, in chickpea genotypes under drought stress during seed filling, a significant and positive association was observed between seed DW at maturity and peak sucrose synthase activity in two genotypes (Kumar and Turner 2009; Turner *et al*. 2009). Relatively, little information exists on the effects of heat stress alone or in combination with drought stress in chickpea. The objectives of this study were to: (i) assess the individual and combined effects of drought and heat stress on the biochemical processes related to seed filling, (ii) investigate how seed-filling processes are affected in chickpea genotypes differing in heat and drought sensitivity, and (iii) determine whether there is any cross-tolerance for these two stresses.

Materials and methods

Genotypes

Six contrasting chickpea (*Cicer arietinum* L.) genotypes – two heat-tolerant (ICC1356, ICC15614), two heat-sensitive (ICC4567, ICC5912), one drought-tolerant (ICC8950) and one drought-sensitive (ICC3776) – with matching phenology were used in this study. The details about origin and phenology of the genotypes are given in Table 1. These genotypes were identified in a screening of the chickpea minicore collection as being heat-tolerant or heat-sensitive (Krishnamurthy *et al*. 2011; Devasirvatham *et al*. 2012), or drought-tolerant or drought-sensitive (Krishnamurthy *et al.* 2010) on the basis of their seed yields under heat or drought stress in the field.

Raising of plants

The six genotypes were sown in 144 earthen pots on either (a) November 2011 or (b) February 2012, and were grown outdoors in a wire-covered shelter to prevent damage from birds and animals at Panjab University, Chandigarh, India (30°44′5.9994″N, 76°47′27.5994″E). In northern India, chickpea is usually sown in November so that temperatures during reproductive development are below 32°C : 20°C (maximum : minimum), whereas sowing in February was to ensure that the plants were exposed to high temperatures (above 32°C : 20°C) during reproductive development. The daily maximum, minimum and mean air temperatures (Fig. 1), and daily maximum, minimum and mean relative humidity (Fig. 1) were recorded between 1 November 2011 and 15 May 2012. When plants were sown in November, the daily maximum and minimum temperatures during seed filling were below 32°C : 20°C, and when plants were sown in February, temperatures were above 32°C : 20°C. Relative humidity during seed filling ranged from 70–85% : 25–48% night : day when plants were sown in November and 40–70% : 11–29% when plants were sown in February (Fig. 1). The photoperiod varied from 11.1 h to 11.5 h when sown in November and 12.0 h to 12.6 h when sown in February.

A sandy loam soil was mixed with sand in a 3 : 1 ratio and then one part of farmyard manure was added to three parts of the soil–sand mixture along with 10 mg kg^{-1} tricalcium phosphate

Table 1. Details about the source, yield and phenology of the chickpea genotypes used in the present study

HT, heat-tolerant; HS, heat-sensitive; DT, drought-tolerant; DS, drought-sensitive

Genotype	Desi or kabuli	100-seed weight	Country source
1356 (HT)	Yellow brown (desi)	14.9	India
15614 (HT)	Yellow brown (desi)	14.6	Tanzania
4567 (HS)	Dark brown (desi)	13.7	India
5912 (HS)	Mosaic (kabuli)	16.4	India
8950 (DT)	Yellow brown (desi)	13.2	India
3776 (DS)	Black (desi)	10.5	Iran

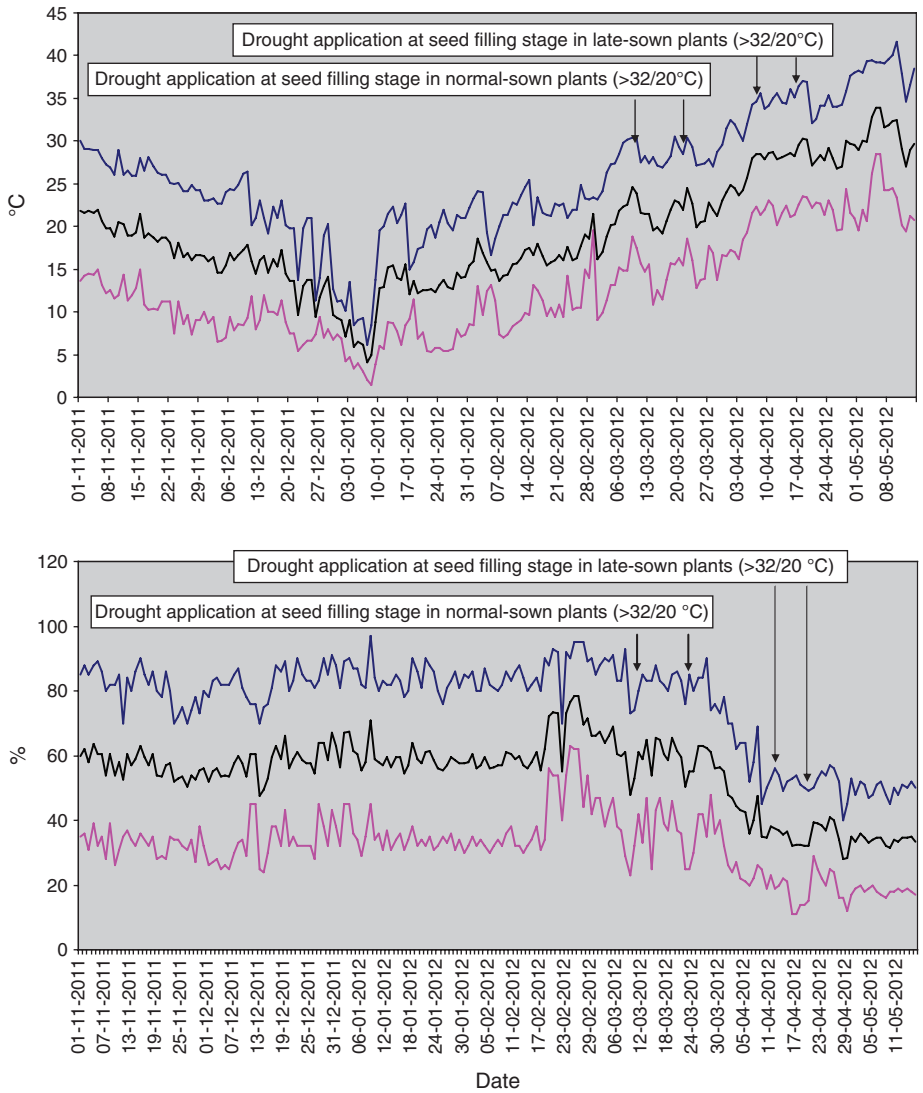

Fig.1. (*a*) Maximum (top line), minimum (bottom line) and mean (middle line) air temperatures, and (*b*) relative humidity at the experimental site from 1 November 2011 to 15 May 2012. The arrows show the time that the drought treatment was imposed (first arrow) and completed (second arrow) on the chickpeas sown in November 2011 (normal sowing) and February 2012 (late sowing).

fertiliser and used to fill earthen pots 300 mm in diameter (8 kg soil capacity). Seeds were inoculated with *Rhizobium ciceri* before sowing. Five seeds were sown in each pot and thinned to three per pot 20 days after sowing. Plants were watered regularly to prevent any water shortage until the water treatments were applied. There were 12 pots per genotype in three replications (a total of 36 pots per genotype) in each of four treatments. The pots were completely randomised.

Drought stress application in normal and late-sown (heat stress) conditions

The plants were kept well watered to maintain ~100% field capacity of the soil until the onset of the seed filling stage. During seed filling (seed size 3–4 mm), when the plants were at 75% podding, water was withheld from half the pots from each sowing time. There were therefore four treatments: (i) normal planting time, watered throughout (controls) every day to maintain ~100% field capacity of the soil; (ii) normal planting time but water withheld (drought stress); (iii) late sowing and watered every day to maintain them at ~100% field capacity of the soil (heat stress); and (iv) late sowing and water withdrawn (combination of heat and drought stress). The water stress continued until the relative leaf water content (RLWC) of leaves on the top three branches) reached 50% of those growing on fully irrigated plants having a RLWC of 85–90%. It was attained in 13 days in the plants sown at the normal planting time and in 7 days in late-sown plants. The soil water content decreased by ~54–57% (DW basis, 7–12 cm depth) in the drought-stressed normally sown plants (from 19.6% to 9%) and late-sown plants (from 19.1% to 8.2%). Thereafter, the water-

stressed plants growing under normal and late sowing conditions were rewatered until seed maturity.

Collection of samples

For biomass and yield parameters, observations were taken from threee plants per genotype and treatment in three replications at maturity. Aboveground biomass, yield and yield components such as pod number, seed number and mean individual seed weight were recorded. The plants were cut at soil level, the number of filled pods was counted, and the seeds were removed and counted before being oven-dried for 3 days at 45°C; average values of the three plants per pot were expressed on a per-plant basis. For stress injury and biochemical analysis, seeds and subtending leaves were collected at 1100 hours randomly from three plants per genotype (the second and third branches from the top) and treatments in three replications at the seed filling stage in the normal and late plantings. The samples of leaves and seeds for analysis were collected at the end of stress period before rewatering.

For collection of leaf and seed samples from drought-stressed plants, we relied upon a fixed value of RLWC as a physiological indicator of water deficit in the plants to obtain homogeneity for comparisons of biochemistry in the genotypes grown at the two different sowing times. The samples for biochemistry were collected from the second and third branches from the top when the RLWC reached 50% of the controls in each genotype under both sowing dates. In the late-sown plants, the samples were collected after the plants had experienced high temperatures (33−39°C : 22−24°C day : night) for at least 7 days consecutively in the heat stress treatment (Treatment iii in the previous section) and in the combination of drought and heat stress treatment (Treatment iv).

Stress injury to leaves

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

Cellular oxidising ability of leaves

This assay measures the activity of dehydrogenases in the cell, indicating the cellular oxidising ability. It was measured as the 2,3,5-triphenyl tetrazolium chloride (TTC) reduction ability, as per the method of Steponkus and Lanphear (1967). Fresh leaf samples (1 g) were cut into 1-cm strips, immersed in an incubation solution (50 mM sodium phosphate, pH 7.4) containing various TTC concentrations and incubated at 25°C in darkness. Since TTC reduction is sensitive to excessive oxygen, the incubation of TTC was done without shaking. After two extractions by 95% ethanol (5 mL each time), the extracts were combined and made up to 10 mL. The formazan formed in green tissues was measured at 530 nm instead of 485 nm to avoid interference by pigments such as chlorophyll (Steponkus and Lanphear 1967). The observations were expressed as absorbance g^{-1} DW.

Chlorophyll and leaf water content of leaves

For estimation of chlorophyll concentration, chlorophyll was extracted by grinding fresh leaves (1 g) in 80% acetone, followed by centrifugation at 1816*g* for 10 min. The absorbance of the supernatant was read at 645 nm and 663 nm, and the total chlorophyll was calculated (Arnon 1949) against 80% acetone as a blank. The chlorophyll content was measured according to Eqns 1–3:

$$Chla = 12.9(Abs_{663}) - 2.69(Abs_{645}) \times \frac{V}{1000 \times W}; \quad (1)$$

$$Chlb = 22.9(Abs_{645}) - 4.68(Abs_{663}) \times \frac{V}{1000 \times W}; \quad (2)$$

$$Total\ Chl = Chl\ a + Chl\ b. \quad (3)$$

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

The RLWC was measured according to the method of Barrs and Weatherley (1962). The fresh leaves were excised into smaller segments, weighed (FW) and then floated on distilled water under low light for 3 h to obtain the turgid weight (TW). Leaf samples were oven-dried at 80°C for 24 h and weighed (DW). RLWC was calculated as shown in Eqn 4:

$$\text{RLWC} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100. \quad (4)$$

The RLWC was expressed as a percentage.

Stomatal conductance

The stomatal conductance (g_s) of fully expanded leaves (from the second or third branches from the top) was measured using a portable leaf porometer (model SC1, Decagon Devices, Pullman, WA, USA) at 1100 hours during the stress period. The units were expressed as mmol m^{-2} s^{-1}.

Leaf photosynthetic function

PSII activity

The photochemical efficiency of the leaves (collected from the second or third branches from the top) was measured as chlorophyll fluorescence using the dark-adapted test of the modulated chlorophyll fluorometer (OS1-FL, Opti-Sciences, Tyngsboro, MA, USA) at 1100 hours. With this system, chlorophyll fluorescence is excited by a 660-nm solid-state light source, with filters blocking radiation at wavelengths above 690 nm. The average intensity of this modulated light was adjusted from 0 μE to 1 μE. Detection was in the 700–750 nm range using a PIN silicon photodiode with appropriate filtering to remove extraneous light. The clamps of the instrument were installed on the leaves to keep them in the dark and to stop the light reaction of photosynthesis for 45 min. After this, the clamps were attached to the optic fibre of the device and the valves of the clamps were opened. After starting the device, the 695-nm modulated light was radiated through the

optic fibre towards the leaf. Subsequently, the F_v/F_m ratio (the maximum quantum yield of PSII photochemistry) was recorded. The leaves tested for chlorophyll fluorescence were also used for the measurement of chlorophyll concentration. The PSII activity was expressed as the F_v/F_m ratio.

Enzymes

The photosynthetic function of leaves (from the second and third branches from the top) was assessed based on the activity of a key photosynthetic enzyme (Rubisco), the sucrose-synthesising enzymes sucrose phosphate synthase and sucrose synthase, the sucrose concentration and a sucrose catabolic enzyme (vacuolar acid invertase), starch phsophorylase (a starch-synthesising enzyme) and β-amylase (a starch-hydrolysing enzyme).

Assay of Rubisco activity in the leaves

The activity of Rubisco was estimated from the leaves by extraction in a precooled pestle and mortar in a buffer containing 50 mM 1,3-bis-tris (hydroxymethyl)methylamino propane (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM phenylmethyl-sulfonyl fluoride, 1 mM benzamindine, 1.5% polyvinyl polypyrrolidone and 3 mM 3-methylbut-2-ene-1-thiol, as per the method of Wang *et al.* (1992). The leaf extract was centrifuged at 29 068*g* for 40 min. The supernatant was quickly desalted at 4°C by passing it through 4-mL Sephadex G-25 columns (Sigma, St Louis, MO, USA) pre-equilibrated with buffer containing 20 mM Hepes–NaOH (pH 7.5), 0.25 mM $MgCl_2$, 0.01% 2-mercaptoethanol, 1 mM EDTA and 0.05% BSA. The desalted extract was assayed immediately. The enzyme activity was assayed by the method of Racker (1962). The enzyme extract was added to the assay medium to a final volume of 1 mL containing 1 M Tris buffer (pH 7.8), 0.006 M NADH, 0.1 M reduced glutathione, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025 M 3-phosphoglycerate kinase, 0.05% α-glycerophosphate dehydrogenase-triose phosphate isomerase, 0.025 M ribulose 1-5 biphosphate, 0.2 M ATP, 0.5 M $MgCl_2$ and 0.5 M $KHCO_3$. The oxidation of NADH was monitored at 340 nm during the conversion of 3-phosphoglycerate to glycerol 3-phosphate using a molar extinction coefficient of 6.22 mM cm^{-1}. One unit was defined as the amount that catalysed the cleavage of 1 µM RuBP per min. The reaction was recorded at 30-s intervals for 3 min at 25°C until a uniform change in absorbance was noticed. The activity was expressed as µmol NADH oxidised g^{-1} DW min^{-1}.

Assay of starch phosphorylase, β-amylase, sucrose synthase and vacuolar acid invertase activities in the leaves and seeds

For extraction of enzymes, the leaf tissue was homogenised in a chilled Hepes buffer containing 50 mM L^{-1} NaOH pH 7, 2 mM L^{-1} $MgCl_2$, 1 mM L^{-1} EDTA and 2 mM L^{-1} DTT according to Dejardin *et al.* (1997). All operations were carried out at 4°C. The homogenate was centrifuged for 20 min at 16 350*g* in a cold centrifuge. The supernatant was quickly desalted at 4°C by passing it through 4-mL G-25 columns (Sephadex) pre-equilibrated with a buffer containing 20 mM Hepes–NaOH (pH 7.5), 0.25 mM $MgCl_2$, 0.01% 2-mercaptoethanol, 1 mM EDTA and 0.05% BSA. The desalted extract was assayed immediately. β-Amylase activity was assayed as described by Shuster and Gifford (1962). The reaction mixture, consisting of 0.2 mL enzyme extract and 1 mL freshly-prepared starch solution (0.2%), was incubated at 30°C for 1 h. The reaction was terminated by adding 1 mL of 3,5-dinitrosalicylic acid (DNSA) reagent. After this, the tubes were placed into boiling water for 10 min and then cooled to room temperature. Two mL of distilled water was added to each tube and the absorbance was recorded at 560 nm using glucose as a standard. A control for every reaction mixture was run to check the level of endogenous sugars. The activity calculated for standard curve of glucose and was expressed as µmoles glucose formed g^{-1} DW.

Starch phosphorylase activity was assayed as per the method described by Baun *et al.* (1970). The enzyme extract (0.2 mL) was incubated with 0.6 mL Tris–maleate buffer (pH 6.5) containing 1 mM NaF followed by the addition of 0.2 mL of 0.05M glucose 1-phosphate. The reaction mixture was incubated for about 1 h at 30°C and the reaction was terminated by adding 0.5 mL cold 5% trichloroacetic acid. The mixture was centrifuged at 29 068*g* to settle protein precipitate and the supernatant was used to estimate inorganic phosphate. For measuring the inorganic phosphate, 0.5 mL of supernatant was added to 3.3 mL distilled water and 1 mL ammonium molybdate reagent (1.5 g ammonium molybdite + 30 mL concentrated HCl, diluted to 100 mL with distilled water). The tubes were shaken well and after ~5 minutes, 0.2 mL of Fiske and Subbarow reagent was added (1.45 g sodium metabisulfite, 50 mg sodium sulfite and 25 mg 1-amino-2-napthol-2-sulfonic acid dissolved in 5 mL water to make a final volume of 10 mL). Blanks were run simultaneously with the heat-inactivated enzyme extract. The mixture was incubated for 15 min at about 30°C and the absorbance was recorded at 660 nm using monopotassium phosphate as a standard. The activity was calculated from the standard curve of monopotassium phosphate and expressed as nmoles of inorganic phosphate min^{-1} g^{-1} DW.

Sucrose synthase activity was assayed as per Hawker *et al.* (1976). The enzyme extract was added to the reaction mixture comprising 0.015 M uridine diphosphate glucose, 0.05 M fructose and 0.2 M Tris–HCl buffer (pH 8.2) containing 0.025 M $MgSO_4$. The abovementioned reaction mixture was incubated at 37°C for 30 min; the reaction was stopped by heating the contents in a boiling water bath for 10 min and then cooling it. Residual fructose was destroyed by adding 0.5 mL of 6% KOH and heating the contents in a boiling water bath for 20 min. After cooling the contents to room temperature, 1 mL of 1% resorcinol solution and 3 mL of 30% hydrochloric acid were added. The contents were incubated for 10 min at 80°C and the intensity of the developed pink colour was read at 490 nm. Blanks were run simultaneously with the heat-inactivated enzyme extract. The concentration of sucrose was calculated from the standard graph prepared by using sucrose as a standard (40–280 mg mL^{-1}). The activity was expressed as µmoles sucrose produced g^{-1} DW h^{-1}.

Vacuolar acid invertase activity was measured as per Nygaard (1977). The reaction mixture was prepared by adding 0.6 mL of a 0.2-M acetate buffer pH 4.8 and 0.3 mL of a 0.4-M sucrose solution (prepared in 0.2 M acetate buffer) in 0.1 mL of enzyme

extract. In control tubes, sucrose was added only when the enzyme preparation had been inactivated by boiling for 5 min. After incubation at 30°C for 30 min, 1 mL of DNSA was added to the reaction mixture. Afterwards, tubes were placed in a boiling water bath for 10 min and then cooled to room temperature. The samples were diluted to 5 mL and absorbance was recorded at 560 nm using glucose as a standard. Blanks were run simultaneously with the heat-inactivated enzyme extract. The activity was calculated from the standard curve of glucose and expressed as μmoles glucose produced g^{-1} DW h^{-1}.

Analysis of sucrose and reducing sugars

Sucrose concentration was measured according to the enzymatic method of Jones *et al.* (1977). Leaf tissue was extracted with 80% ethanol at 80°C three times for 1.5 h for each extraction. The extracts were pooled, evaporated at 40°C in a forced-draught oven and subsequently used for sucrose assays. Aliquots of 200 μL from standard sucrose and samples were added to 1 mL of a reaction mixture comprising an imidazole buffer 100 mM (pH 6.9; 40 mM imidazole base, 60 mM imidazole–HCl), 0.4 mM $NADP^+$, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02% (w/v) BSA, 20 μg mL^{-1} yeast invertase (EC3.2.1.26), 2 μg mL^{-1} yeast hexokinase (EC 2.7.1.1) and 1 μg mL^{-1} yeast phospho-glucoisomerase (EC 5.3.1.9) and incubated for 30 min at 25°C to allow conversion of glucose and fructose to glucose 6-phosphate. The absorption was read at 340 nm. Eighty-five μL of glucose-6-phosphate dehydrogenase (70 units mL^{-1}) was added, mixed well and re-read after ~5 min when the absorbance became constant. Blanks were run with 200 μL of the extract and 1 mL of the reaction mixture without invertase. The readings obtained from each sample were converted to sucrose concentrations using a standard curve and expressed as μmoles sucrose g^{-1} DW.

To estimate reducing sugars, 1 mL DNSA reagent was added to 1 mL ethanol extract (prepared as above for sucrose estimation). The reaction mixture was boiled for 12 min, 2 mL distilled water was then added and the absorbance was recorded at 560 nm against a blank containing 80% ethanol in place of the ethanol extract. The concentration of reducing sugars was calculated from a standard curve plotted with known concentrations of glucose (Sumner and Howell 1935) and expressed as μmoles glucose g^{-1} DW.

Statistical analyses

Data were analysed by ANOVA using three factorial experimental designs using VSN Genstat v. 14 statistical software. The principal components analysis was conducted using Genstat. Standard errors and LSDs ($P < 0.05$) for genotypes, treatments and their interaction are presented in the Tables 1–6 and Figs 2–7.

Results

Phenology and biomass

Days to flowering, podding and maturity were accelerated markedly in heat-stressed plants of all genotypes (Tables 2–4). Drought stress reduced the days to maturity. When drought stress was imposed at the podding stage in heat-stressed plants at the time of seed filling, days to maturity decreased further. The sensitive genotypes for heat and drought matured significantly earlier than the tolerant genotypes, which reduced their seed yield to a significantly higher extent.

Table 2. Days to flowering in six chickpea genotypes in controls, and under drought stress, heat stress and combined stress

LSD ($P < 0.05$) for water × sowing × genotype: 2.9. Values having the same letters within a column do not differ significantly from each other. HT, heat-tolerant; HS, heat sensitive; DT, drought-tolerant; DS, drought-sensitive

Genotypes	Control	Drought stress	Heat stress	Heat + drought stress
1356 (HT)	61 ± 2.3a	61 ± 2.3a	44 ± 2.2a	44 ± 2.2a
15614 (HT)	59 ± 2.1a	59 ± 2.1a	42 ± 2.5a	42 ± 2.5a
4567 (HS)	61 ± 2.4a	61 ± 2.4a	45 ± 2.4a	45 ± 2.4a
5912 (HS)	58 ± 2.2a	58 ± 2.2a	43 ± 2.6a	43 ± 2.6a
8950 (DT)	60 ± 2.5a	60 ± 2.5a	42 ± 2.2a	42 ± 2.2a
3776 (DS)	62 ± 2.3a	62 ± 2.3a	42 ± 2.3a	42 ± 2.3a

Table 3. Days to podding in six chickpea genotypes in controls, and under drought stress, heat stress and combined stress

LSD ($P < 0.05$) for water × sowing × genotype: 3.0. Values having the same letters within a column do not differ significantly from each other. HT, heat-tolerant; HS, heat-sensitive; DT, drought-tolerant; DS, drought-sensitive

Genotypes	Control	Drought stress	Heat stress	Heat + drought stress
1356 (HT)	111 ± 2.1a	111 ± 2.1a	60 ± 2.1a	60 ± 2.1a
15614 (HT)	109 ± 2.5a	109 ± 2.5a	58 ± 2.5a	58 ± 2.5a
4567 (HS)	111 ± 2.4a	111 ± 2.4a	58 ± 2.2a	58 ± 2.2a
5912 (HS)	108 ± 2.4a	108 ± 2.4a	58 ± 2.7a	58 ± 2.7a
8950 (DT)	110 ± 2.5a	110 ± 2.5a	60 ± 2.5a	60 ± 2.5a
3776 (DS)	110 ± 2.3a	110 ± 2.3a	59 ± 2.2a	59 ± 2.2a

Table 4. Days to maturity in six chickpea genotypes in controls, and under drought stress, heat stress and combined stress

LSD ($P < 0.05$) for water × sowing × genotype: 3.1. Values having the same letters within a column do not differ significantly from each other. HT, heat-tolerant; HS, heat-sensitive; DT, drought-tolerant; DS, drought-sensitive

Genotypes	Control	Drought stress	Heat stress	Heat + drought stress
1356 (HT)	157 ± 2.4a	123 ± 2.5b	86 ± 2.1a	78 ± 2.6a
15614 (HT)	157 ± 2.1a	121 ± 2.6b	84 ± 2.5a	78 ± 2.2a
4567 (HS)	154 ± 2.5a	126 ± 2.5a	85 ± 2.3a	73 ± 2.5b
5912 (HS)	154 ± 2.5a	123 ± 2.3b	82 ± 2.6b	72 ± 2.4b
8950 (DT)	157 ± 2.7a	126 ± 2.7b	86 ± 2.2a	80 ± 2.3a
3776 (DS)	154 ± 2.5a	122 ± 2.4bc	85 ± 2.5a	69 ± 2.5c

Heat stress reduced total plant biomass (Table 5) more than drought stress, with a greater impact on genotypes that were sensitive to both individual stresses. The combination of stresses increased the inhibition of growth markedly in all genotypes with less effect on tolerant (58% reduction) than on sensitive (75% reduction) genotypes.

Tissue damage to leaves

Drought stress caused more damage to leaf membranes (31–95%) than heat stress (20–84%), more so in sensitive genotypes. The

Table 5. Aboveground biomass (g per plant) at maturity in adequately watered controls, and under heat stress, drought stress and combined stress in six genotypes of chickpea contrasting for heat tolerance (HT) and heat sensitivity (HS) as well as drought tolerance (DT) and drought sensitivity (DS)

LSD ($P < 0.05$) for interaction (water × sowing × genotype): 0.16. Values with different small letters in rows indicate significant differences in the response of a genotype to various treatments. Values with different capital letters in columns indicate significant differences in the response of genotypes to a treatment

Genotypes	Control	Drought stress	Heat stress	Heat + drought stress
1356 (HT)	7.09 ± 0.13aD	5.4 ± 0.14bC	3.57 ± 0.12cC	2.83 ± 0.16dB
15614 (HT)	5.83 ± 0.15aE	4.4 ± 0.13bD	3.41 ± 0.14cC	2.73 ± 0.13dC
4567 (HS)	7.75 ± 0.16aB	5.07 ± 0.16bB	3.65 ± 0.13cB	2.31 ± 0.14dD
5912 (HS)	7.94 ± 0.13aA	5.01 ± 0.15bB	3.60 ± 0.12cB	2.32 ± 0.13dD
8950 (DT)	7.68 ± 0.14aB	6.58 ± 0.14bA	5.84 ± 0.14cA	3.02 ± 0.15dA
3776 (DS)	7.41 ± 0.15aC	3.55 ± 0.15bE	2.82 ± 0.15cD	1.24 ± 0.16dE

Table 6. Correlations and probability values between leaf and seed metabolism and aboveground biomass and seed yield at maturity

Significant correlations are shown in bold

Trait	Seed weight per plant	*P*-value	Biomass per plant	*P*-value
Aboveground biomass	**0.80**	0.000		
Shoot weight per plant	**0.84**	0.000	**0.95**	0.000
Seed weight per plant	–	–	**0.80**	0.000
Pod number per plant	**0.90**	0.000	**0.92**	0.000
Seed number per plant	**0.95**	0.000	**0.79**	0.000
Seed size	**0.94**	0.000	**0.86**	0.000
Stomatal conductance	**0.90**	0.000	**0.70**	0.000
Cellular oxidising ability	0.20	0.347	–0.20	0.361
Leaf tissue damage	**–0.85**	0.000	**–0.82**	0.000
Leaf chlorophyll	**0.97**	0.000	**0.81**	0.000
Leaf PSII function	**0.70**	0.000	0.58	0.003
Leaf Rubisco	**0.71**	0.000	0.41	0.047
Leaf sucrose synthase	**0.80**	0.000	**0.76**	0.000
Seed sucrose synthase	**0.95**	0.000	**0.76**	0.000
Leaf invertase	**0.64**	0.001	0.41	0.047
Seed invertase	0.51	0.012	0.23	0.269
Leaf starch phosphorylase	**0.63**	0.001	0.43	0.037
Seed starch phosphorylase	**0.85**	0.000	**0.76**	0.000
Leaf β-amylase	**0.64**	0.001	0.35	0.094
Seed β-amylase	**0.86**	0.000	**0.70**	0.000
Leaf starch	0.54	0.006	0.30	0.150
Seed starch	**0.88**	0.000	**0.85**	0.000
Leaf sucrose	**0.95**	0.000	**0.90**	0.000
Seed sucrose	**0.93**	0.000	**0.80**	0.000
Reducing sugars (leaf)	0.26	0.228	0.26	0.215
Reducing sugars (seed)	0.41	0.047	0.28	0.189

extent of membrane damage was higher in both heat- and drought-sensitive genotypes compared with the controls. The combination of drought and heat increased membrane damage, with the greatest effect seen in a heat-sensitive genotype (ICC5912; 2.1-fold) and a drought-sensitive genotype (ICC3776; 2.5-fold). The damage was significantly lower in the drought-tolerant (65%) and heat-tolerant genotypes (50–57%) than in the sensitive genotypes.

Cellular oxidising ability

Heat stress increased cellular oxidising ability (Fig. 2) in all genotypes; the maximum increase was observed in the drought-sensitive genotype ICC3776. In contrast, drought stress increased cellular oxidising ability in the tolerant genotypes and reduced it in the sensitive genotypes. The combined heat and drought stress significantly inhibited the process in all genotypes compared with the controls.

Photosynthetic function

The g_s varied from 46 mmol m^{-2} s^{-1} to 68 mmol m^{-2} s^{-1} in the control plants. Heat stress reduced g_s compared with the controls by an average of 47% across all genotypes, whereas drought reduced g_s by 93% on average (Fig. 2). The combination of heat and drought stress did not decrease g_s further. The tolerant genotypes for drought and heat had significantly higher values of g_s than sensitive genotypes under individual and combined application of the two stresses.

Drought stress suppressed PSII function more than heat stress in all genotypes (Fig. 2). Combining the two stresses further inhibited PSII function. Tolerant genotypes, especially the drought-tolerant genotype ICC8950, had significantly higher PSII function compared with other genotypes under the heat, drought and combined stress treatments, but not in the controls.

The loss of chlorophyll was significantly higher under drought stress (36% loss) than under heat stress (26% loss) compared with the controls, especially in drought- and heat-sensitive genotypes, whereas the tolerant genotypes retained significantly higher chlorophyll (Fig. 2). Combining the two stresses increased chlorophyll loss to 44% compared with the controls across all genotypes, with greater impact on the sensitive genotypes.

In all genotypes, heat stress increased Rubisco activity compared with the controls, whereas drought stress decreased Rubisco activity, with a significantly greater decrease in drought- and heat-sensitive genotypes (Fig. 2). The combination of stresses severely inhibited Rubisco activity in all genotypes, with a significantly greater inhibition in the sensitive genotypes. The drought-tolerant genotype ICC8950 had the highest Rubisco enzyme activity under the combined heat and drought stress treatment. Increases in Rubisco activity were highly and positively correlated with seed yield per plant (Table 6).

Starch metabolism in leaves and seeds

Since starch and starch metabolism in the leaves affect sucrose availability to the developing seeds, the activities of enzymes related to these metabolic pathways were assessed in both these organs. In the leaves of heat-stressed plants, the starch concentration was 35% higher on average across genotypes compared with the controls, but decreased by 23% in leaves of drought-stressed plants (Fig. 3). When the two stresses were combined, the starch concentration in leaves decreased even further (by 44%) compared with the controls. With the combined stresses, the tolerant genotypes accumulated significantly more starch than the sensitive genotypes in leaves. A drought-sensitive genotype, ICC3776, had the lowest starch concentration in its leaves with the combined heat and drought stresses. The starch concentration in seeds

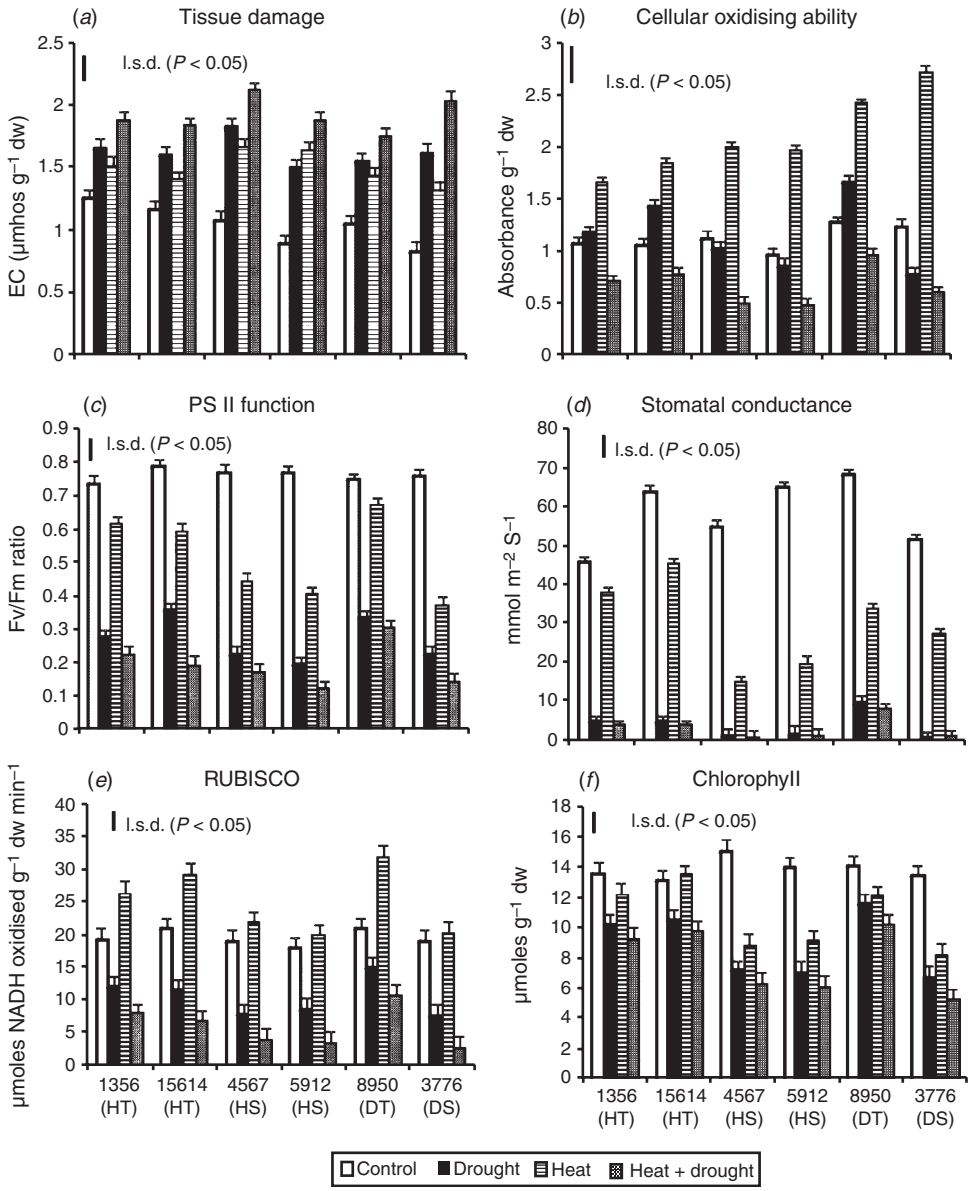

Fig. 2. (*a*) Tissue damage, (*b*) cellular respiration, (*c*) PSII function, (*d*) stomatal conductance, (*e*) Rubisco activity and (*f*) chlorophyll concentration in the leaves of six genotypes of chickpea contrasting for heat tolerance (HT), and heat sensitivity (HS), and drought tolerance (DT) and sensitivity (DS) in unstressed controls, and under heat stress, drought stress and combined stress. LSD ($P < 0.05$): tissue damage (water × sowing × genotype): 0.14; cellular viability (water × sowing × genotype): 0.16; PSII function (water × sowing × genotype): 0.061; stomatal conductance (water × sowing × genotype): 4.20; Rubisco (water × sowing × genotype): 2.69; chlorophyll (water × sowing × genotype): 1.8. Values represent means ± s.e. ($n = 3$); LSD ($P < 0.05$) for the interaction is indicated as a single vertical bar in the chart area.

decreased both in plants under drought stress and those under heat stress (Fig. 3). Drought stress caused a greater inhibition of starch accumulation in the seeds of the sensitive genotypes than did heat stress. The combination of two stresses substantially decreased starch accumulation in the seeds of all the genotypes.

The activity of the starch-synthesising enzyme (starch phosphorylase) increased in leaves under heat stress but decreased significantly in the drought stress treatment (Fig. 3). The combination of the two stresses markedly decreased the activity of starch phosphorylase. A drought-tolerant genotype, ICC8950, had the highest starch-synthesising enzyme activity in leaves under both the individual and the combined application of drought and heat. In seeds, drought stress inhibited starch phosphorylase activity more than heat stress in all genotypes (Fig. 3). Under the combined stresses, the activity of the enzyme in the seed was severely inhibited, particularly in the heat- and drought-sensitive genotypes.

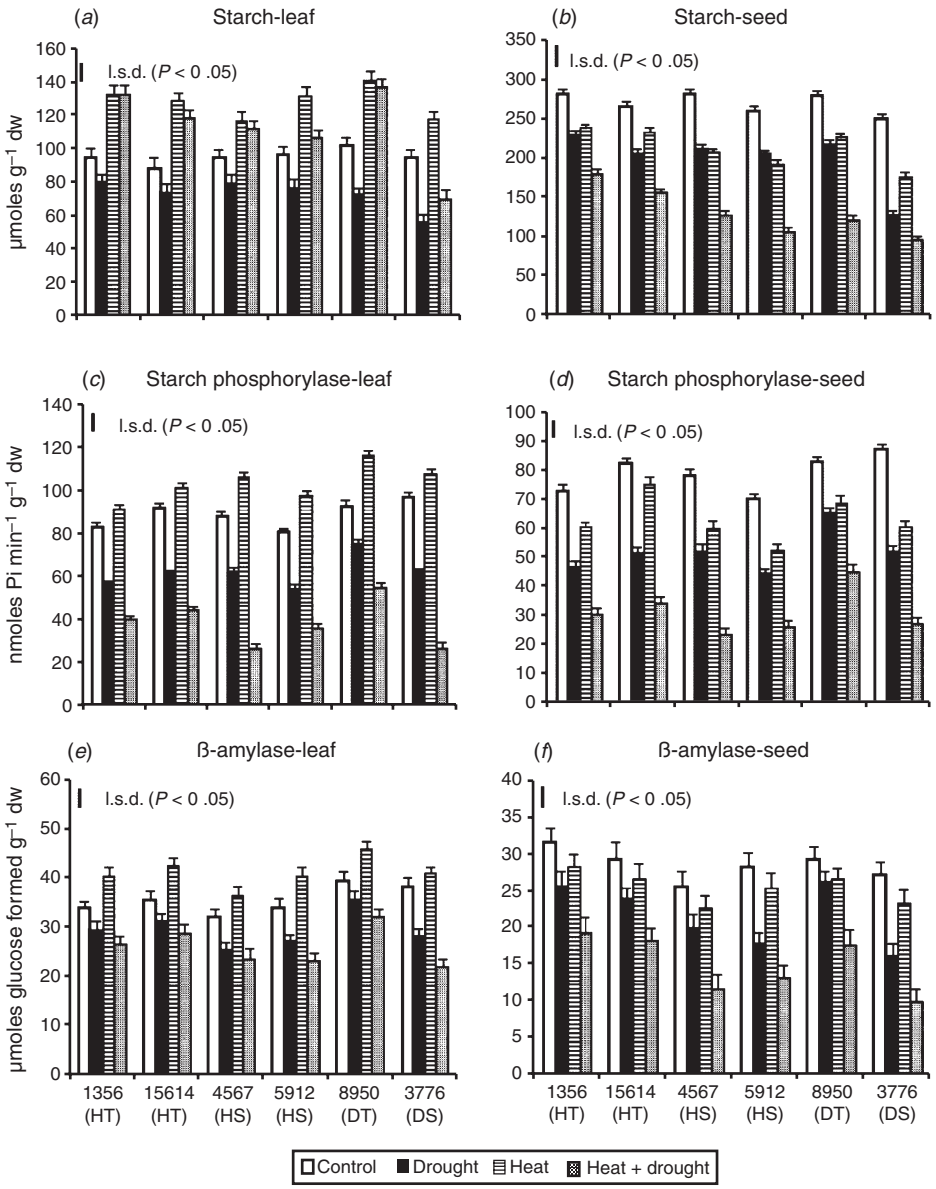

Fig. 3. (*a*, *b*) Starch content, (*c*, *d*) starch phosophorylase activity and (*e*, *f*) β-amylase activity in (*a*, *c*, *e*) the leaves and (*b*, *d*, *f*) seeds of six genotypes of chickpea contrasting for heat tolerance (HT) and heat sensitivity (HS), and drought tolerance (DT) and sensitivity (DS) in unstressed controls, and under heat stress, drought stress and combined stress. LSD ($P < 0.05$): leaf starch (water × sowing × genotype): 14.2; seed starch (water × sowing × genotype): 20.9; leaf starch phosphorylase (water × sowing × genotype): 6.16; seed starch phosphorylase (water × sowing × genotype): 5.69); leaf β-amylase (water × sowing × genotype): 4.88; seed β-amylase (water × sowing × genotype): 5.27. Values represent means ± s.e. ($n = 3$); LSD ($P < 0.05$) for the interaction is indicated as a single vertical bar in the chart area.

With heat stress, the activity of β-amylase, which hydrolyses starch, increased significantly in the leaves of all the genotypes compared with the controls, but it was inhibited by drought stress and the combination of drought and heat stress (Fig. 3). Heat-tolerant (ICC15614) and drought-tolerant (ICC8950) genotypes had significantly higher activity in the leaves than other genotypes under the combination of stresses. In seeds, the β-amylase activity decreased under drought and under heat stress – to a greater extent under drought stress – in all genotypes (Fig. 3). Combining the stresses further inhibited the β-amylase activity in the seeds, especially in the sensitive genotypes.

Sucrose metabolism in leaves and seeds

Both leaves and seeds contribute towards sucrose synthesis for seed filling; hence it was considered worthwhile to compare both these organs for sucrose metabolism. In the controls, the

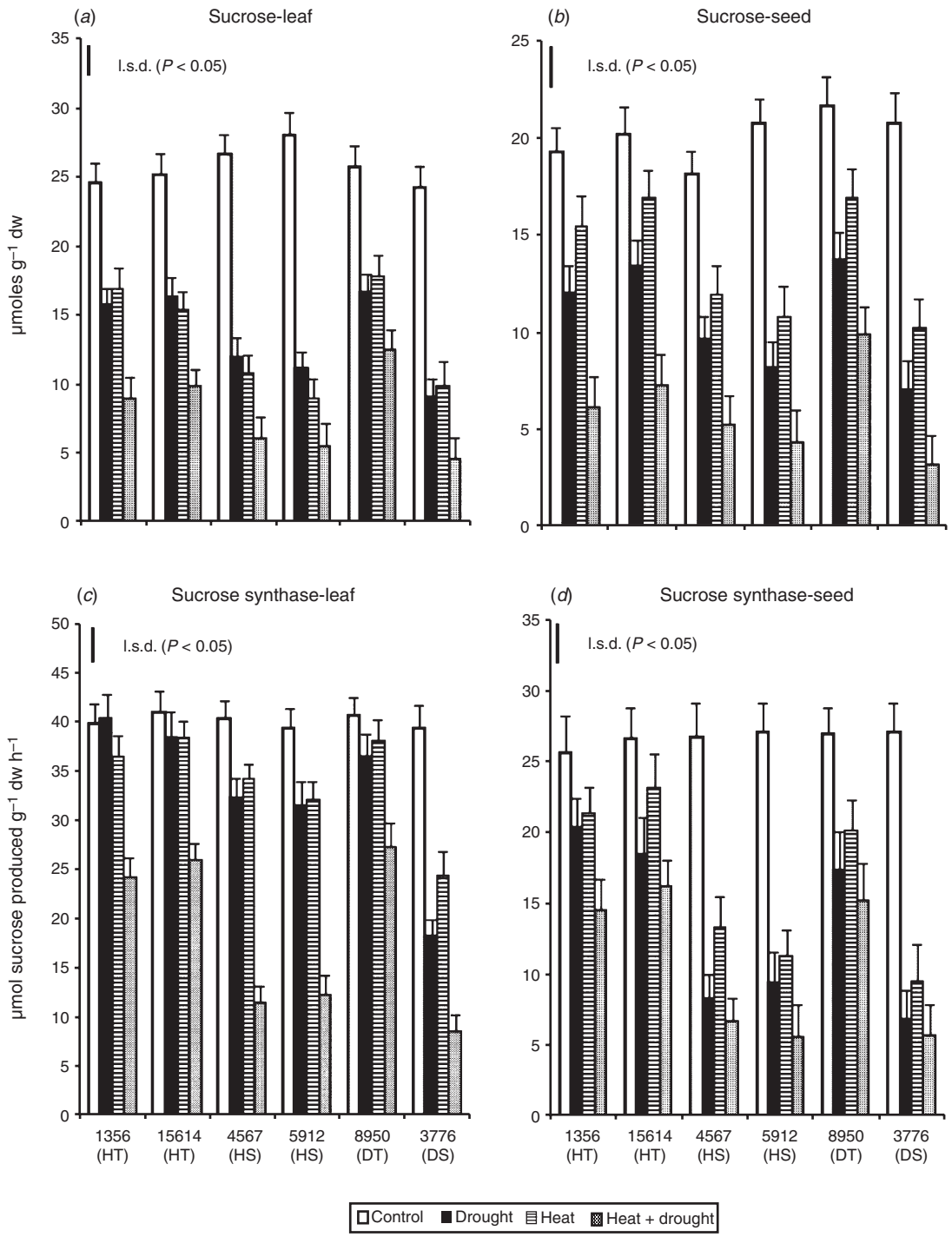

Fig. 4. (*a*, *b*) Sucrose content and (*c*, *d*) sucrose synthase activity in (*a*, *c*) the leaves and (*b*, *d*) seeds of six genotypes of chickpea contrasting for heat tolerance (HT) and heat sensitivity (HS), and drought tolerance (DT) and sensitivity (DS) in unstressed controls, and under heat stress, drought stress and combined stress. LSD ($P < 0.05$): leaf sucrose (water × sowing × genotype): 3.62; seed sucrose (water × sowing × genotype): 4.11; leaf sucrose synthase (water × sowing × genotype): 6.87; seed sucrose synthase (water × sowing × genotype): 5.16. Values represent means ± s.e. ($n = 3$); LSD ($P < 0.05$) for the interaction is indicated as a single vertical bar in the chart area.

sucrose concentration in the leaves was 26.3 µmol g^{-1} DW and it decreased to 22.3 µmol g^{-1} DW in the seeds averaged across all genotypes (Fig. 4). In leaves, the sucrose concentration decreased slightly more (54%) under drought stress than under heat stress (48%) in all genotypes. Under the combined heat and drought stress, sucrose concentration was severely inhibited (69% on

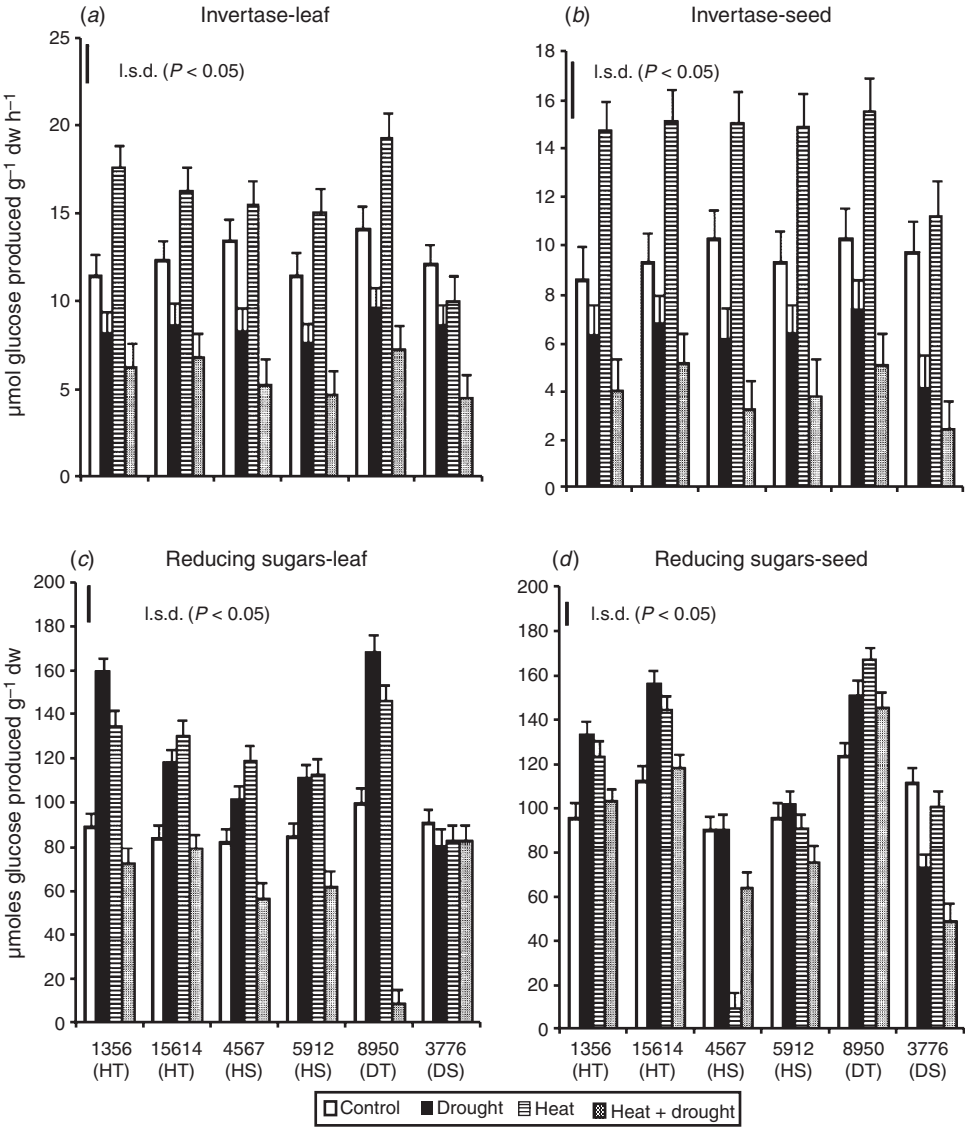

Fig. 5. (*a*, *b*) Invertase activity and (*c*, *d*) reducing sugar concentrations in (*a*, *c*) the leaves and (*b*, *d*) seeds of six genotypes of chickpea contrasting for heat tolerance (HT) and heat sensitivity (HS), and drought tolerance (DT) and sensitivity (DS) in unstressed controls, and under heat stress, drought stress and combined stress. LSD ($P < 0.05$): leaf invertase (water × sowing × genotype): 3.05; seed invertase (water × sowing × genotype): 2.76; reducing sugars in the leaf (water × sowing × genotype): 18.5; reducing sugars in the seed (water × sowing × genotype): 17.7. Values represent means ± s.e. ($n = 3$); LSD ($P < 0.05$) for the interaction is indicated as a single vertical bar in the chart area.

average) in the leaves, with a greater decrease in sensitive compared with tolerant genotypes. The drought-tolerant genotype ICC8950 had a significantly higher sucrose concentration than sensitive genotypes in the leaves when combined heat and drought stress was imposed. Similarly, the sucrose concentration in seeds decreased more under drought stress than heat stress (Fig. 4). The sucrose concentration of the seeds in the sensitive genotypes was about 60% of that in the tolerant genotypes in the individual and combined stress treatments.

The activity of sucrose synthase, a sucrose-synthesising enzyme, was 40 µmol g^{-1} DW h^{-1} in the leaves of the genotypes when not exposed to stress, but decreased under drought stress as well as heat stress, particularly in the heat- and drought-sensitive genotypes (Fig. 4). Under the combination of stresses, sucrose synthase activity in the leaves was lower than under each individual stress in all the genotypes, particularly in the sensitive genotypes. In seeds, the sucrose synthase activity was lower than in the leaves and as in leaves, drought stress inhibited sucrose synthase activity more than heat stress (Fig. 4). Combining the two stresses further inhibited the sucrose synthase activity of the seeds, especially in the sensitive genotypes. Compared with the controls, invertase activity increased under heat stress, but decreased under drought stress in the leaves and seeds of all genotypes except ICC3776 (Fig. 5). Invertase activity increased more in drought- and heat-tolerant genotypes under

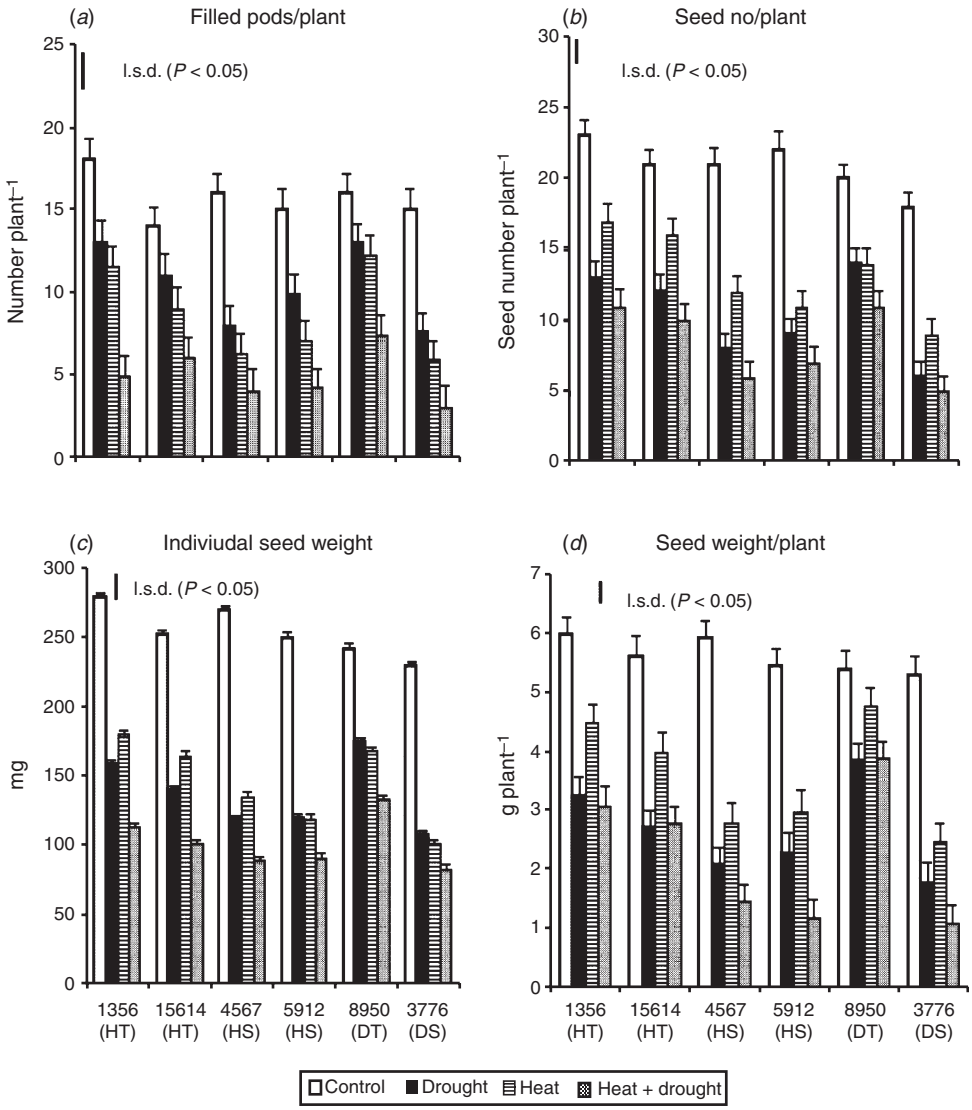

Fig. 6. (*a*) Filled pods per plant, (*b*) seed number per plant, (*c*) individual seed weight (seed size) and (*d*) seed weight per plant of six genotypes of chickpea contrasting for heat tolerance (HT) and heat sensitivity (HS), and drought tolerance (DT) and sensitivity (DS) in unstressed controls, and under heat stress, drought stress and combined stress. LSD ($P < 0.05$): filled pods per plant (water × sowing × genotype): 2.87; seed number per plant (water × sowing × genotype): 2.42; individual seed weight (water × sowing × genotype): 9.32; seed weight per plant (water × sowing × genotype): 0.68. Values represent means ± s.e. ($n = 3$); LSD ($P < 0.05$) for the interaction is indicated as a single vertical bar in the chart area.

heat stress. The combined application of the stresses reduced invertase activity in leaves by about 50% in the tolerant genotypes and by more than 60% in the sensitive genotypes.

The leaves and seeds of tolerant genotypes had higher concentrations of reducing sugars than sensitive genotypes in plants under heat stress and under drought stress (Fig. 5). With the combined heat and drought stress, there was a reduction in the accumulation of reducing sugars in the leaves, more so in sensitive genotypes; in the seeds, the reducing sugar concentration was only reduced in the sensitive genotypes. The drought-tolerant genotype ICC8950 had the most reducing sugars in both the leaves and seeds.

Yield-associated traits

The exposure to heat or drought stress significantly reduced pod and seed numbers and seed weight per plant, which was exacerbated under the combined stress (Fig. 6). All heat-stressed plants, except for the drought-tolerant genotype ICC8950, produced fewer filled pods than drought-stressed plants. Under the combined heat and drought stress treatment, pod number substantially decreased, especially in the heat- and drought-sensitive genotypes. There were one or two (mean 1.3) seeds per pod in the controls, but drought reduced the number to one seed per pod, whereas heat stress and the combined stresses

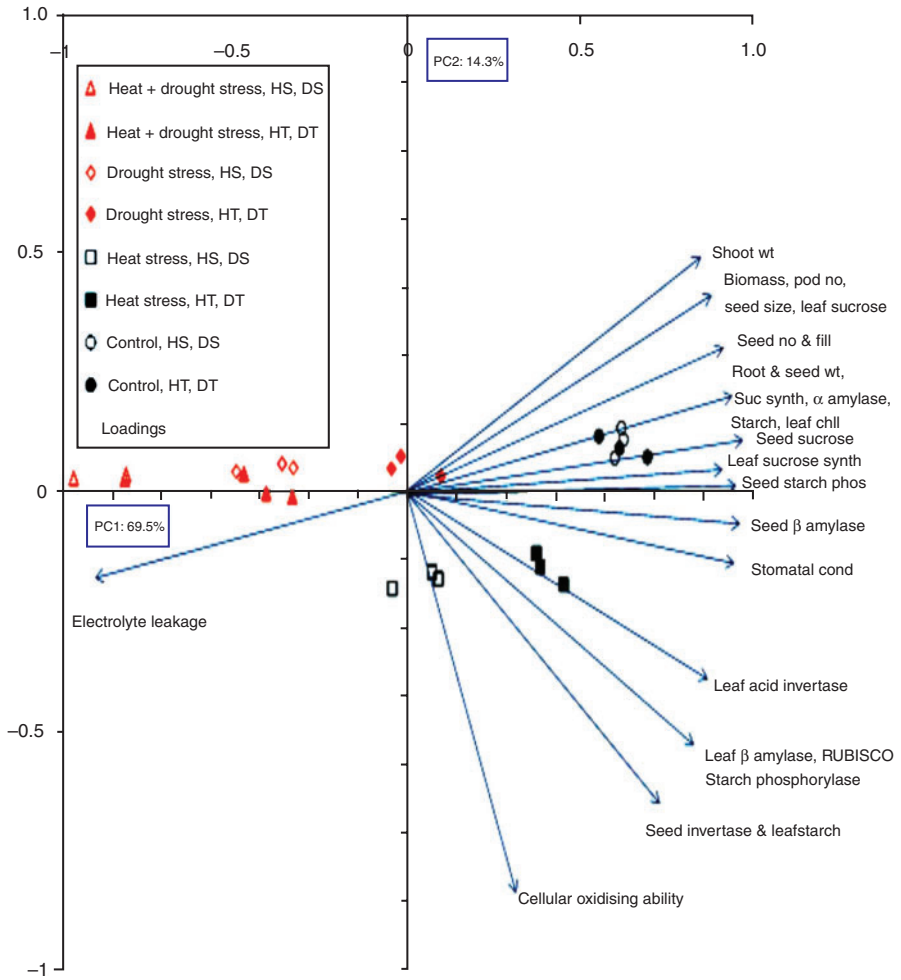

Fig. 7. Principal component analysis of all variables tested in leaves and seeds of six genotypes of chickpea contrasting for heat tolerance (HT) and heat sensitivity (HS), and drought tolerance (DT) and sensitivity (DS) in unstressed controls, and under heat stress, drought stress and combined stress (Heat + Drought). PC1, Principal Component 1; PC2, Principal Component 2.

maintained the seed number of 1.3 seeds per pod (data not shown). Thus the drought-stressed plants, except the drought-tolerant genotype ICC8950, produced fewer seeds per plant than heat-stressed plants (Fig. 6) because they produced fewer pods per plant and fewer seeds per pod. Under the combination of the two stresses, tolerant genotypes produced half the seed number compared with the control, while sensitive genotypes produced one-third that of the controls. Individual seed weight (seed size) varied from 230 to 280 mg in the controls (Fig. 6). Heat reduced the seed size by 33% in the tolerant genotypes and by 52% in the sensitive genotypes, whereas drought reduced the seed size by 40% in the tolerant genotypes and by 54% in the sensitive genotypes. Under the combined heat and drought stress treatment, seed size decreased by almost two-thirds in sensitive genotypes, whereas the tolerant genotypes were reduced by 55%. The well watered controls had a seed yield of 5.6 g per plant on average across genotypes (Fig. 6). Seed weight per plant decreased more in drought-stressed plants than in heat-stressed plants. With the combined heat and drought stress, seed weight was reduced by 40% in the tolerant genotypes and by over 70% in the sensitive genotypes. The drought-tolerant genotype ICC8950 yielded more than the heat-tolerant genotypes when exposed to both stresses in combination.

Principal components analysis and correlations

Combined principal components analysis (Fig. 7) effectively summarised treatment differences; accounting for 84.1% of variance in the two components, Principal Component 1 (PC1) and Principal Component (PC2), and separating the four drought and heat combinations in stress order along PC1. PC1 described plant productivity (biomass, pod and seed number and seed size), g_s, chlorophyll concentration, seed and leaf carbohydrate metabolism, contrasting these with tissue damage estimated by electrolyte leakage. Invertase, leaf β-amylase, Rubisco and starch phosphorylase activities were located on PC2, as indicated by the vectors in the lower right quadrant of Fig. 7, although PC2 was dominated by cellular oxidising ability. Narrow vector angles in the PC1-dominating variables are described in the arc from leaf β-amylase to shoot weight in Fig. 7 and are indicative of

strong correlations between these variables, highlighted in Table 6, for seed and biomass production. Indeed, of all the variables listed in Table 5, only cellular oxidising ability and leaf reducing sugar concentration were not correlated with seed productivity.

The terminal drought treatments were located on the left of PC1, contrasting with the well watered treatments on the right. Similarly, combined heat and drought stress was consistently more stressful than drought stress, indicated by more negative PC1 scores in each instance. Consequently, productivity, photosynthetic and metabolic activity increased, whereas cell damage decreased from left to right in the following order: heat plus drought stress, to drought stress, to heat stress, to no stress treatment (control). Under stress conditions, there were consistent differences in PC1 scores between tolerant and sensitive genotypes for all the variables tested compared with control conditions. Thus, drought- and heat-tolerant genotypes had more positive PC1 scores than their respective sensitive counterparts ($P < 0.001$) in the drought stress and the heat stress treatments. Moreover, the drought-tolerant genotype ICC8950 was particularly robust, with higher PC1 scores ($P < 0.05$) than the heat-tolerant genotypes in all the stress treatments.

Discussion

Our findings indicate that high temperatures in drought-stressed chickpea plants during seed filling adversely affected the biochemical processes related to seed filling, resulting in marked reductions in the quality and quantity of seeds. Variations existed among the contrasting genotypes in this regard, which were attributed to differential sensitivity of the leaf and seed functions to stress. The reduction in seed yield due to these stresses, when applied alone or in combination, occurred as a result of physiological and metabolic impairment of the photosynthetic pathway and leaves that closed stomata, and increased cellular oxidation, thereby restricting the supply of photoassimilates and the accumulation of carbohydrates and other reserves in the developing seeds.

Leaf function

Leaves have a primary role in the production and transport of photoassimilates; their photosynthetic status at the time of seed development determines the extent of seed filling. Aboveground plant biomass decreased more in heat-stressed plants as a result of longer exposure to high temperatures compared with drought stress. Reductions in biomass by heat or drought stress, applied individually or in combination, have been observed in other crops (Barnabás *et al*. 2008) and are related to inhibited expression of growth-related metabolism (Rollins *et al*. 2013).

Tissue damage to leaves (measured as increased electrolyte leakage) was significantly higher in drought-stressed than in heat-stressed plants, but intensified under their combination. Electrolyte leakage from stressed tissues is an indicator of damage to cellular membranes resulting from membrane instability caused by changes in lipid–protein configuration, which leads to impaired leaf function and affects various cellular processes (Earnshaw 1993). Membranes may become damaged by heat as well as drought either from direct or indirect effects, which basically disrupt the structural organisation of membranes, causing leakage of vital ions or other molecules, which impairs cellular function (Conde *et al*. 2011). These effects are similar to others seen previously in chickpea (Kumar *et al*. 2012), Kentucky bluegrass (*Poa pratensis* L., Liu *et al*. 2008) and reported in perennial ryegrass (*Lolium perenne* L., Jiang and Huang 2001) under heat stress conditions.

Photosynthetic function in leaves was inhibited in all genotypes under combined heat and drought stress, as indicated by reductions in g_s, chlorophyll concentration, PSII function and Rubisco activity (a primary enzyme for photosynthesis). Chlorophyll loss results in a loss of photosynthetic efficiency, which occurred to a greater extent in drought-stressed plants than in heat-stressed plants and intensified with the combination of heat and drought stress. Chlorophyll is stored in the thylakoid membranes; stresses can disrupt these membranes by direct or indirect effects such as lipid peroxidation of the chloroplast membranes, which damages chlorophyll molecules (Kotak *et al*. 2007; Ristic *et al*. 2007; Djanaguiraman *et al*. 2010). Inhibited biosynthesis or degradation of chlorophyll (Tewari and Tripathy 1998) or disorganisation of chloroplasts due to photooxidation might be other reasons for the reduction in chlorophyll concentration. Our observations on chlorophyll loss are similar to those of previous studies on chickpea subjected to drought (Kashiwagi *et al*. 2010) and heat stress (Kumar *et al*. 2012), and to combined heat and drought stress in bentgrass (*Agrostis stolonifera* L.) (McCann and Huang 2007).

PSII function governs the light reaction or electron flow of photosynthesis and is reported to be more sensitive to heat stress than to drought stress (Havaux 1992). In contrast, in our study, drought stress caused more inhibition than heat stress, and was further inhibited under the combined stress. In previous studies on chickpea, PSII function was inhibited to a higher extent by drought (Macar and Ekmekci 2008) than by heat stress (Kumar *et al*. 2012). PSII function depends upon the fluidity of thylakoid membranes and electron dynamics, both of which can be affected by these stresses, depending upon the extent of water loss or inactivation of the metabolic pathway of carbon fixation (Havaux 1992). The combined effects of heat and drought stress severely reduced PSII function, which matches observations on *Lotus japonicus* (Regel) K. Larsen (Sainz *et al*. 2010). These stresses together can degrade vital components of PSII, such as D1, D2 and CP47 proteins, to inhibit photosynthetic activity (Sainz *et al*. 2010).

Drought affects photosynthesis either through pathway regulation by stomatal closure and decreasing the flow of CO_2 into mesophyll tissue (Chaves *et al*. 2003), or by directly impairing the metabolic activity of enzymes (Farquhar *et al*. 1989). The components associated with Rubisco activity (Parry *et al*. 2002) such as RuBP and Rubisco protein content also decrease with water deficit, which contributes to photosynthetic inhibition (Bota *et al*. 2004). Rubisco activity indicated carboxylation efficiency during stress, which might possibly influence sucrose generation in leaves and its availability to the seeds, affecting their weight and hence yield. In the present study, heat stress increased Rubisco activity and starch concentration, whereas drought stress decreased these traits, indicating a variation in the response to drought

stress and to heat stress. Sucrose production decreased under drought stress and under heat stress, indicating photosynthetic inhibition; combining these two stresses exacerbated these effects due to the reduced availability of CO_2 because of stomatal closure and inactivation of Rubisco (Carmo-Silva *et al*. 2012). These observations suggest that the photosynthetic function of leaves was impaired by either drought or heat stress applied individually, and became more severe in the presence of both stresses.

Seed function

Disruptions in photosynthesis due to heat and drought stress, applied alone or in combination, affected the production of sucrose and starch in the leaves and the seeds. Biochemical conversions related to starch and sucrose metabolism in leaves operate in tandem with those in developing seeds to determine the extent of seed filling. We examined these aspects both in leaves and seeds, which revealed varied expression of the enzymes under individual and combined application of these stresses.

In leaves, relative to the controls, starch concentration increased with heat stress but decreased under drought, in accordance with the activity of the starch-synthesising enzyme, starch phosphorylase. A reduction in starch concentration has been reported in drought-stressed chickpea plants (Basu *et al*. 2007). Similar responses have been observed in other legume species, such as pigeonpea (*Cajanus cajan* L.; Keller and Ludlow 1993) and soybean (*Glycine max* (L.) Merr.; Liu *et al*. 2004). In our study, leaf starch concentration increased under heat stress, perhaps due to diversion of triose-phosphate to starch synthesis rather than sucrose synthesis as a result of restricted sucrose transport to seeds (Yang *et al*. 2004). In contrast to our findings, some previous studies have observed reductions in starch concentration as a result of heat stress (e.g. in creeping bentgrass (Liu and Huang 2000) and mulberry (*Morus nigra* L.; Chaitanya *et al*. 2001) leaves), which might be due to inhibitory effects on starch-synthesising enzymes. Severe inhibition in starch concentrations under the combined stresses might be attributed to the marked reduction in starch phosphorylase activity observed here. In rice (*Oryza sativa* L.), the expression of starch-synthesising enzymes decreased under drought and high temperature conditions, which accounted for the reduced starch concentration (Wang *et al*. 2006*b*).

The rise in the activity of β-amylase under heat stress but not under drought stress suggested increased degradation of starch under heat stress, which contributed towards more reducing sugars in heat-stressed leaves. These observations differ from those of drought-stressed pigenonpea (Keller and Ludlow 1993) and barley (*Hordeum vulgare* L.) leaves (Jacobsen *et al*. 1986), which increased amylase activity. Similarly, in heat-stressed plants, rises in β-amylase activity have been reported (Kaplan *et al*. 2006).The reducing sugars produced due to the action of amylases during stress conditions can serve various functions, including energy storage, osmoregulation and signalling, which assist plants to adapt to environmental stresses (Anderson and Kohorn 2001).

Sucrose is a major and vital photoassimilate that is imported by developing sinks such as seeds from the leaves (Yang *et al*. 2004). Impaired generation and transport to seeds due to stress is detrimental to seed filling, which largely depends upon sucrose import (Yang *et al*. 2004). In our study, the reduction in sucrose concentration in the leaves of drought-stressed plants is similar to findings in soybean (Liu *et al*. 2004), peach (*Prunus persica* (L.) Batsch.; Lo Bianco *et al*. 2003) and common bean (*Phaseolus vulgaris* L.; Castrillo 1992). The reduction in sucrose concentration was associated with reduced enzyme activity related to its synthesis such as sucrose synthase or increased use of sucrose by the action of hydrolases such as invertase (Cornic and Massacci 1996), or a combination of these factors. A reduction in activity of sucrose synthase has been reported previously in water-stressed bean plants (Castrillo 1992). In contrast, Fu *et al*. (2010) reported increased sucrose concentration in water-stressed tall fescue (*Festuca arundinacea* Schreb.), which was attributed to increased activity of sucrose-synthesising enzymes in leaves. Similarly, in pigeonpea, the sucrose-synthesising enzymes sucrose synthase and sucrose phosphate synthase increased under drought stress (Keller and Ludlow 1993). The inhibited activity of these enzymes might impede sucrose and starch synthesis and their partitioning under drought stress (Haupt-Herting and Fock 2002). Compared with the controls, heat-stressed chickpea plants also showed a reduction in sucrose, but to a lesser extent than drought-stressed plants, with a concomitant reduction in sucrose synthase activity, which is similar to observations in heat-stressed mulberry leaves (Chaitanya *et al*. 2001). Under the combined heat and drought stress, sucrose concentration decreased markedly and was associated with an inhibition of sucrose synthesis. This finding is in contrast to observations in *Arabidopsis thaliana* (L.) Heynh. by Rizhsky *et al*. (2004), where sucrose accumulated under the combination of stresses. These differences may arise from different species, plant age, intensity and duration of stress, which all varied in the different experiments.

Carbohydrates are synthesised in source leaves and are translocated to sink tissues as sucrose to sustain growth, or to be stored as sucrose or starch. Sucrose in the sinks is irreversibly catalysed to glucose and fructose by different types of invertase isozymes: vacuolar, cell-wall bound and neutral invertases. Cell wall and vacuolar invertases are glycosylate forms with an acid pH optimum, whereas cytosolic invertase is likely to be a nonglycosylated form with a neutral or alkaline pH (Roitsch and Gonzalez 2004). Invertases hydrolyse sucrose into glucose and fructose, thereby playing a key role in primary metabolism and plant development (Ruan 2012). In chickpea genotypes, heat stress increased the invertase activity compared with the controls, whereas drought stress inhibited sucrose hydrolysis. By breaking sucrose, these enzymes provide hexoses (an energy source) to power cellular processes; the starting molecules convert to numerous metabolites and building blocks for synthesising essential polymers including starch, cellulose and proteins. Particularly in stressed leaves, invertase makes reducing sugars available for osmoregulation (Kameli and Losel 1995). Sucrose synthesis and hydrolysis operate together to maintain photosynthetic processes; any impairment in sucrose use may inhibit sucrose generation (Nguyen-Quoc and Foyer 2001). In contrast to our study, drought did not affect invertase activity in soybean leaves but decreased starch and sucrose

concentrations and increased hexose concentrations (Liu *et al*. 2004). As in our study, in water-stressed leaves of tomato (*Solanum lycopersicum* L.), sucrose synthase and invertase activity decreased significantly to reduce sucrose levels (Bhatt *et al*. 2009). The increase in reducing sugars in heat-stressed leaves can be attributed to the degradation of starch and sucrose, as indicated by higher activity of β-amylase and invertase. When the two stresses were combined, there was a marked reduction in reducing sugars, which appears to be due to a considerable reduction in β-amylase and invertase activity, suggesting extreme inhibition of carbohydrate metabolism.

These observations suggest that drought alone and in combination with heat stress severely impaired photosynthetic assimilation into starch as well as sucrose in all genotypes, although the extent varied, depending upon their stress sensitivity. Consequently, the availability of sucrose to developing seeds was markedly inhibited, which may be coupled with downregulation of sucrose transporters located in leaves and seeds (Qin *et al*. 2008), and which may further disrupt sucrose accessibility to seeds. In the absence of adequate sucrose, the biochemical processes occurring in seeds, which contribute to carbohydrate accumulation, are likely to be affected. Seed weight, a function of rate and duration of filling, decreased in stressed plants because both these traits decreased (data not shown), resulting in smaller seeds. Previous findings on the effects of environmental constraints like drought and high temperatures on seed development have suggested that impaired seed-filling processes affect seed quality (Triboï *et al*. 2000; Larmure *et al*. 2005). Seed starch possibly decreased because of restrictions in assimilate supply to developing seeds, as indicated by low sucrose concentrations in the leaves and seeds of stressed plants. Additionally, starch synthesis was directly inhibited, as shown by lower activities of starch phosphorylase. In cereal grains developing under the influence of water stress, reduced starch concentration has been attributed to the reduced capacity of the endosperm due to fewer amyloplasts (Jones *et al*. 1996). A similar possibility might exist for storage cells in chickpea seeds. We observed reduced activity of invertase (a sucrolytic enzyme) and β-amylase (a starch-hydrolysing enzyme) under the combined heat and drought stress, which may decrease the availability of glucose precursors for starch and sucrose synthesis in seeds. Moreover, reductions in starch phosphorylase activity as well as sucrose synthase activity also contributed towards reduced starch and sucrose accumulation in seeds. These findings are similar to those of Hawker and Jenner (1993), who observed a reduction in the activity of starch-synthesising enzymes in wheat grains exposed to heat stress. Seed growth in chickpea was positively correlated to sucrose synthase activity (Kumar and Turner 2009; Turner *et al*. 2009), suggesting that sucrose is a vital molecule for growing seeds. Similar results were obtained by Ahmadi and Baker (2001) in wheat grains developing under water stress, which was attributed to less availability of substrates for starch and sucrose synthesis. The detrimental effects of the combined stresses on the seed yield of chickpea are similar to those observed in wheat (Shah and Paulsen 2003; Balla *et al*. 2011). The reduction in starch concentration in the seeds of the stressed plants, especially those subjected to combined stress, indicated a marked reduction in seed quality in chickpea.

Genotypes

The heat-tolerant and heat-sensitive, and the drought-sensitive and drought-tolerant genotypes were selected on the basis of their seed yields under the specific environmental conditions of heat or drought in the field (Krishnamurthy *et al*. 2010, 2011). In the present study, the seed yields of the heat-tolerant genotypes were lower than those of the unstressed controls under heat stress, but were higher than those of the heat-sensitive genotypes. Likewise, drought stress reduced the yield of the drought-tolerant genotypes compared with the unstressed controls, but not to the same degree as the drought-sensitive genotypes. Thus the present study confirms the classification of the genotypes into tolerant or sensitive to heat and drought. What is of interest is that the heat-tolerant genotypes were also tolerant of the drought stress and *vice versa*. This suggests partial cross-tolerance to heat and drought. This is confirmed by the performance of the genotypes to the combined heat and drought stress. Again, the heat-tolerant and drought-tolerant genotypes yielded at least twice the yields of the heat- and drought-sensitive genotypes to the combined heat and drought stress. The differences in yield were associated with differences in pod and seed number, as observed previously (Davies *et al*. 1999; Leport *et al*. 1998). Both heat and drought individually and in combination reduced seed size (individual seed weight) compared with the unstressed controls, suggesting that the stresses reduced carbon assimilation and transport to the seed. Limitations in photosynthesis involving the reduction in activity of Rubisco as well as sucrose metabolism enzymes under stress conditions have been reported as compromising seed yield (Egli and Bruening 2004; Ashraf and Harris 2013). The observations on contrasting chickpea genotypes indicated that, compared with sensitive genotypes, tolerant genotypes maintained higher stomatal conductance, chlorophyll concentration and photosynthetic function under similar conditions of heat or drought stress, applied individually or in combination, suggesting that the tolerant genotypes have a higher ability to resist a loss of photosynthetic ability under stress situations. Previous studies also indicated that under drought situations, drought-tolerant genotypes of chickpea have higher RLWC (Deshmukh and Mate 2013), g_s (Yordanov *et al*. 2003), membrane stability (Almeselmani *et al*. 2011), cellular respiration (McCann and Huang 2008) and PSII function (Mishra *et al*. 2012). Likewise, heat-tolerant genotypes of other plant species had less leaf damage in regard to these traits (Srinivasan *et al*. 1996; Singh *et al*. 2007; Kumar *et al*. 2012). Moreover, the activities of Rubisco, and the starch- and sucrose-synthesising enzymes of tolerant chickpea genotypes increased significantly under heat, drought and their combination, indicating a stress-tolerant carbohydrate metabolism. Previous studies also showed that tolerant genotypes of other plant species had higher activity of Rubisco (Ji *et al*. 2012), starch synthase (Sumesh *et al*. 2008) and sucrose synthase (Saeedipour 2011) under both heat stress and drought stress conditions. Interestingly, a drought-tolerant genotype in our study, ICC8950, had a relatively higher degree of tolerance to the combined effects of heat and drought than the heat-tolerant genotypes, suggesting partial cross-tolerance of these two stresses, as indicated previously in chickpea (Canci and Toker 2009). This might be attributed to the carbon

metabolism enzymes examined here, which are possibly tolerant to both stresses in this genotype. This aspect needs further investigation using more contrasting chickpea genotypes.

Conclusions

Our studies have indicated that heat and drought stress alone in combination impaired starch and sucrose metabolism in leaves and seeds, resulting in poor availability of sucrose to growing seeds. Drought had higher impact than heat stress, and the combined effects of drought and heat were more similar to those of drought than those of heat alone. In combination, these stresses inhibited seed filling, which resulted in smaller and fewer seeds and hence reduced seed yields. These effects were more severe in heat- and drought-sensitive genotypes compared with drought-tolerant genotypes. From the measurements of seed yield and observations on the biochemical mechanisms governing seed filling, we conclude that compared with heat, drought stress had a greater effect on these processes. The effect of drought during seed filling was exacerbated in the presence of heat stress (at temperatures >32°C : 20°C). These stresses appear to hinder similar biochemical events governing seed filling, but to varying extents, which become intense under the combination of heat and drought stress. The drought-tolerant genotype used in this study was more tolerant to the individual and combined effects of heat and drought than the two heat-tolerant genotypes. We suggest that a study with a wider range of genotypes is warranted to determine whether this is true in a larger gene pool.

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