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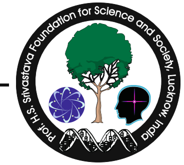

RESEARCH ARTICLE

Proline induces heat tolerance in chickpea (*Cicer arietinum* L.) plants by protecting vital enzymes of carbon and antioxidative metabolism

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Abstract Chickpea is a heat sensitive crop hence its potential yield is considerably reduced under high temperatures exceeding 35 °C. In the present study, we evaluated the efficacy of proline in countering the damage caused by heat stress to growth and to enzymes of carbon and antioxidative metabolism in chickpea. The chickpea seeds were raised without (control) and with proline (10 μM) at temperatures of 30/25 °C, 35/30 °C, 40/35 °C and 45/40 °C as day/ night (12 h/12 h) in a growth chamber. The shoot and root length at 40/35 °C decreased by 46 and 37 %, respectively over control while at 45/40 °C, a decrease of 63 and 47 %, respectively over control was observed. In the plants growing in the presence of 10 μM proline at 40/35 °C and 45/40 °C, the shoot length showed improvement of 32 and 53 %, respectively over untreated plants, while the root growth was improved by 22 and 26 %, respectively. The stress injury (as membrane damage) increased with elevation of temperatures while cellular respiration, chlorophyll content and relative leaf water content reduced as the temperature increased to 45/40 °C. The endogenous proline was elevated to 46 μmol g^{-1} dw at 40/35 °C but declined to 19 μmol g^{-1} dw in plants growing at 45/40 °C that was associated with considerable inhibition of growth at this temperature. The oxidative damage measured as malondialdehyde and hydrogen peroxide content increased manifolds in heat stressed plants coupled with inhibition in the activities of enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and levels of non-enzymatic (ascorbic acid, glutathione, proline) antioxidants. The enzymes associated with carbon fixation (RUBISCO), sucrose synthesis (sucrose phosphate synthase) and sucrose hydrolysis (invertase) were strongly inhibited at 45/40 °C. The plants growing in the presence of proline accumulated proline up to 63 μmol g^{-1} dw and showed less injury to membranes, had improved content of chlorophyll and water, especially at 45/40 °C. Additionally, the oxidative injury was significantly reduced coupled with elevated levels of enzymatic and non-enzymatic antioxidants. A significant improvement was also noticed in the activities of enzymes of carbon metabolism in proline-treated plants. We report here that proline imparts partial heat tolerance to chickpea's growth by reducing the cellular injury and protection of some vital enzymes related to carbon and oxidative metabolism and exogenous application of proline appears to have a countering effect against elevated high temperatures on chickpea.

Keywords Chickpea · Carbon fixation · Heat stress · Oxidative stress · Proline

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Introduction

The atmospheric temperatures are rising due to potential climatic changes (Cutforth 2000) that are proving to be a concern for agricultural crops growing in arid and semi-arid regions (Wahid et al. 2007). Moreover, the rising temperatures may result in altered geographical distribution and growing season of agriculturally crops by causing the threshold temperature for the commencing the season and crop maturity to reach earlier (Porter 2005). Heat stress can impair the overall normal growth and development of the

plants causing reduction in their production potential leading to severe yield losses (Hall 2004). High temperature can accelerate the rate of plant development (Gan et al. 2004), hasten the reproductive growth, shorten the duration of reproductive growth, affect the flowering and pod filling stages (Hall 2004, Boote et al. 2005).

At cellular and sub-cellular levels, heat stress can cause several alterations, which depend upon the growth stage, intensity and duration of heat stress (Sung et al. 2003). Heat stress can directly result in denaturation of proteins and enzymes (Kepova et al. 2005), membrane damage (Liu and Huang 2000) and can indirectly result in inactivation of enzymes located in the mitochondria and chloroplasts, reduction in protein synthesis and disruption of their membranes (Howarth 2005). One of the prominent effects of heat stress includes oxidative damage due to production of reactive oxygen species like superoxides, lipid peroxides and hydrogen peroxide (Rivero et al. 2001, Yin et al. 2008). To deal with oxidative damage caused by heat stress, the plants activate several enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and non-enzymatic (ascorbic acid, glutathione) antioxidants as reported in wheat (Balla et al. 2007), strawberry (Wang and Zheng 2001) and rice (Cao et al. 2008). Heat stress is reported to inhibit photosynthesis by impairing the functioning of ribulose 1,5 bisphospahe carboxylase (RUBISCO) and sucrose metabolizing enzymes (Chaitanya et al. 2001; Tian et al. 2006).

Chickpea is a cool-season legume of northern region of India, which is also being cultivated in warm season environment of central and southern parts of the country. Due to changing climate, the exposure of chickpea to high temperature in terms of intensity and duration is expected to increase leading to reduction in its potential yield. Previous reports have indicated adverse effects of high temperature on chickpea (Summerfield et al. 1984; Wang et al. 2006). Thus, effective measures are needed to counter the negative effects of high temperature on this crop.

One of the ways to deal with adverse effects of heat stress may involve exploring some molecules that have the potential to protect the plants from the harmful effects of high temperature. Proline, an amino acid, which is elevated in response to diverse types of abiotic stresses (Verbruggen and Hermans 2008) is one such molecule that has several roles such as turgor generation, storage of carbon and nitrogen, as partial antioxidant (Smirnoff and Cumbes 1989), molecular chaperone stabilizing the structure of proteins, maintenance of cytosolic pH, balance of redox status and as part of stress signal (Maggio et al. 2002) influencing adaptive responses (Verbruggen and Hermans 2008). Previous studies report that exogenous proline application may improve the tolerance against different types of abiotic stresses such as osmotic (Beumer et al. 1994), salt (Hoque et al. 2007a, b) and chilling (Posmyk and Janas 2007), but not heat stress. Hence, in the present study, we aimed at exploring the (a) association of proline with heat stress response of plants using chickpea as a model and (b) mechanism of proline in imparting protection against heat stress.

Materials and methods

Chickpea (*Cicer arietinum* L; cultivar GPF2) seeds were treated with 0.1 % mercuric chloride and grown hydroponically at temperatures of 30/20 °C, 35/25 °C, 40/30 °C and 45/35 °C as day/ night (12 h/12 h) in a growth chamber in the absence (control) or presence of 10 μM proline. The concentrations of proline were optimized using a range from 5, 10 and 15 μM on growth and stress injury. The plants growing at a temperature of 30/20 °C were treated as controls. The seeds were counted for germination everyday and the seedling growth was observed on 10th day. Based upon the findings on growth, the treatment of 10 μM proline was found to be the best. Hence, we focused only on this treatment for our subsequent observations on shoots for analysis of the following parameters on 10th day:

Stress injury The stress injury was measured using some indicators like electrolyte leakage (Premchandra et al. (1990), total chlorophyll content (Arnon 1949), 2,3,5 triphenyl tetrazolium chloride (TTC) reduction ability (Steponkus and Lanphear 1967) and relative leaf water content (Barrs and Weatherley (1962), which have been described previously (Nayyar and Gupta 2006).

Enzymes of carbon and carbohydrate metabolism

The activities of ribulose 1,5, bisphosphate carboxylase/ oxygenase (RUBISCO) was assayed as per the methods of Racker (1962) while for assaying the activity of invertase and sucrose phosphate synthase, the methods of Hawker et al. (1976) was used.

Oxidative damage The stress-induced oxidative injury was measured as lipid peroxidation (malondialdehyde content) and hydrogen peroxide content according to the methods of Heath and Packer (1968) and Mukherjee and Choudhuri (1983), respectively. The methods have been described in detail previously (Nayyar and Gupta 2006).

The antioxidants such as enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and non-enzymatic (ascorbic acid, glutathione, proline) were analyzed from the shoots of control and stressed

plants as follows. The frozen tissue was homogenized in 4 ml solution containing 50 mM phosphate buffer (pH 7.0), 1 % (w/v) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid (ASA). The homogenate was centrifuged at 15,000 g for 30 min, supernatant was collected and used for enzyme assays. The superoxide activity was assayed as per the method of Giannopolities and Ries (1977). The activity of catalase was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 according to the method of (Change and Maehly 1955). The ascorbate peroxidase was assayed as a decrease in absorbance at 290 nm for 1 min as per the method of Nakano and Asada 1981). The activity of glutathione reductase (GR) was assayed as described by Foyer and Halliwell (1976). The ascorbic acid content was measured by the method of Mukherjee and Choudhuri (1983). The leaves were extracted with 10 ml of 6 % trichloroacetic acid. The extract was mixed with 2 ml of 2 % dinitrophenylhydrazine (in acidic medium) followed by addition of 1 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 with its known concentration. The glutathione content was measured by using fresh leaf tissue that was homogenized in 2 ml of 2 % metaphosphoric acid and centrifuged at 17,000 g for 10 min. The 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), 100 µl DTNB (6 mmol/l), 100 µl distilled water and 100 µl of extract was prepared and stabilized at 25 °C for 3–4 min. Later 10 µl of glutathione reductase was added and the absorbance was measured at 412 nm. Glutathione was calculated from a standard graph as described by Griffth (1980).

The proline content was estimated using the acid ninhydrin method (Bates et al. 1973). The leaf tissue was homogenized with 6 ml of 3 % (w/v) sulfosalicylic acid aqueous solution and the homogenate was filtered through Whatman No. 1 filter paper. Two ml of the filtered extract was taken for the analysis to which 2 ml acid ninhydrin and 2 ml of glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1 h and the reaction was finished in an ice bath. Four ml of toluene was added to the reaction mixture and the organic phase was extracted, in which a toluene soluble reddish chromophore was obtained, which was read at 520 nm using toluene as blank by UV-visible spectrophotometer.

Antioxidants The enzymatic and non-enzymatic antioxidants were estimated from the leaves as follows. The leaves were frozen and then ground in 4 ml solution containing 50 mM phosphate buffer (pH 7.0), 1 % (w/v) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid (ASA). The homogenate was centrifuged at 15,000 g for 30 min, and supernatant was collected and used for enzyme assays. The superoxide activity was assayed as per 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 the enzyme to reach an inhibition of 50 % NBT reduction rate by monitoring the absorbance at 560 nm. The activity of catalase was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 according to the method of (Change and Maehly 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2. The ascorbate peroxidase was assayed as a decrease in absorbance at 290 nm for 1 min as per the method of Nakano and Asada (1981). The assay mixture consisted of 0.5 mM ascorbic acid, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.0), and 0.15 ml enzyme extract. The activity of glutathione reductase (GR) was assayed as described by 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.

All the observations were replicated three times and analyzed statistically for Tukey's test with SPSS software.

Results

On 7th day, the germination was reduced to 61 % at 40/35 °C and to 24 % at 45/40 °C compared to 100 % in control (30/25 C) (Table 1). With 5 µM and 15 µM proline application, no significant change was observed in germination response at these temperature while with 10 µM proline, the germination improved to 88 and 76 % at 40/35 and 45/40 °C, respectively.

The shoot length (Table 1) decreased by 46 and 63 % at 40/35 and 45/40 °C, respectively. With 5 µM proline application, a slight improvement was observed in the shoot length at 45/40 °C. With 10 µM proline treatment, an increase of 32 and 36 % occurred in the shoot length at 40/35 °C and 45/40 °C, respectively over untreated plants growing at these temperatures. The treatment with 15 µM proline resulted in improvement in shoot length only by 12–13 % over untreated plants.

Table 1 Effect of proline (5–15 μM) application on germination and seedling growth (10th day) in heat stressed chickpea seedlings. Values with same letters in the same column are not different significantly at *P*<0.05 (Tukey's LSD test)

Treatment	Germination %	Shoot length (cm)	Root length (cm)
30/25 °C	100 a	4.60a	6.13a
35/30 °C	100a	4.29a	6.01a
40/35 °C	61c	2.64 c	3.87c
45/40 °C	24d	1.76 e	3.08e
30/25 °C+5 μM Proline	100a	4.56a	6.23a
35/30 °C+5 μM Proline	100a	4.31a	6.11a
40/35 °C+5 μM Proline	74.5c	2.61c	3.81c
45/40 °C+5 μM Proline	62d	1.91d	3.15e
30/25 °C+10 μM Proline	100a	4.53a	6.16a
35/30 °C+10 μM Proline	100a	4.39a	6.09a
40/35 °C+10 μM Proline	88b	3.50 b	4.72b
45/40 °C+10 μM Proline	76c	2.76c	3.88c
30/25 °C+15 μM Proline	100a	4.37 °	6.01a
35/30 °C+15 μM Proline	100a	4.31 °	5.94a
40/35 °C+15 μM Proline	76c	2.70c	4.30c
45/40 °C+15 μM Proline	66d	2.00d	3.50d

The root length (Table 1) decreased at 40/35 °C by 37 % while at higher temperature (45/35 °C), 47 % inhibition was observed over control. Proline at 5 μM concentration did not cause any significant change in root length at high temperatures while 10 μM proline resulted in 21 % and 20 % improvement at 40/35 and 45/40 °C, respectively over plants not treated with proline. With 15 μM proline application, an improvement of 13 % at 40/35 °C and 11 % at 45/40 °C was reported in root growth over untreated controls.

Stress injury At temperature of 40/35 °C, the membrane damage in the shoots measured as electrolyte leakage (EL) increased to 26 % and it elevated further to 36 % at 45/40 °C compared to 9 % in control (Table 2). With 10 μM proline application, the EL decreased to 18 % at 40/35 °C and 24 % at 45/40 °C.

Cellular respiration (measured as 2,3,5-triphenyl tetrazolium chloride (TTC) reduction test) The cellular respiration in the shoots showed 39 % increase over control at 40/35 °C (Table 2) but declined by 34 % at 45/40 °C over the previous temperature. With proline application, the cellular respiration decreased slightly at 40/35 °C but increased by 26 % at 45/40 °C relative to the untreated plants growing at this temperature**.**

Total chlorophyll and relative leaf water content The total chlorophyll content (Table 2) decreased by 28 and 46 % at 40/35 and 45/40 °C, respectively over control. The proline treated plants showed improvement of 18 % at 40/35 °C and 44 % at 45/40 °C over the untreated plants. The relative leaf water content decreased to 76 % at 40/35 °C and to 67 % at 45/40 °C compared to 87 % in controls (Table 2). The proline treated plants, especially those growing at 45/40 °C showed significant improvement in leaf water content over the untreated plants.

Oxidative damage The oxidative stress assessed as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content

Table 2 Effect of proline (10 μM) application on electrolyte leakage, tissue viability, relative leaf water content and total chlorophyll content in heat stressed Chickpea seedlings. Values with same letters in the same column are not different significantly at *P*<0.05 (Tukey's LSD test)

Treatment	Electrolyte leakage (%)	Tissue viability (TTC reduction (A $_{530/g}$)	Relative leaf water content (%)	Total chlorophyll (mg/g fw)
30/25 °C	9.4e	0.46c	87.3a	3.96a
35/30 °C	18.9c	0.52b	86.4a	3.69a
40/35 °C	26.3b	0.64a	76.4b	2.85b
45/40 °C	36.4a	0.42c	67.4c	2.13b
30/25 °C+10 μM Proline	13.3d	0.48c	88.4a	3.86a
35/30 °C+10 μM Proline	16.6c	0.55b	88.1a	3.67a
40/35 °C+10 μM Proline	18.4c	0.58b	80.3b	3.3a
45/40 °C+10 μM Proline	24.6b	0.53b	76.7b	3.07a

(Fig. 1) increased with elevation of temperature. At 40/35 °C, the MDA content increased by 1.6 folds while at 45/40 °C, an increase of 2.7 folds occurred over the control (Fig. 1a). With proline application, the MDA content in shoots decreased by 20 % at 40/35 °C while 32 % reduction was observed at 45/40 °C compared to those growing without proline at these temperatures.

Hydrogen peroxide (Fig. 1b) content showed 1.4 and 2.6 folds increase over control at 40/35 and 45/40 °C, respectively. With proline application, a reduction of 11 and 20 % occurred in hydrogen peroxide content at 40/35 °C and 45/40 °C, respectively compared to untreated plants.

Enzymatic antioxidants The activity of superoxide dismutase (SOD; Fig. 2a) increased by 35 % at 40/35 °C but decreased by 24 % at 45/40 °C compared to control. With proline application, a significant increase (17 %) in SOD activity was observed in plants growing at 45/40 °C over the plants growing without proline. With elevation of temperature, the activity of catalase (CAT; Fig. 2b) showed greater increase than SOD over the control. Thus, at 40/35 °C the activity increased by 71 % over control but decreased significantly (43 % over control) at higher temperature (45/40 °C). The proline treated plants possessed 19 % higher activity at 40/35 °C over untreated plants growing at the same temperature. At 45/40 °C, an increase of 26 % was observed in CAT activity with proline application over untreated plants.

The activity of ascorbate peroxidase (APX; Fig. 3a) increased by 90 % at 40/35 °C over the control. With further elevation of temperature, the activity showed appreciable decrease over the previous temperature. Proline application improved the activity by 16 % in plants

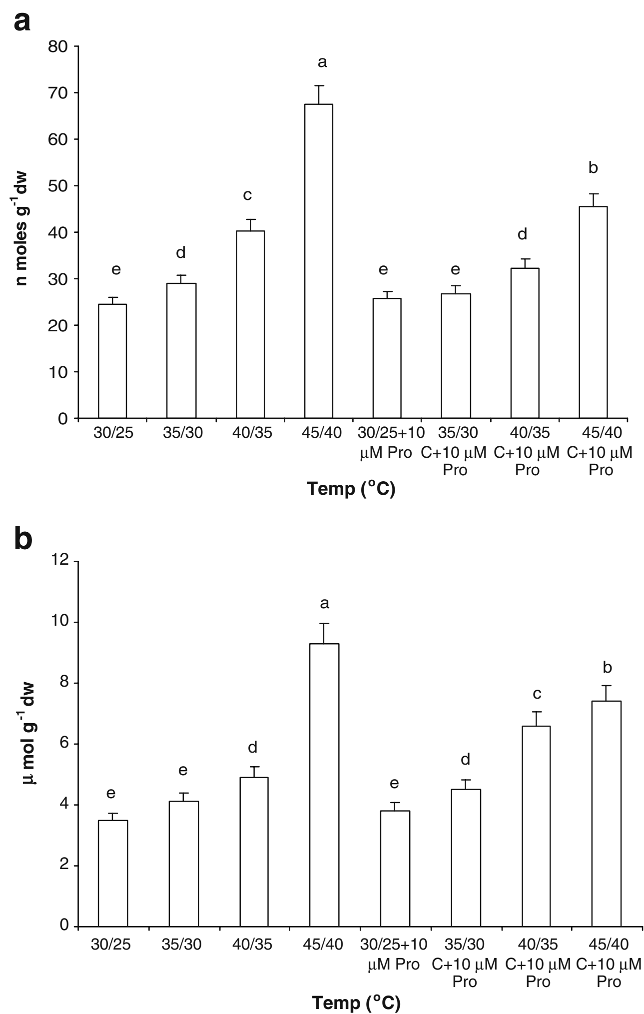

Fig. 1 Effect of proline (10 µM) application on malondialdehyde (**a**) and hydrogen peroxide (**b**) content in heat stressed chickpea seedlings. Values with same letters are not different significantly at $P<0.05$ (Tukey's LSD test)

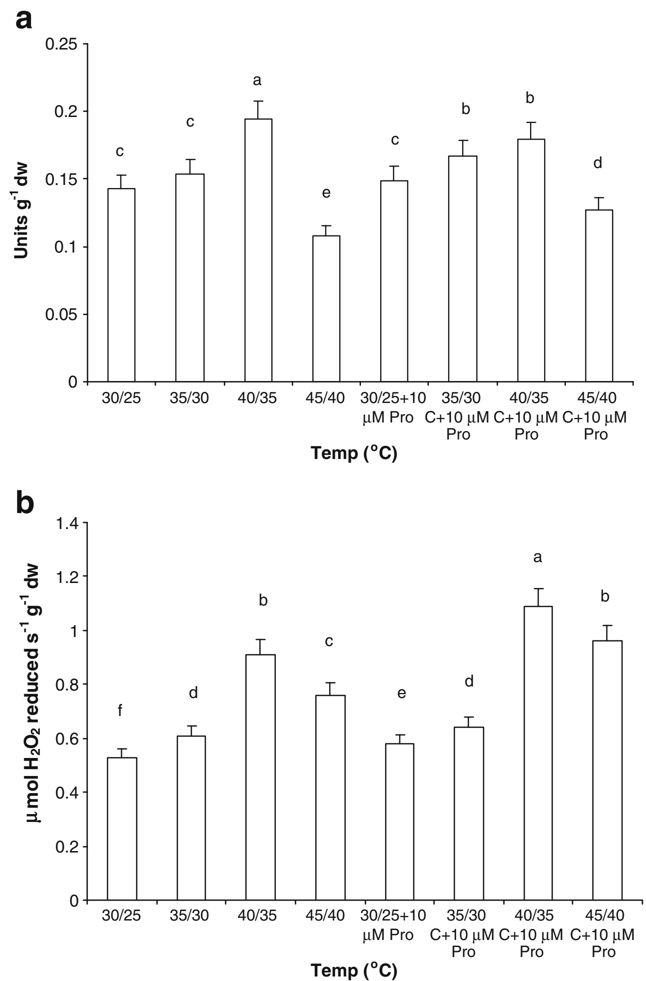

Fig. 2 Effect of proline (10 µM) application on activities of superoxide dismutase (**a**) and catalase (**b**) activity in heat stressed chickpea seedlings. Values with same letters are not different significantly at $P<0.05$ (Tukey's LSD test)

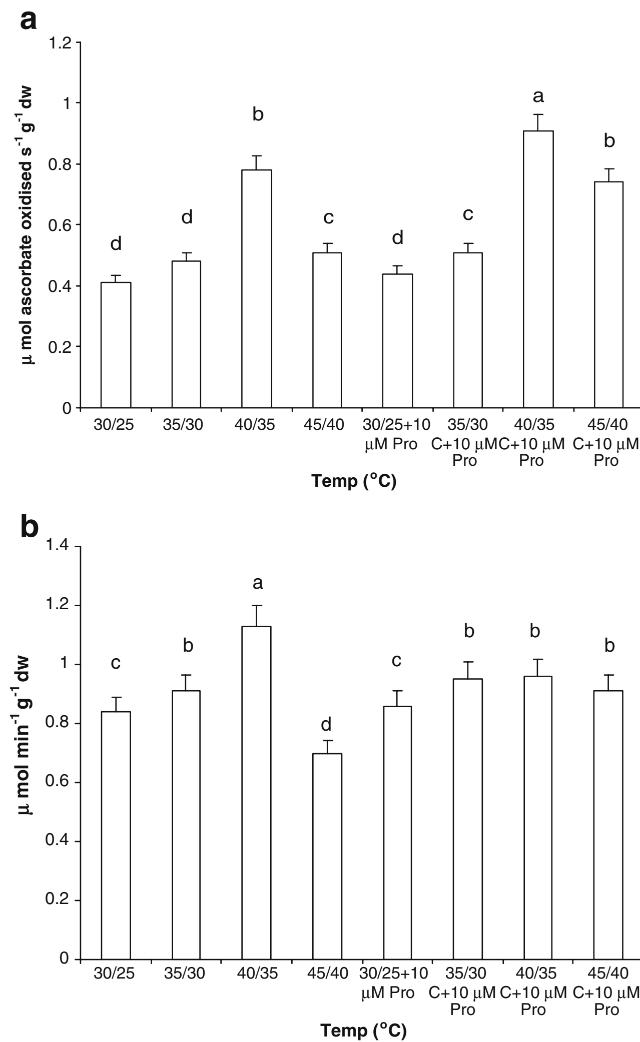

Fig. 3 Effect of proline (10 μM) application on ascorbate peroxidase (a) and glutathione reductase (b) activity in heat stressed chickpea seedlings. Values with same letters are not different significantly at $P<0.05$ (Tukey's LSD test)

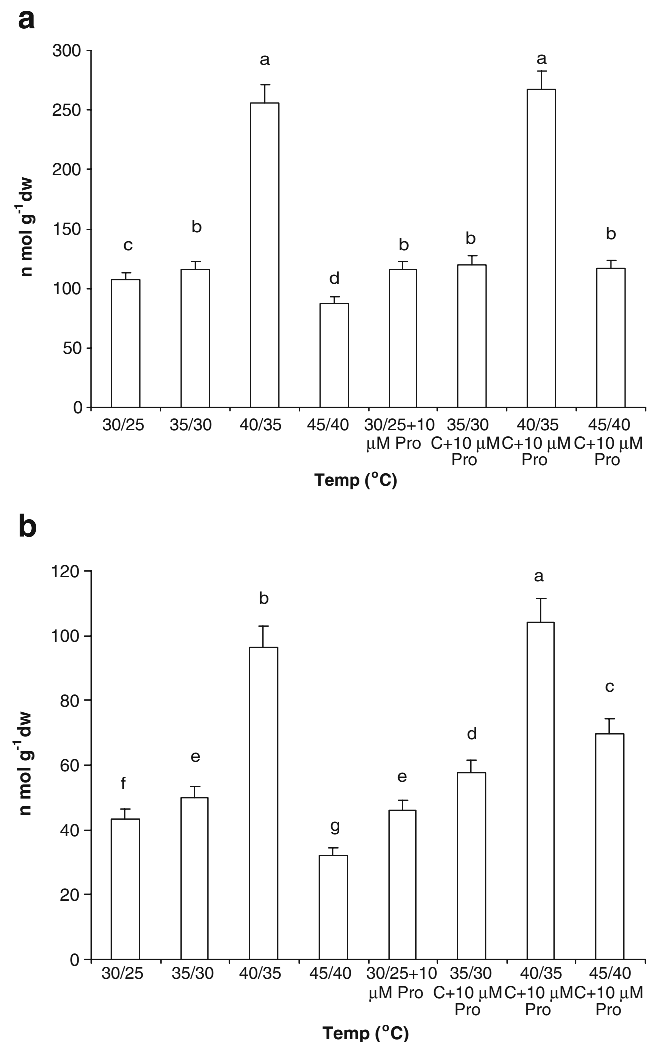

Fig. 4 Effect of proline (10 μM) application on ascorbic acid (a) and glutathione (b) content in heat stressed chickpea seedlings. Values with same letters are not different significantly at $P<0.05$ (Tukey's LSD test)

growing at 40/45 and 45 % in those growing at 45/40 °C compared to plants growing without proline treatment at these temperatures. The activity of glutathione reductase (GR; Fig. 3b) increased by 34 % over control in plants at a temperature of 40/35 °C but It declined at higher temperature significantly. With proline application, an increase of 15 and 30 % was observed in GR activity in plants growing at 40/35 and 45/40 °C, respectively without proline.

Non-enzymatic antioxidants

The endogenous profile of non-enzymatic antioxidants ascorbate (ASC) and glutathione (GSH) was recorded. The ASC (Fig. 4a) content elevated by more than 2 folds at 40/35 °C but declined by 2.9 folds at 45/40 °C compared to the previous temperature. The proline-treated plants growing at 45/40 °C showed 33 % improvement in ASC content over the untreated plants growing at this temperature. The endogenous level of GSH (Fig. 4b) increased by 2.2 folds at 40/35 °C over the control while it decreased by 1.8 folds at 45/40 °C over the previous temperature. In proline-applied plants, the GSH content increased by 8 % at 35/30 °C and by 14 % at 40/35 °C over the plants growing without proline. The plants growing in the presence of proline at 45/40 °C showed 30 % increase in GSH over the untreated plants.

Proline

The endogenous proline (Fig. 5c) content showed increase by 3.4 folds at 40/35 °C but decreased by 2.3 folds at 45/40 °C compared to the preceding temperature. The exogenous application of proline raised its endogenous levels substantially in all the treatments indicating its uptake.

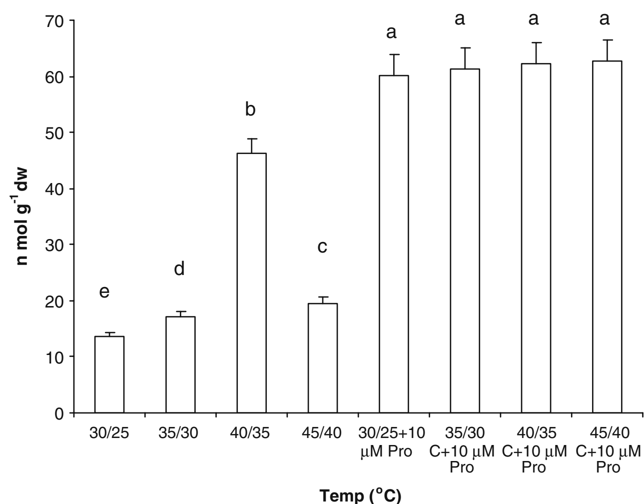

Fig. 5 Effect of proline (10 μM) application on proline content in heat stressed chickpea seedlings. Values with same letters are not different significantly at $P<0.05$ (Tukey's LSD test)

Effects of heat stress on carbon metabolism

The activity of ribulose 1,5 bisphosphate carboxylase (RUBISCO) decreased by 20 % over control at 40/35 °C while at 45/40 °C, 47 % decrease was observed in its activity (Fig. 6a). With proline application to plants growing at 45/40 °C, the activity was raised by 28 % over the untreated plants. The activity of sucrose phosphate synthase (Fig. 6b) increased by 19 % at 40/35 °C but decreased by 33 % over control at 45/40 °C. In proline treated plants, the activity was improved by 19 % over the untreated plants at this temperature. The invertase activity (Fig. 6c) in plants growing at 40/35 °C showed 31 % elevation while a decrease of 35 % was observed at 45/40 °C over control. With proline treatment, the activity showed 36 % increase at 45/40 °C over the untreated plants.

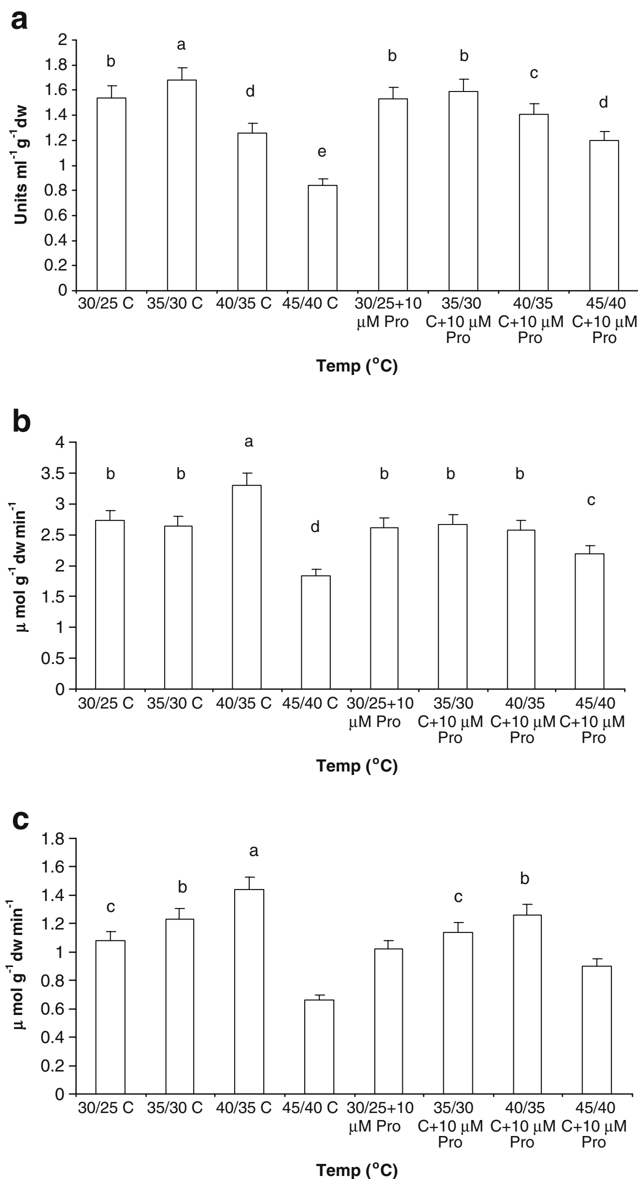

Fig. 6 Effect of proline (10 μM) application on ribulose 1,5 bisphosphate carboxylase (**a**), sucrose synthase (**b**) and invertase (**c**) in heat stressed chickpea seedlings. Values with same letters are not different significantly at $P<0.05$ (Tukey's LSD test)

Discussion

Effects of heat stress

Chickpea is sensitive to high temperature stress (Wang et al. 2006), hence the present studies were undertaken to find out the extent of damage caused by heat stress to chickpea plants at early vegetative growth and to probe the involvement of proline in mediating its heat sensitivity.

Our observations indicated that the germination and growth of the chickpea seedlings were significantly inhibited with increase in temperature to 40/35 °C and 45/40 °C. Pertinently, the growth of shoots was impaired to a greater extent than those of roots; the underlying reasons for this differential sensitivity of both the organs to heat stress need to be investigated. Due to greater inhibitory effect of heat stress on shoots, we focused our subsequent observations only on these organs to find out (a) the causes of damage to growth by elevated temperature and (b) involvement of proline in countering this damage. The stress injury was recorded in terms of increase in electrolyte leakage (EL), decrease in tissue viability, chlorophyll and leaf water status. The elevation of temperature resulted in increase in EL indicating membrane injury, which is reported to be a direct consequence of high temperature (Coria et al. 1998). The EL has been reported as a useful indicator of heat stress injury to plants in some earlier

studies (Liu and Huang 2000; Gulen and Eris 2004) and our findings in this context match with the similar ones reported in heat-stressed strawberry (Gulen and Eris 2004) and rice (Sohn and Back 2007) plants. The cellular respiration was assessed using 2,3,5 triphenyl tetrazolium chloride (TTC) reduction assay that indicates cellular respiration. The respiration may get affected at supra-optimal temperatures due to direct inhibitory effects of heat stress on enzymes (Salvucci and Crafts-Brandner 2004). The decrease in viability of leaf tissue at 45/40 °C in our case is in agreement with the observations on wheat (Wang and Nguyen 1989) and potato (Coria et al. 1998) plants exposed to heat stress. Our observations on decrease in leaf water content are in concurrence with the findings on wheat (Sairam et al. 2000), turfgrass (Jiang and Huang 2001) and Kentucky bluegrass (Liu et al. 2008) plants subjected to heat stress. The drop in leaf water status observed here can be attributed to reduction in hydraulic conductivity of the roots by heat stress, as reported earlier in tomato (Morales et al. 2003). The chlorophyll reduction occurred in heat-stressed chickpea plants that was similar to the observations on wheat (Almeselmani et al. 2009) and rice (Sohn and Back 2007) plants experiencing stressful high temperatures. The damage to pigments due to elevated temperature had earlier been ascribed to photo-oxidation of chlorophyll (Guo et al. 2006).

The oxidative stress was measured as malondialdehyde (MDA) and hydrogen (H_2O_2) peroxide, which increased with high temperature, especially MDA content showed higher increase implying greater damage to the membranes. The elevation of MDA due to heat stress in the present case is in accordance with the findings on heat-stressed plants of cotton (Mahan and Mauget 2005) and lily (Yin et al. 2008). The increase in hydrogen peroxide by high temperature in our studies is similar to the findings of Sairam et al. (1998) on wheat plants and Ma et al. (2008) in case of apple plants growing at high temperature. On the other hand in cotton plants growing at 38 and 45 °C, no significant change occurred in the levels of MDA and H_2O_2 molecules suggesting its greater tolerance to high temperatures. The activity of enzymatic antioxidants such as superoxide dismutase (removes superoxides to form hydrogen peroxide), catalase (beaks down hydrogen peroxide), ascorbate peroxide (uses ascorbate as a substrate to neutralize hydrogen peroxide) and glutathione reductase (reduces glutathione disulfide (GSSG) to the sulfhydryl from (GSH), which is an important cellular antioxidant) showed elevation in plants growing at 40/35 °C but decreased at 45/40 °C compared to the controls. The increase in the activity of these antioxidants at 40/35 °C matches with the observations on wheat plants subjected to high temperature of 35 °C (Dash and Mohanty 2002; Almeselmani et al. 2009). The elevation of enzymes at 40/35 °C might possibly be due to activation of defense mechanisms against oxidative stress, which at higher degree appear to fail leading to damage to membranes, chlorophyll and hence growth. The decrease in activity of these antioxidants possibly occurred due to their denaturation by high temperature (Salvucci and Crafts-Brandner 2004) and is similar to the findings on wheat (Almeselmani et al. 2009) and mulberry (Chaitanya et al. 2001) plants growing at high temperatures.

The ascorbic acid and glutathione content also elevated at 40/35 °C but decreased at 45/40 °C. The decrease in ascorbic acid and glutathione levels due to high temperature in our case is similar to the findings on heat-stressed wheat (Dash and Mohanty 2002) and apple (Ma et al. 2008) plants. The reduction in content of these antioxidants at stressful temperature could possibly occur due to inhibition in their regeneration because of impaired ascorbate/glutathione cycle by high temperature (Dash and Mohanty 2002).

The stressed chickpea plants showed reduced activities of ribulose 1,5 bisphosphate carboxylase (RUBISCO) and sucrose phosphate synthase (SPS) that is in agreement with the observations on wheat plants subjected to high temperature of 40 °C, which was attributed to enzyme inactivation and inhibition of photosynthesis (Demirevska-Kepova et al. (2000) plants. Similarly, in the leaves of the heat-stressed (40 °C) mulberry plants, a decrease in activity of RUBISCO and SPS was observed (Chaitanya et al. 2001). The increase in invertase activity at mild heat stress (40/35 °C) is in accordance with the findings on developing anthers of tomato plants experiencing high temperature stress of 32 °/26 °C (day/night; Pressman et al. 2006). On the other hand, the reduction in invertase activity at 45/40 °C in our studies was similar to the observations on grains of rice plants subjected to heat stress (Tian et al. 2006). It appears that at mild heat stress (40/35 °C), the demand for sucrose as well as its breakdown products namely glucose and fructose increases to meet the elevated energy requirements while with further increase in temperature, the enzymes get denatured or inactivated thereby affecting the overall carbon metabolism and consequently reducing the growth.

The endogenous proline content showed 2.8 times increase at 40/35 °C but declined appreciably at 45/40 °C. Earlier studies on red microalga by Chang and Lee (1999) reported elevation of proline at 35 °C, which was associated with increase in the activity of proline biosynthetic enzymes. The decrease in proline content in our studies is similar to the findings on cotton plants growing at 45/35 °C. On the other hand, in case of french bean, an increase in proline content was reported in plants growing even at 46–48 °C (Nagesh Babu and Devaraj 2008) suggesting a species-specific variation in proline-accumulation ability at stressful temperatures. In wheat plants, the reduction in proline content by high temperature was attributed to inhibition in

the activity of proline biosynthetic enzymes namely pyrroline-5-carboxylate synthetase (P5CS) and ornithine aminotransferase (OAT) (Song et al. 2005) that might be the situation in our case too, which needs to be examined. Elevated proline levels are reported to prevent denaturation of enzymes at high temperature (Dionisio-Sese et al. 1999); its decline at higher temperature in our case was related to onset of heat injury, inhibition of growth along with decrease in activity levels of antioxidants and sucrose metabolizing enzymes.

Considering our observations and keeping in view the earlier reports on decrease in endogenous proline content due to high temperature, we hypothesized that diminution of proline content might increase the heat sensitivity of chickpea plants causing the associated metabolic damage and restriction in its growth. Taking into consideration this, we exogenously provided proline to the plants growing at high temperatures to test the effectiveness of this molecule in imparting protection against heat stress. The mechanism of proline's effect in countering the heat stress was also examined using certain parameters related to stress injury, antioxidants and enzymes of sucrose metabolism.

Exogenous application of proline

Here, we found that supplementation of proline to the heat-stressed chickpea plants enhanced the proline accumulation to about 63 μmol g^{-1} dw that improved the growth at stressful temperature (45/35 °C) compared to the plants growing without proline at the same temperature. Previous studies have demonstrated that proline application confers protection to the plants growing under different types of abiotic stresses such as osmotic (Beumer et al. 1994), salt (Hoque et al. 2007a, b) and cold (Posmyk and Janas 2007) stresses. Our findings demonstrate the protective effects of proline against heat stress not reported so far to the best of our knowledge, at least in case of chickpea plants. The mechanism of proline action in imparting heat stress might involve several cellular sites. For example, we noticed that the proline-treated heat-stressed plants experienced reduction in stress injury measured as decrease in damage to membranes, improvement of chlorophyll content and tissue viability. Additionally, the proline treated plants also maintained greater leaf water content than those growing without it. In an earlier study on grapevine (*Vitis vinefera* L.) plants experiencing oxidative stress, the damage to membranes was reported to be reduced by proline application (Ozden et al. 2009). In our studies, the proline-treated plants were able to retain greater leaf water status that may be attributed to elevated endogenous proline that possibly improved the turgor content. In a previous study, the exogenous proline application was reported to increase the stomatal resistance in *Vicia faba* plants (Rajagopal 1981) that might be one of the additional reasons for raising the water content of heat stressed chickpea plants in our studies. Our observations in this regard are similar to those of Bandurska (1998) who reported reduction in membrane damage and increase in leaf water content with 0.1 M proline treatment to barley genotypes growing under water deficit conditions. Ben Ahmed et al. (2010) also reported that proline application resulted in improvement of the leaf water status of olive plants subjected to salt stress. The chlorophyll damage due to heat stress in our studies was significantly improved in proline-treated plants, which might be the result of enhancement of leaf water status to some extent and possibly reduced photo-oxidation. These observations are similar to those of Shaddad (1990) who reported improvement in pigment content (chlorophyll and carotenoids) in salt-stressed barley seedlings growing in the presence of proline. Similarly, Shevyakova et al. (2010) also observed increase in chlorophyll content with proline application to ice (*Mesembryanthemum crystallinum*) plants subjected to salt stress. These responses indicate that the elevated proline content was able to confer stability to membranes of the cell and those of organelles such as chloroplast, as well as to respiratory metabolism.

Moreover, the proline treated chickpea plants experienced less oxidative damage as indicated by decrease in production of MDA and H_2O_2 molecules at 40/35 °C and 45/40 °C temperatures. The larger effect of proline in reducing MDA production indicates its significant role in preventing the membrane damage. In salt-stressed cucumber plants treated with proline, Huang et al. (2009) reported decrease in oxidative stress as malondialdehyde content. The decrease in extent of oxidative stress in proline-treated seedlings might have occurred due to elevation in levels of antioxidants. In this regard, our findings are in line with the observations on salt-stressed cultured tobacco cells (Hoque et al. (2007a, b), olive plants (Ben Ahmed et al. 2010) and selenium-stressed bean plants (Aggarwal et al. 2010) where proline application resulted in reduction in oxidative damage. In these cases, the stressed plants showed improvement in the activities of enzymatic antioxidants such as superoxide dismutase, catalase, ascorbate peroxidase and polyphenol oxidase. In contrast to our results, the proline treated cucumber plants had lower superoxide dismutase activity, showed no effect on catalase and ascorbate peroxidase activity but possessed increased peroxidase activity. Thus, the heat-stressed plants treated with proline experienced less oxidative damage to their membranes as well as cells, most likely due to stabilization of enzymatic and non-enzymatic antioxidative systems.

The carbon metabolism in proline-treated plants appears to be modulated through enhanced activities of vital enzymes such as RUBISCO, sucrose phosphate synthase and invertase, though to different degrees, suggesting their

differential sensitivity to heat stress or proline response. Earlier studies in vitro reported that proline retarded the de-naturation of RUBISCO isolated from rice leaves at 45 °C (Dionisio-Sese et al. 1999).

In conclusion, the present study showed for the first time that exogenous application of proline reverses retardation of the growth of chickpea plants under heat stress, indicating that depletion of proline might be one of the crucial reasons for growth retardation at higher temperatures.

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