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Effect of varying high temperatures during reproductive growth on reproductive function, oxidative stress and seed yield in chickpea genotypes differing in heat sensitivity

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The mechanisms affecting the heat sensitivity of chickpea are largely unknown. Heat-tolerant (ICCV07110, ICCV92944) and heat-sensitive (ICC14183, ICC5912) chickpea genotypes were sown in February in the soil-filled pots. At the time of flowering, these were subjected to varying day/night temperatures of 30/20, 35/25, 40/30 and 45/35°C in the growth chambers (12 h light/12 h dark; light intensity, 250 μmol m^{-2} s^{-1}, 80% relative humidity). The pollen viability, pollen germination, tube growth, pollen load and stigma receptivity decreased with increases in temperatures to 45/35°C. The heat-tolerant genotypes experienced significantly less damage to pollen and stigma function. Membrane integrity, chlorophyll content, photochemical efficiency and cellular oxidizing ability were inhibited by the increase in temperature, with greater impacts on the sensitive genotypes. Oxidative injury as lipid peroxidation and hydrogen peroxide content was significantly greater in sensitive genotypes at 40/30 and 45/35°C. Enzymatic and non-enzymatic antioxidants showed increased levels at 40/30°C, but decreased considerably at 45/35°C. Heat-tolerant genotypes possessed greater activity of ascorbate peroxidase and glutathione reductase, along with higher levels of ascorbate and reduced glutathione at 40/30 and 45/35°C. Biomass, pod set and yield were not affected significantly at 35/25°C, but began to decrease at 40/30°C and were lowest at 45/35°C. The sensitive genotypes were not able to set any pods at 45/35°C, whereas the tolerant genotypes produced only few fertile pods at this temperature. It was concluded that heat stress leads to loss of pollen as well as stigma function and induces oxidative stress in the leaves that cause failure of fertilization and damage to the leaves, respectively.

Keywords: chickpea; heat stress; reproductive function

Introduction

Global temperatures are increasing possibly due to climate change (Cutforth 2000), which would have detrimental effects on agricultural crops being grown in arid and semi-arid regions (Wahid et al. 2007). In addition, increased temperature is likely to cause changes in the geographical distribution and growing season of crops by

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causing the threshold temperatures to reach earlier maturity (Porter 2005). Depending upon its intensity, duration and stage of exposure (Wahid et al. 2007), heat stress can adversely affect the rate of growth and development of plants (Gan et al. 2004).

Reproductive growth is sensitive to heat stress, which results in various abnormal effects such as: (1) impaired micro-sporogenesis and mega-sporogenesis (Peet et al. 1998; Porch and Jahn 2001; Young et al. 2004), (2) loss of pollen viability (Kafizadeh et al. 2008), (3) poor pollen germination (Porch and Jahn 2001), (4) inhibited pollen tube growth (Pressman et al. 2006; Kafizadeh et al. 2008), (5) the absence of pollen on stigma surface and loss of stigma receptivity (Jagadish et al. 2007), (6) loss of ovule function (Gross and Kigel 1994), (7) impaired fertilization (Dupuis and Dumas 1990), (8) limited embryogenesis (Zinn et al. 2010), and (9) reduced ovule number and increased ovule abortion (Whittle et al. 2009) leading to poor seed set (Young et al. 2004). In addition, heat stress may accelerate reproductive growth, thereby reducing the duration of this stage and limiting the yield potential (Boote et al. 2005). The relative sensitivity of reproductive stages such as flowering and seed filling to heat stress may vary according to the crop species (Sung et al. 2003).

Heat stress can cause several cellular abnormalities such as denaturation of proteins and enzymes (Kepova et al. 2005), damage to membranes (Liu and Huang 2000), inactivation of enzymes in the mitochondria and chloroplasts, inhibition of protein synthesis and disorganization of their membranes (Howarth 2005) leading to cell death. Oxidative stress is a common adverse effect of heat stress in cells because of the production of superoxides, lipid peroxides and hydrogen peroxide (Yin et al. 2008). To counter the oxidative damage, the heat-stressed cells activate many enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and non-enzymatic (ascorbic acid, glutathione) antioxidants, as observed in strawberry (Wang SY and Zheng 2001), wheat (Balla et al. 2007) and rice (Cao et al. 2008). The expression of these antioxidants may differ depending upon the severity of stress and genotype (Wahid et al. 2007).

Chickpea is a cool-season food legume cultivated during the winter season in northern India (Nayyar et al. 2005) and parts of Western Australia (Clarke and Siddique 2004). The late-sown crop is exposed to high temperatures ($>35^{\circ}C$) at its reproductive stage in the months of February and March. Moreover, it is also being grown in the warm-season environment of central and southern India, where its chances of experiencing supra-optimal temperatures during the reproductive stage are much higher. In other parts of the world also, chickpea is reported to suffer due to heat stress during its reproductive stage (Summerfield et al. 1984; Wang J et al. 2006).

One of the ways to find out the basis of heat sensitivity in crop species is to examine the response of differentially sensitive genotypes to high temperature at the reproductive stage. Because chickpea possesses large genetic variation for heat tolerance (Krishnamurthy et al. 2011), it was possible to identify some genotypes having contrasting heat sensitivity used in our studies. To date, no studies exist regarding the effect of varying high temperatures on chickpea reproductive function and oxidative damage, which constituted the objective of this study. Here, we subjected tolerant and sensitive chickpea genotypes to different high temperatures during their reproductive phase under controlled conditions to assess damage to leaves, reproductive function and seed yield.

Materials and methods

The heat-tolerant (ICCV07110, ICCV92944) and heat-sensitive (ICC14183, ICC5912) chickpea genotypes were procured from the germplasm bank of International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India and sown in soil-filled pots. The seeds were inoculated with Rhizobium and raised in pots (15 cm diameter) with loamy soil (3 kg pot^{-1}; pH 6.2; organic matter, 0.77%; available-N, 30 mg kg^{-1}; available-P, 4 mg kg^{-1}; K, 108 mg kg^{-1}; Ca, 0.417 meq 100 g^{-1} soil; Mg, 0.084 meq 100 g^{-1} soil). Two plants per pot were maintained throughout the experiment. The plants were sown on 10 February 2011 and irrigated frequently to maintain sufficient moisture all the time to avoid water stress interference. The plants were grown under natural conditions having a temperature profile shown in Figure 1. At the time of 50% flowering (3–14 April 2011 depending upon the genotype), the plants were subjected to varying day/night temperatures of 30/20, 35/25, 40/30 and 45/35°C in growth chambers (12 h light/12 h dark; light intensity, 250 μmol m^{-2} s^{-1}; 80% relative humidity). The plants remained at these temperatures thereafter and were harvested on 21 April 2011. The freshly opened flowers of the plants after 2 days of exposure were collected from these plants and examined for pollen and stigma function. The upper two to three leaves of the plants were subjected to analysis for stress injury and oxidative stress after 8 days of exposure. The methods are as follows.

Pollen viability

Pollen viability was tested on 200 pollen grains with 0.5% acetocarmine/Alexander stain. Pollen was collected from between three and five flowers open on the same day.

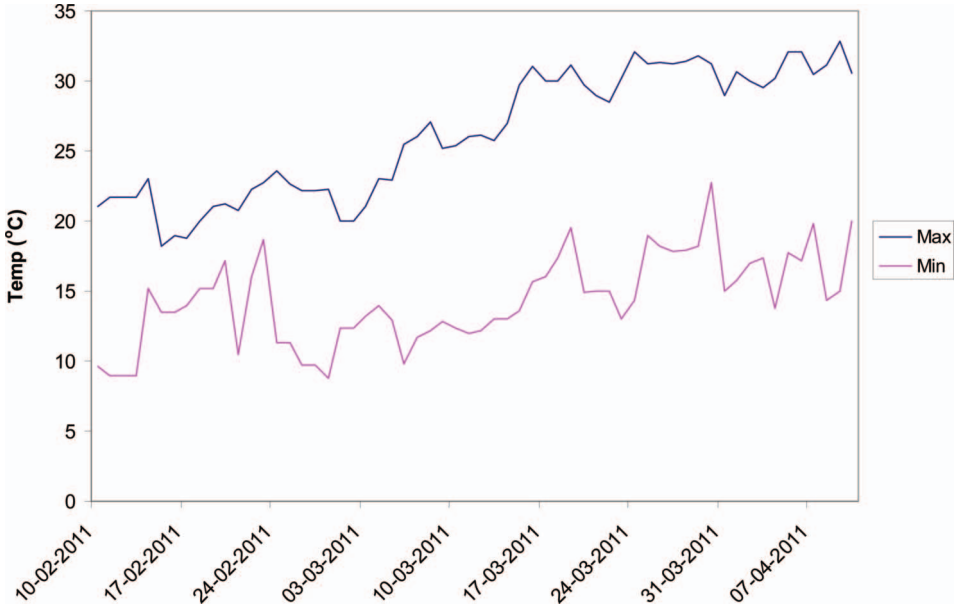

Figure 1. Temperature profile of the natural environment of the potted plants from date of sowing to attainment of 50% flowering in various genotypes. Thereafter, the plants were subjected to controlled temperature conditions (details are given in Materials and methods).

Pollen grains were bulked and thereafter examined for their viability (Alexander 1969). The criteria for selecting viable pollen were pollen size, pollen shape (triangular or spherical) and intensity of stain taken up by pollen.

Stigma receptivity

Stigma receptivity describes the competence of the stigma to hold pollen grains and allow them to germinate in germination media. To detect stigma receptivity, an esterase test was carried out using α-napthyl acetate as the substrate in the azo-coupling reaction with fast blue B, as modified by Mattson et al. (1974). Stigmas were removed one day before flower opening, immersed in a solution containing α napthyl acetate and fast blue B in phosphate buffer, at 37°C for 15 min. The reddish brown colour that developed on the surface of the stigma was scored on a 1–5 scale (1 = low receptivity and 5 = high receptivity).

Pollen germination and pollen tube growth (in vivo)

Aniline blue staining was done to assess pollen germination on stigma and to trace the pollen tube in the style and ovary. Five flowers were collected on the day of opening or anthesis to consecutive three days after anthesis and fixed in acetic alcohol (1:3) for 24 h and cleared in 8 N NaOH for 6 h at 60°C. The gynoecium was stained overnight with aniline blue solution (0.1% in 0.1 mM Na_3PO_4). The stained gynoecia were mounted on aniline blue/10% glycerine (1:1) and observed usingt the Epi-Fluorescent method in a FLUPHOT microscope (Nikon, Japan) with an excitation filter UV 330–380, dichroic mirror DM-400 and absorption filter 420K (Dumas and Knox 1983).

Electrolyte leakage of leaves

Fresh leaf samples (1 g) were washed three times with deionized water to remove surface-adhered electrolytes. Samples were placed in closed vials containing 10 mL of deionized water and incubated at 25°C on a rotary shaker for 24 h and the electrical conductivity of the solution (L_1) was determined. Leaf samples were then autoclaved at 120°C for 20 min and the final electrical conductivity (L_2) was obtained after equilibration at 25°C. Electrolyte leakage was defined as follows: EL (%) = $(L_1/L_2) \times 100$ (Premchandra et al. 1990).

Cellular oxidizing ability of leaves

The cellular oxidizing ability was measured as the 2,3,5-triphenyl tetrazolium chloride (TTC) reduction ability, as per the modified method of Steponkus and Lanphear (1967). Fresh leaf samples (1 g) were cut into 1 cm strips, immersed in incubation solution (50 mM sodium phosphate, pH 7.4) containing various TTC concentrations, and incubated at 25°C in darkness. Because TTC reduction is sensitive to excessive oxygen, the incubation of TTC was carried out 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).

Chlorophyll content of leaves

Fresh leaves (1 g) were extracted with 80% acetone and centrifuged at 5000 ***g*** for 10 min. The absorbance of the supernatant was read at 645 and 663 nm and calculated for total chlorophyll (Arnon 1949).

Oxidative damage in leaves

Leaf samples (1 g) were homogenized in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 ***g*** for 5 min. The supernatant was treated as extract. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content. A 1-mL aliquot of supernatant of leaf extract was mixed with 4 mL of 20% (v/v) TCA containing 0.5% (v/v) thiobarbituric acid. The mixture was heated at 100°C for 30 min, quickly cooled and then centrifuged at 10 000 ***g*** for 10 min. The absorbance of the supernatant was read at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

To measure hydrogen peroxide (H_2O_2) content, leaf samples (1 g) were homogesnied in 5 mL chilled acetone (80%) and filtered with a Whatman filter paper. The filtrate was treated as extract. One millilitre of the extracted solution was mixed with 0.1 mL of 5% titanium dioxide in 20% (v/v) H_2SO_4 and 0.2 mL of ammonia, the mixture was then centrifuged at 806 ***g*** for 10 min, the supernatant was discarded and the precipitate was dissolved in 3 mL of 2 M H_2SO_4 and the absorbance was recorded at 415 nm. The concentration of H_2O_2 was determined using a standard curve plotted with a known concentration of H_2O_2 (Mukherjee and Choudhuri 1983).

Antioxidants (enzymatic and non-enzymatic) in leaves

For enzyme extracts and assays, leaf samples (1 g) were frozen and then ground in 4 mL solution containing 50 mM phosphate buffer (pH 7.0) and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 15 000 ***g*** for 30 min and the supernatant was collected and used in the enzyme assays.

Superoxide dismutase activity was measured according to the method of Giannopolities and Ries (1977). The assay medium contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM *p*-nitro blue tetrazolium chloride (NBT), 2 mM riboflavin, 0.1 mM EDTA and 5 mL enzyme extract. One unit of enzyme activity was determined as the amount of enzyme needed to reach 50% inhibition in the NBT reduction rate by monitoring the absorbance at 560 nm.

Catalase activity was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Change and Maehly 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2.

Ascorbate peroxidase activity was measured according to the method of Nakano and Asada (1981). A leaf sample (1 g) was homogenized in 1 mL of 50 mM phosphate buffer (pH 7.8) containing 5 mM ascorbate, 5 mM dithiothreitol, 5 mM EDTA, 100 mM NaCl and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenized material was centrifuged at 15 000 ***g*** for 15 min at 4°C. The reaction was initiated by adding H_2O_2 to a final concentration of 44 μM. The reaction rate was monitored by decrease in absorbance at 290 nm. The rate constant was calculated using the extinction coefficient of 2.7 mM^{-1} cm^{-1}.

Glutathione reductase (GR) activity was assayed according to Foyer and Halliwell (1976). The oxidized glutathione (GSSG)-dependent oxidation of NADPH was followed at 340 nm in a 1-mL reaction mixture containing 100 mM sodium phosphate buffer (pH 7.8), 0.5 mM GSSG, 50 μL extract and 0.1 mM NADPH.

Ascorbic acid was measured by the method of Mukherjee and Choudhuri (1983). A leaf sample (1 g) was extracted with 10 mL of 6% TCA. The extract was mixed with 2 mL of 2% dinitrophenylhydrazine (in acidic medium) followed by the addition of one drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling at room temperature, 5 mL of 80% (v/v) H_2SO_4 was added to the mixture at 0°C. The absorbance was recorded at 530 nm. The concentration of ascorbic acid was calculated from a standard curve plotted using known concentration of ascorbic acid.

For measurement of glutathione content, a fresh leaf sample (1 g) was homogenized in 2 mL of 2% metaphosphoric acid and centrifuged at 17 000 ***g*** for 10 min. Aliquots of the supernatant were neutralized by adding 0.6 mL of 10% sodium citrate to 0.9 mL of the extract. A total volume of 1 mL of assay containing 700 μL NADPH (0.3 mmol L^{-1}), 100 μL 5, 5′-Dithio-bis(2-nitrobenzoic acid (DTNB) (6 mmol L^{-1}), 100 μL distilled water and 100 μL of extract was prepared and stabilized at 25°C for 3–4 min. Later, 10 μL GR was added and the absorbance was measured at 412 nm. Glutathione was calculated from a standard graph as described by Griffith (1980). The extraction and measurement of proline was carried out as per the method of Bates et al. (1973). Fresh leaf samples (1 g) were extracted with 3% sulfosalicylic acid. Extracts (2 mL) were held for 1 h in boiling water by adding 2 mL ninhydrin and 2 mL glacial acetic acid, after which cold toluene (4 mL) was added. Proline content was measured by a spectrophotometer at 520 nm and calculated from a standard curve plotted using known concentration of proline.

Photochemical efficiency in leaves

Photochemical efficiency was measured as the chlorophyll fluorescence using a chlorofluorometer. The clamps of the instrument were installed on the leaves to keep the leaves 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 Fv/Fm ratio that appeared on the instrument was recorded.

Biomass and yield

Five plants from each genotype were harvested at maturity, sun-dried and weighed for total dry biomass (above ground). The flowers were tagged and examined for pod set (%) in plants growing at different temperatures. The number of fertile and infertile pods, seed weight and harvest index were recorded from the mature plants.

Statistics

The observations were replicated three times and the data were subjected to analysis of variance (ANOVA) and least significant difference (LSD; $p < 0.05$) with SPSS software.

Results

Growth and yield

Plant biomass

Compared with the control, the total plant dry biomass (above ground dry weight) was largely unaffected at 35/25°C in both tolerant and sensitive genotypes and showed a slight increase at this temperature (Table 1). At 40/30°C, the biomass decreased in all genotypes, with a greater decline observed for the sensitive genotypes. At 45/35°C, the sensitive genotypes showed a 19–22% decrease over the tolerant genotypes. In addition, these genotypes experienced greater chlorosis and necrosis of their leaves at these temperatures.

Total pods per plant

Plants growing at 35/25°C experienced a small increase in the number of pods but the contrasting genotypes did not differ significantly from each other at this temperature (Table 1). At 40/30°C, the number of pods decreased compared with the previous temperatures. Thus, at these temperatures, the sensitive genotypes produced 28–37% fewer pods than the tolerant genotypes. At 45/35°C, the sensitive

Table 1. Growth and yield of tolerant (T) and sensitive (S) chickpea genotypes at varying temperatures.

Parameters	30/20°C	35/25°C	40/30°C	45/35°C
Dry biomass (g plant^{-1})				
ICCV07110 (T)	6.76 ± 1.3aB	7.18 ± 0.81aA	6.23 ± 0.18aB	5.11 ± 0.17aC
ICCV92944 (T)	6.96 ± 1.1aB	7.94 ± 0.87aA	6.34 ± 0.21aB	5.24 ± 0.14aC
ICC14183 (S)	7.11 ± 1.6aA	7.61 ± 0.92aA	5.86 ± 0.16bB	4.23 ± 0/18bC
ICC5912 (S)	6.56 ± 1.4aB	7.24 ± 0.78aA	5.72 ± 0.20bC	4.06 ± 0.20bD
Total pods plant^{-1}				
ICCV07110 (T)	19.4 ± 0.16aB	22.4 ± 0.23aA	16.3 ± 0.17aC	3.1 ± 0.64bD
ICCV92944 (T)	21.5 ± 0.19aB	24.6 ± 0.27aA	18.2 ± 0.19aC	4.5 ± 0.71aD
ICC14183 (S)	20.8 ± 0.20aA	22.3 ± 0.22aA	13.1 ± 0.16b B	0
ICC5912 (S)	20.4 ± 0.26aA	23.4 ± 0.25aA	11.2 ± 0.23b B	0
Unfilled pods plant^{-1}				
ICCV07110 (T)	0	0	0.8 ± 0.2bB	1.5 ± 0.3aA
ICCV92944 (T)	0	0	1.1 ± 0.3bA	1.3 ± 0.4aA
ICC14183 (S)	0	0	3.0 ± 0.5a	0
ICC5912 (S)	0	0	3.9 ± 0.5a	0
Seed dry mass (g plant^{-1})				
ICCV07110 (T)	4.32 ± 0.71aA	5.03 ± 0.27aA	3.21 ± 0.21aB	0.57 ± 0.17bC
ICCV92944 (T)	4.98 ± 0.64aA	5.20 ± 0.17aA	3.41 ± 0.25aB	0.86 ± 0.21aC
ICC14183 (S)	4.60 ± 0.84aA	5.12 ± 0.0.21aA	2.13 ± 0.18bB	0
ICC5912 (S)	4.21 ± 0.91aA	4.96 ± 0.26aA	1.86 ± 0.19bB	0
Harvest index				
ICCV07110 (T)	63.9 ± 0.38bB	70.0 ± 0.53aA	51.5 ± 0.31aC	11.1 ± 1.6bD
ICCV92944 (T)	71.5 ± 0.46aA	65.4 ± 0.59aB	53.7 ± 0.42aC	16.1 ± 1.8aD
ICC14183 (S)	64.6 ± 0.59bA	67.2 ± 0.62aA	34.5 ± 0.4bB	0
ICC5912 (S)	64.1 ± 0.48bA	68.0 ± 0.66aA	31.3 ± 0.52bB	0

Note: Values with different lower case (columns) and upper case (horizontal) letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean ± SE ($n = 3$).

genotypes did not produce any pods compared with 3–4.5 pods for the tolerant genotypes. Moreover, the tolerant genotypes also produced some infertile pods at this temperature.

Unfilled pods

Although the temperatures 30/20 and 35/25°C did not yield any unfilled pods, the number of unfilled pods increased at 40/30°C (day and night temperature regime) with a greater impact on the sensitive genotypes (Table 1). At 45/35°C, the number of infertile pods increased in tolerant genotypes, whereas no pod set occurred in the sensitive genotypes at this temperature.

Seed weight per plant

Plants grown at 35/25°C showed increased seed weight compared with those grown at 30/20°C (Table 1). The genotypes did not differ significantly from each other at this temperature. At 40/30°C, the sensitive genotypes experienced a 37–45% reduction in seed weight compared with the tolerant genotypes. At 45/35°C, the tolerant genotypes also experienced considerable reduction in seed weight, whereas the sensitive genotypes were unable to produce any pods and seeds.

Harvest index

The harvest index was found to increase slightly at 35/25°C in all genotypes compared with lower temperatures (Table 1). At 40/30°C, it decreased by 35–41% in the sensitive genotypes over the tolerant genotypes, whereas at 45/35°C, no pods were produced by the former.

Stress injury

The chlorophyll content (Table 2) was unaffected at 35/25°C but decreased at 40/30°C by 23–30% in the sensitive genotypes compared with the tolerant genotypes. At 45/35°C, the sensitive genotypes possessed 22–32% lower chlorophyll content than the tolerant genotypes. Damage to membranes increased with high temperatures in all the genotypes (Table 2). The sensitive genotypes experienced significantly greater damage than the tolerant genotypes. Cellular oxidizing ability was found to increase with increase in temperature. The tolerant genotypes had 19–26% greater oxidizing ability than the sensitive genotypes at 40/30°C. The difference between these genotypes was larger (31–34%) at 45/35°C (Table 2). Photochemical efficiency decreased as the temperature increased in all the genotypes with greater inhibition in sensitive genotypes. At 40/30 and 45/35°C, a reduction of 37–46% and 33–53%, respectively, was observed in photochemical efficiency of the sensitive genotypes over the tolerant genotypes (Table 2).

Reproductive function

Pollen viability

Pollen viability was not affected until 35/25°C, but decreased at 40/30°C to a similar extent in both tolerant and sensitive genotypes. Viability was reduced to a minimum

Table 2. Chlorophyll content, membrane damage, cellular oxidizing ability and photochemical efficiency in tolerant (T) and sensitive (S) chickpea genotypes at varying temperatures.

Parameters	30/20°C	35/25°C	40/30°C	45/35°C
Chlorophyll (mg g^{-1} fw)				
ICCV07110 (T)	5.42 ± 0.31aA	5.11 ± 0.26aA	4.31 ± 0.38aB	3.11 ± 0.30aC
ICCV92944 (T)	4.64 ± 0.42aA	4.87 ± 0.29bA	4.45 ± 0.31aB	3.41 ± 0.26aC
ICC14183 (S)	5.31 ± 0.37aA	5.34 ± 0.45aA	3.31 ± 0.40bB	2.64 ± 0.39bB
ICC5912 (S)	4.86 ± 0.34aA	5.16 ± 0.40aA	3.09 ± 0.32bB	2.31 ± 0.28bB
Membrane damage (%)				
ICCV07110 (T)	10.4 (1.01 ± 0.06) aC	11.5 (0.96 ± 0.06) aC	19.6 (1.2 ± 0.03) bB	22.6 (1.3 ± 0.07) bA
ICCV92944 (T)	8.9 (0.97 ± 0.05) aC	10.3 (0.92 ± 0.07) aC	16.6 (1.0 ± 0.03) bB	20.6 (1.1 ± 0.06) c A
ICC14183 (S)	9.1 (0.98 ± 0.07) aB	11.3 (0.93 ± 0.07) aB	26.4 (1.4 ± 0.04) aA	30.4 (1.4 ± 0.03) aA
ICC5912 (S)	10.1 (1.01 ± 0.06) aB	10.6 (1.0 ± 0.08) aB	28.3 (1.4 ± 0.03) aA	33.3 (1.4 ± 0.04) aA
Cellular oxidizing ability (A_{530} g^{-1} fw)				
ICCV07110 (T)	0.18 ± 0.05aC	0.23 ± 0.07aC	0.38 ± 0.06aA	0.26 ± 0.05aB
ICCV92944 (T)	0.16 ± 0.07aD	0.23 ± 0.06aC	0.41 ± 0.08aA	0.29 ± 0.07aB
ICC14183 (S)	0.20 ± 0.08aC	0.27 ± 0.07aB	0.30 ± 0.07bA	0.19 ± 0.06bC
ICC5912 (S)	0.16 ± 0.06aB	0.22 ± 0.08aB	0.33 ± 0.06bA	0.20 ± 0.05bB
Photochemical efficiency (Fv/Fm ratio)				
ICCV07110 (T)	1.3 ± 0.13aA	1.6 ± 0.17aA	0.98 ± 0.11aB	0.59 ± 0.13aC
ICCV92944 (T)	1.5 ± 0.16aA	1.3 ± 0.13bA	0.91 ± 0.16aB	0.52 ± 0.15aD
ICC14183 (S)	1.5 ± 0.15aA	1.5 ± 0.14aA	0.61 ± 0.14bB	0.39 ± 0.11bC
ICC5912 (S)	1.4 ± 0.16aA	1.6 ± 0.16aA	0.52 ± 0.14bB	0.31 ± 0.15bC

Note: Figures in parentheses are log transformed. Values with different lower casae (columns) and upper case (rows) letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean ± SE ($n = 3$).

level in both the genotypes at 45/35°C, with greater inhibition in the sensitive genotypes (Figure 2).

Pollen germination

Pollen germination was inhibited in plants growing at high temperatures (Table 3). At 40/30°C, tolerant genotypes had 71–76% germination compared with 51–56% in sensitive genotypes. At 45/35°C, germination was reduced markedly in both the genotypes with larger impact on sensitive genotypes (Figure 2).

Pollen tube growth

With increased temperatures, pollen tube growth was inhibited significantly; the sensitive genotypes experienced a greater impact (Table 3). The differences between the tolerant and sensitive genotypes were distinctive at 40/30 and 45/35°C, clearly indicating the higher sensitivity of pollen function in sensitive genotypes (Figure 2).

Stigma receptivity

Compared with 30/20°C, the stigma receptivity (Table 3) was unaffected at 35/25°C, but showed significant reduction at 40/30 and 45/35°C. Stigma receptivity was found to decrease to a greater extent in sensitive genotypes at these temperatures (Figure 2).

Pod set

The pod set (%) was not affected at 35/25°C in either genotype (Table 3). At 40/30°C, the pod set was inhibited considerably, with tolerant genotypes showing a better pod set (51–56%) compared with the sensitive genotypes (28–35%). At 45/35°C, the pod set occurred only in the tolerant genotypes, while the sensitive genotypes did not set any pods at this temperature, indicating impaired fertilization. No pollen tube growth into the ovules of the sensitive genotypes was observed (Figure 2).

Oxidative stress and antioxidants

Oxidative stress was measured in terms of MDA and H_2O_2 content (Figure 3). At 40/30°C, the MDA content was higher by 29–32% in the sensitive genotypes. At the higher temperature of 45/35°C, the MDA content showed a 40–49% increase in the sensitive genotypes compared with the tolerant genotypes. The H_2O_2 content was not significantly different among the genotypes at 35/25°C, whereas at 40/30°C, the sensitive genotypes possessed 18–21% higher hydrogen peroxide content than the tolerant genotypes. At 45/35°C, the sensitive genotypes showed an almost two-fold increase in H_2O_2 compared with the tolerant genotypes.

The activity and levels of antioxidants were increased significantly as the temperatures increased to 40/30°C, but decreased at 45/35°C in all genotypes. Regarding enzymatic antioxidants, the activity of superoxide dismutase (SOD) and catalase (CAT) differed only slightly among the genotypes at 40/30 and 45/35°C (Figure 4). However, the activity of ascorbate peroxidase (APX) was reduced by 38–49% in the sensitive genotypes at 40/30°C and by 30–46% at 45/35°C relative to the tolerant genotypes (Figure 5). The GR activity in the sensitive genotypes decreased

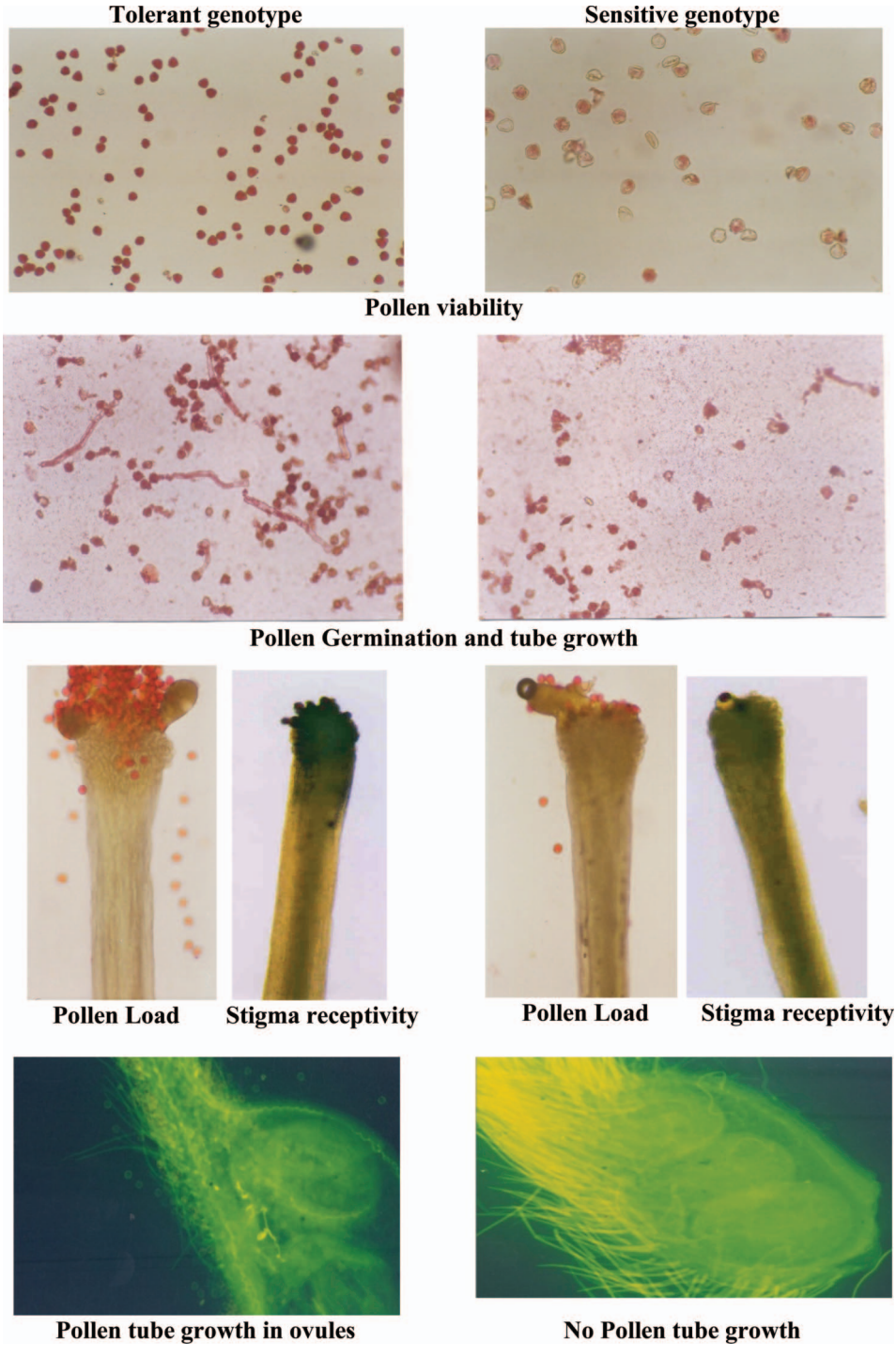

Figure 2. Pollen viability, pollen germination, pollen load, stigma receptivity and pollen tube growth in ovules in tolerant (ICCV92944) and sensitive (ICC5912) chickpea genotypes. Flowers were collected from the plants growing at 45/35°C.

Table 3. Pollen function, stigma receptivity and pod set in tolerant and sensitive chickpea genotypes at varying temperatures.

Parameters	30/20°C	35/25°C	40/30°C	45/35°C
Pollen viability (%)				
ICCV07110 (T)	89.6 (1.23 ± 0.30aA)	90.3 (1.23 ± 0.05aA)	82.6 (1.14 ± 0.04aB)	34.1 (0.64 ± 0.08aC)
ICCV92944 (T)	90.1 (1.25 ± 0.24aA)	88.6 (1.22 ± 0.04aA)	80.8 (1.16 ± 0.03aB)	42.6 (0.71 ± 0.09aC)
ICC14183 (S)	91.3 (1.27 ± 0.26aA)	85.3 (1.26 ± 0.06 aB)	82.0 (1.14 ± 0.04aB)	29.8 (0.57 ± 0.07bC)
ICC5912 (S)	90.6 (1.26 ± 0.21aA)	82.1 (1.16 ± 0.04bB)	80.6 (1.15 ± 0.05aB)	24.6 (0.52 ± 0.07bC)
Pollen germination (%)				
ICCV07110 (T)	87.6 (1.21 ± 0.05aA)	85.4 (1.15 ± 0.04aA)	71.4 (1.06 ± 0.04aB)	38.3 (0.67 ± 0.04aC)
ICCV92944 (T)	89.4 (1.24 ± 0.04aA)	87.4 (1.15 ± 0.05aA)	76.1 (1.10 ± 0.03aB)	42.4 (0.71 ± 0.05aC)
ICC14183 (S)	88.1 (1.22 ± 0.06aA)	84.3 (1.17 ± 0.05aA)	56.3 (0.97 ± 0.04bB)	20.3 (0.47 ± 0.05bC)
ICC5912 (S)	90.2 (1.26 ± 0.05aA)	84.1 (1.15 ± 0.05aA)	51.3 (0.94 ± 0.05bB)	17.6 (0.44 ± 0.04bB)
Pollen tube size (μm)				
ICCV07110 (T)	17.6 ± 1.8aA	20.3 ± 1.7aA	14.4 ± 1.3aB	5.9 ± 0.7aC
ICCV92944 (T)	18.3 ± 1.6aA	17.6 ± 1.8aA	16.3 ± 1.6aA	6.4 ± .0.9aB
ICC14183 (S)	16.9 ± 1.8aA	19.3 ± 1.5aA	10.3 ± 1.9bB	1.8 ± 0.7bC
ICC5912 (S)	17.2 ± 1.3aA	18.6 ± 1.8aA	8.4 ± 1.4bB	1.1 ± 0.6bC
Stigma receptivity (esterase test) (1–5 scale)				
ICCV07110 (T)	4.6 (0.74 ± 0.04aA)	4.1 (0.71 ± 0.04aA)	2.6 (0.56 ± 0.04aB)	1.6 (0.41 ± 0.04aC)
ICCV92944 (T)	4.1 (0.71 ± 0.03aA)	4.4 (0.72 ± 0.03aA)	3.1 (0.61 ± 0.03aB)	1.4 (0.37 ± 0.03aC)
ICC14183 (S)	4.4 (0.73 ± 0.06aA)	4.6 (0.74 ± 0.05aA)	1.8 (0.45 ± 0.04bB)	1.1 (0.32 ± 0.04bB)
ICC5912 (S)	4.2 (0.71 ± 0.05aA)	4.3 (0.72 ± 0.04aA)	1.7 (0.43 ± 0.05bB)	0.8 (0.24 ± 0.04bC)
Pod set (%)				
ICCV07110 (T)	87.5 (1.21 ± 0.04aA)	89.2 (1.19 ± 0.03aA)	51.5 (0.79 ± 0.05aB)	18.3 (0.44 ± 0.05aC)
ICCV92944 (T)	85.4 (1.17 ± 0.05aA)	86.4 (1.15 ± 0.04aA)	56.4 (0.84 ± 0.04aB)	23.4 (0.50 ± 0.06aC)
ICC14183 (S)	86.1 (1.19 ± 0.05aA)	87.5 (1.20 ± 0.04aA)	35.6 (0.63 ± 0.04 bB)	0
ICC5912 (S)	88.2 (1.21 ± 0.04aA)	85.2 (1.16 ± 0.04aA)	28.1 (0.53 ± 0.05 cB)	0

Note: Figures in parentheses are arcsin transformed except for stigma receptivity (log transformed). Values with different lower case (columns) and upper case (rows) letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean ± SE ($n = 3$).

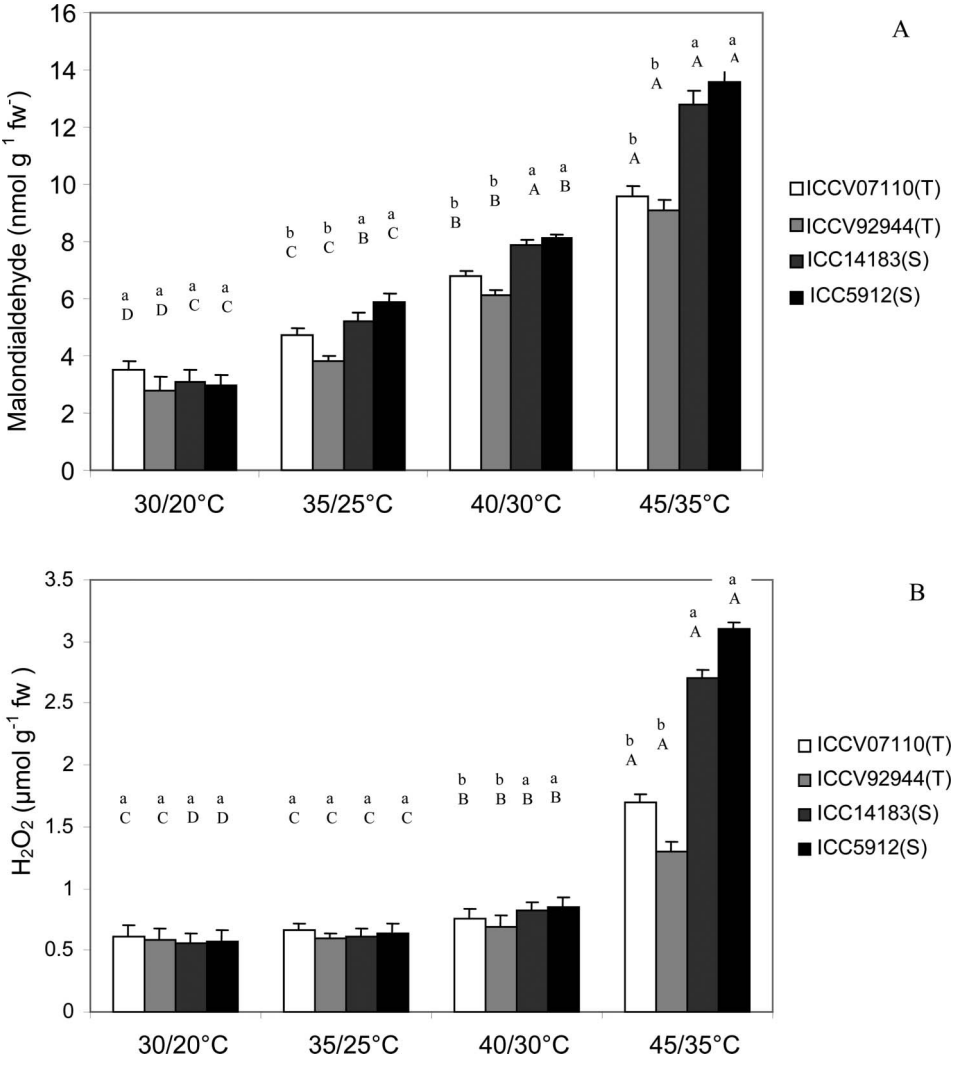

Figure 3. Malondialdehyde (A) and hydrogen peroxide (B) content in tolerant (T) and sensitive (S) chickpea genotypes at varying temperatures. Values with different lower case and upper case letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean $\pm$ SE ($n = 3$).

by 43–50% at 40/30°C and by 44–49% at 45/35°C compared with the tolerant genotypes.

The non-enzymatic antioxidants (Figure 6A,B) ascorbate and reduced glutathione showed a significant increase at 35/25°C relative to the lower temperatures. Their levels increased further at 40/30°C in both genotype categories. At this temperature, the sensitive genotypes had 13–18% lower ascorbate than the tolerant genotypes. At 45/35°C, the ascorbate content was 28–32% lower in the sensitive genotypes than in the tolerant genotypes. The reduced glutathione content was also significantly lower (24–33% at 40/30°C and 37–44% at 45/35°C) in the sensitive genotypes compared with the tolerant genotypes. Endogenous proline (Figure 6C)

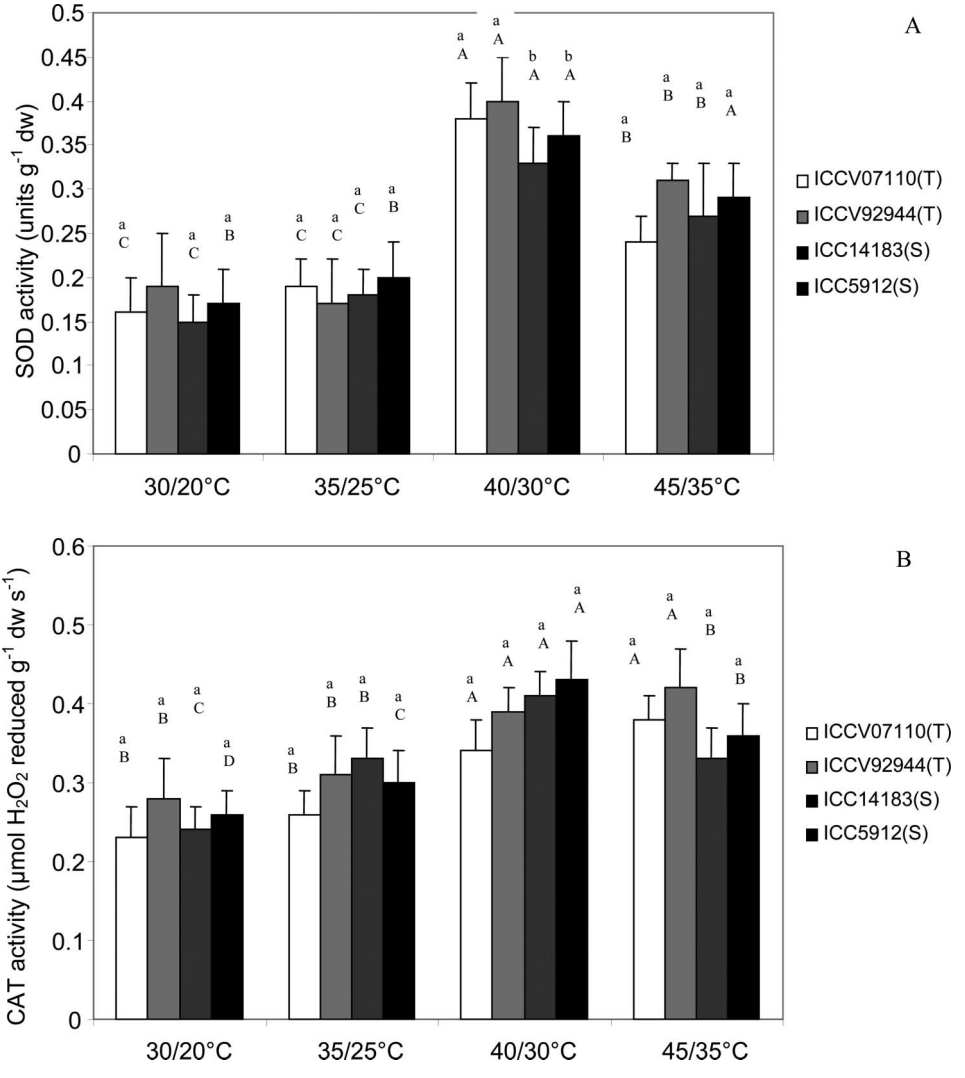

Figure 4. Superoxide dismutase (SOD; A) and catalase (CAT; B) activities in tolerant (T) and sensitive (S) chickpea genotypes at varying temperatures. Values with different lower case and upper case letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean $\pm$ SE ($n = 3$).

increased with temperature up to 40/30°C to the same extent in tolerant and sensitive genotypes, but proline decreased significantly at 45/35°C. No significant differences in proline content were observed between the tolerant and sensitive genotypes at this temperature.

Discussion

Chickpea exhibits optimum growth between 15 and 30°C and is sensitive to supra-optimal temperatures, especially during its reproductive phase, and consequently experiences yield losses at temperatures >35°C (Wang J et al. 2006). Because the

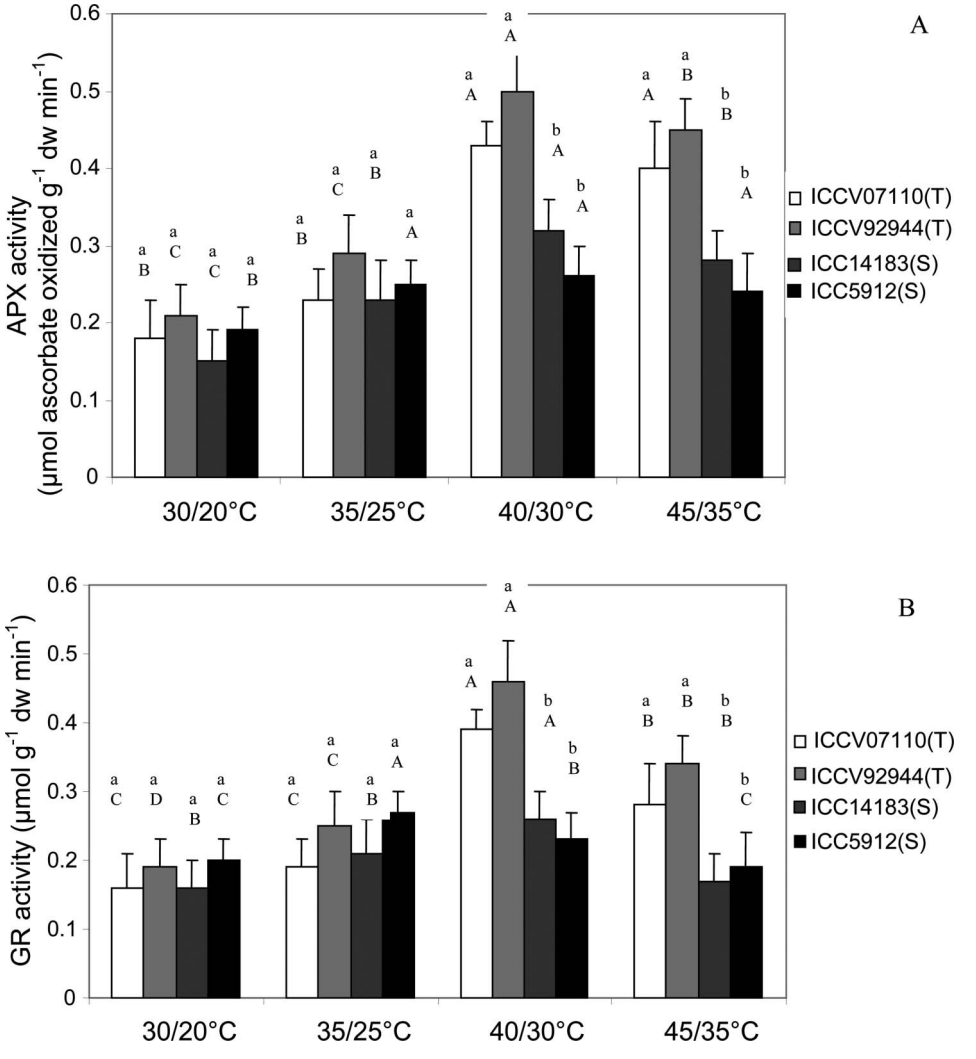

Figure 5. Ascorbate peroxidase (APX; A) and glutathione reductase (GR; B) activities in tolerant (T) and sensitive (S) chickpea genotypes at varying temperatures. Values with different lower case and upper case letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean $\pm$ SE ($n = 3$).

reasons affecting chickpea heat sensitivity have not been reported earlier, in this study we examined the reproductive function and leaf damage due to increased temperatures in differentially sensitive genotypes.

The symptoms of heat stress became particularly evident in the sensitive genotypes at 40/30°C in the form of enhanced chlorosis of leaves, as evidenced by the decrease in chlorophyll content. The leaves also experienced damage to membranes and tissue viability at 40/30°C that intensified at 45/35°C in all the genotypes, with greater impact on the sensitive genotypes. The appearance of chlorosis in heat-stressed chickpea plants is similar to previous observations on mungbean (Kumar et al. 2011a,b), wheat (Almeselmani et al. 2009) and maize (Karim et al. 1997).

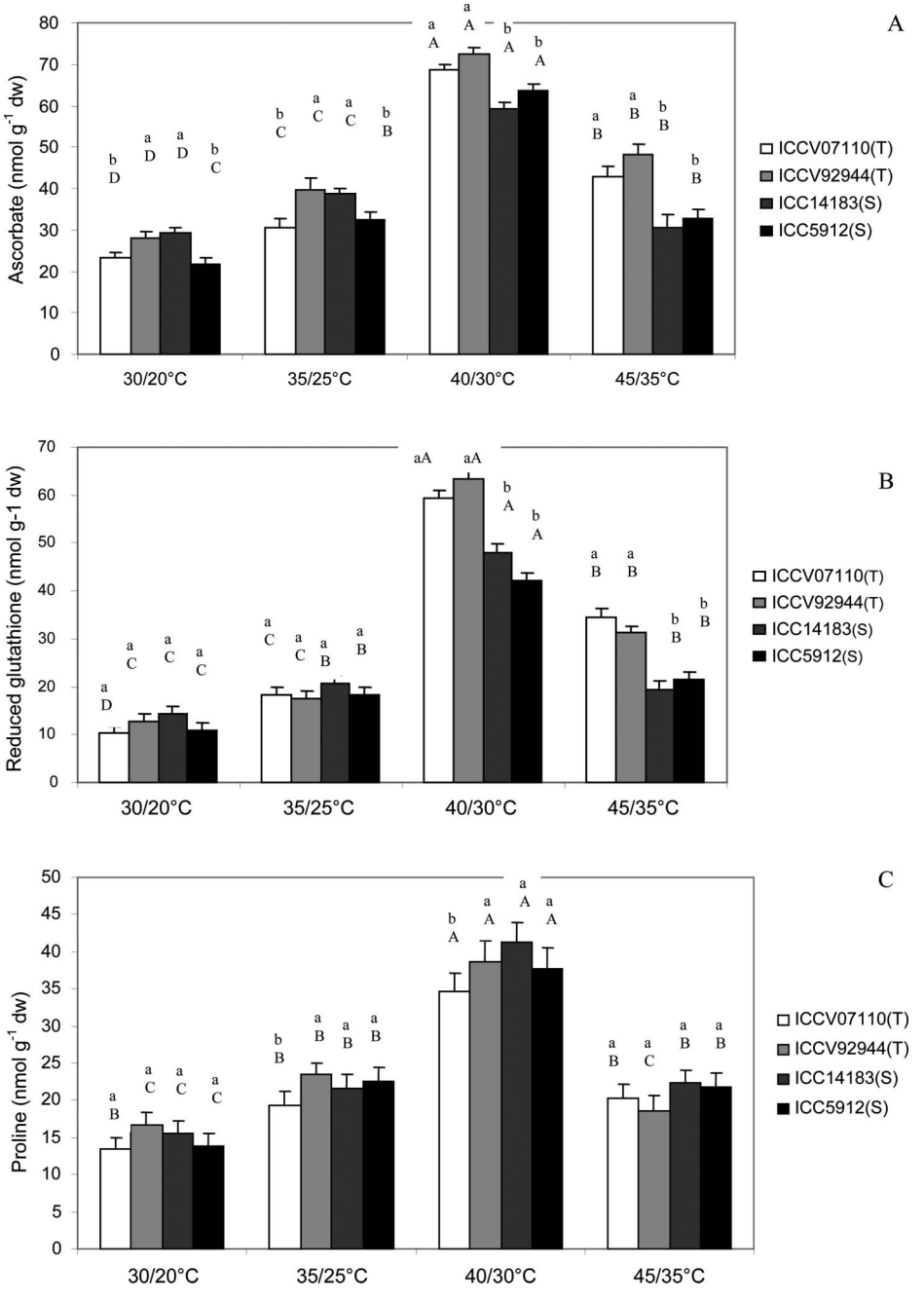

Figure 6. Ascorbate (A), reduced glutathione (B) and proline (C) content in tolerant and sensitive chickpea genotypes at varying temperatures. Values with different lower case and upper case letters represent significant variations among genotypes and temperatures, respectively (Tukey's LSD test; $p < 0.05$). Mean $\pm$ SE ($n = 3$).

The loss of chlorophyll may be attributed to photo-oxidative stress or inhibition of chlorophyll synthesis (Guo et al. 2006) due to heat stress. The injury to membranes observed in our studies could be the direct effect of high temperature on membranes

or the consequence of oxidative stress, in line with previous observations on rice (Sohn and Back 2007) and wheat (Almseselmani et al. 2009). The cellular oxidizing ability was reduced appreciably at 45/35°C suggesting impairment in respiration and energy generation, possibly due to inactivation of the enzymes (Salvucci and Crafts-Brandner 2004), which match with previous studies on heat stress in potato (Coria et al. 1998) and wheat (Wang WC and Nguyen 1989). The photochemical efficiency was reduced to a significantly greater level in the sensitive genotypes at 40/30 and 45/35°C that which indicated damage to the functional status of the photosynthetic apparatus (Yamane et al. 1997). Our results are in agreement with those of Petkova et al. (2007) in bean, in which heat-tolerant genotypes had a greater Fv/Fm ratio. Thus, in our studies, the heat-tolerant chickpea genotypes retained significantly greater chlorophyll content and photochemical efficiency, had higher cellular viability, and experienced less membrane damage than the sensitive genotypes at higher temperatures. It was apparent from these observations that the tolerant genotypes were able to minimize heat stress injury to the leaf tissues to a greater extent than the sensitive genotypes.

The major reason for reduced yields due to heat stress was failure to set pods at high temperatures, especially by the heat-sensitive genotypes. We examined the pollen function under varying temperatures and found impairment with increased temperature. Pollen viability decreased at 45/35°C in both categories of genotype, which is similar to the responses of other plant species such as *Phaseolus vulgaris* (Porch and Jahn 2001), tomato (Pressman et al. 2006) and pepper (Kafizadeh et al. 2008). The reduction in pollen viability might be related to their developmental impairment (Porch and Jahn 2001) or lack of sufficient starch at maturity (Pressman et al. 2006). The pollen of sensitive genotypes experienced greater loss of viability possibly due to the higher abnormalities as cited above, which need to be examined further. Pollen germination in plants growing at 40/30 and 45/35°C was inhibited both under in vivo and in vitro conditions with a greater impact on the sensitive genotypes. Poor pollen germination could be the result of undernourished pollen during development due to stress, as reported in tomato (Pressman et al. 2006) and *Brassica* species (Young et al. 2004). Previous studies have reported that the tapetal layer in the anthers, which provides nutrients to the developing pollen, is the target of thermal stress, as reported in cowpea (Ahmed et al. 1992), chickpea (Kumar et al. 2010) and *P. vulgaris* (Porch and Jahn 2001). Disorganization of the tapetum leads to developmental and functional abnormalities of the pollen (Porch and Jahn 2001; Suzuki et al. 2001). Pollen load was inhibited severely at high temperature in the sensitive genotypes, suggesting some restrictions in the dehiscence of anthers, as reported in cowpea (Ahmed et al. 1992), and also been reported previously in chickpea subjected to cold stress (Kumar et al. 2010). Pollen adhesion to the stigma surface has been reported to decline with increases in temperature, as observed in sweet cherry (*Prunus avium*) by Hedhly et al. (2003).

Stigma receptivity also decreased with the increase in temperature in both tolerant and sensitive genotypes, which is similar to earlier observations on crop species such as chickpea (Kumar et al. 2010) and sweet cherry (*Prunus avium*; Hedhly et al. 2003) exposed to temperature stress. Stigma receptivity is based upon the expression of esterases (Shivanna and Sastri 1981) on the surface of the stigma, which appeared to be inactivated in stressed plants. Eventually, the fertilization process was impaired in infertile flowers due to restriction in pollen tube growth to reach the ovules, which is similar to observations on chickpea subjected to cold

(Nayyar et al. 2005) and drought stress (Fang et al. 2010), as well as cotton subjected to heat stress (Snider et al. 2011). Lack of nutrients from the style for the growing pollen tube, possibly due to lack of transport, might also contribute towards impaired tube growth (Nayyar et al. 2005; Snider et al. 2011). We previously reported reduced sucrose levels in the style of cold-stressed chickpea that possibly caused starvation of pollen tubes (Nayyar et al. 2005). Such reasons need to be investigated under heat stress also and will be part of our future investigation. It is possible that heat stress might have also affected mega-gametophyte in chickpea as reported in *Brassica napus* (Young et al. 2004), which requires to be investigated in our case.

Oxidative injury is one of the primary causes of damage by heat stress to plant cells, as reported in tomato (Rivero et al. 2004) and chickpea (Kaushal et al. 2011), which is manifested in increases in chlorophyll loss, membrane leakage and damage to macromolecules (Wahid et al. 2007). We recorded an increase in oxidative stress (measured as MDA and H_2O_2 content) at 40/30°C, reaching a maximum at 45/35°C. In this context, our findings are similar to those of other studies on heat-stressed tomato (Rivero et al. 2004) and creeping bentgrass (Liu and Huang 2000). The tolerant chickpea genotypes were seen to have experienced less oxidative damage, especially to their membranes as indicated by lower MDA content than in sensitive genotypes. Our findings in this regard are comparable with observations on contrasting wheat genotypes exposed to heat stress (Almeselmani et al. 2009).

To defend against oxidative stress, cells produce enzymatic and non-enzymatic antioxidants (Mittler 2002). In our studies, plants exposed to 40/30°C showed an appreciable increase in these antioxidants in both tolerant and sensitive genotypes. Comparatively, the tolerant genotypes differed from the sensitive genotypes with respect to a greater increase in the activity of APX and GR among the enzymatic antioxidants, as well as ascorbic acid and reduced glutathione content among the non-enzymatic antioxidants, pertinently at 45/35°C. The enzymes APX and GR are components of the ascorbate–glutathione cycle and are implicated in the removal of H_2O_2. Their elevation in the tolerant genotypes reflects the efficient detoxification of H_2O_2 compared with the sensitive genotypes which possibly reduced heat-stress-induced damage in the former. By contrast, no significant differences existed for SOD and CAT activities between the tolerant and sensitive genotypes at 40/30 and 45/35°C. SOD removes peroxides while converting them to H_2O_2, which is quickly acted upon by catalase to convert into water and oxygen. It appears that at extreme temperatures, the expression of antioxidants is inactivated or inhibited. Tolerant genotypes are able to maintain APX and GR at higher levels, which might be significant in the differential heat sensitivity of the contrasting genotypes. Our findings are slightly different from those on heat-stressed wheat in which the tolerant genotypes possessed greater activity levels of all the antioxidants such as SOD, APX, CAT, GR and peroxidase (Almeselmani et al. 2009). These findings are similar to those of Sairam et al. (2000) on wheat in which the tolerant genotypes had greater APX activity in a heat-tolerant genotype compared with a sensitive genotype.

Conclusion

In conclusion, heat-tolerant chickpea genotypes possessed better functioning of male and female components, coupled with greater antioxidative ability in terms of the components of the ascorbate–glutathione cycle relative to the heat-sensitive genotypes under high temperature stress. For screening of heat tolerance at

reproductive stage in chickpea, pollen germination, pollen tube growth and per cent pod set can be employed.

Acknowledgement

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