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ORIGINAL PAPER

Growth and metabolic responses of contrasting chickpea (*Cicer arietinum* L.) genotypes to chilling stress at reproductive phase

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Abstract Chilling stress (<10°C) at reproductive phase of chickpea results in abortion of flowers and pods leading to poor yield. The metabolic causes associated with cold sensitivity of chickpea are not well understood. Hence, in the present study, we evaluated four chickpea genotypes (ICC 16348, ICC 16349, PBG1 and GPF2) having contrasting cold sensitivity for their reproductive growth and metabolism subjected to cold stress (average day temperature: 17.6°C; average night temperature: 4.9°C). Genotypes ICC 16348 and ICC 16349 showed flowering and set pods, while PBG1 and GPF2 failed to do so during the stress conditions indicating the former to be cold tolerant. The stress injury in the leaves such as increase in electrolyte leakage, decrease in chlorophyll content and relative leaf water content was significantly less in ICC 16348 and ICC 16349 genotypes. The analysis of carbohydrates indicated total sugars and starch to be present in greater content in ICC 16348 and ICC 16349 relative to PBG1 and GPF2 genotypes. The enzymes related to carbohydrate metabolism such as β-amylase, invertase and sucrose synthase showed significantly higher activity in the leaves of ICC 16348 and ICC 16349 compared to the other two genotypes. PBG1 and GPF2 genotypes experienced greater oxidative stress measured as malondialdehyde and hydrogen peroxide. ICCV 16348 and ICC 16349 possessed significantly higher levels of enzymatic (superoxide dismutase, catalase, ascorbate peroxidase) and non-enzymatic antioxidants (proline and ascorbic acid) relative to PBG1 and GPF2. Particularly, proline and ascorbic acid were markedly higher in cold-tolerant genotypes compared to the sensitive ones suggesting their deciding role in governing the cold tolerance.

Keywords Chickpea · Chilling · Reproductive growth · Enzymes · Proline · Yield

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Introduction

Low temperatures (especially, day temperature < 20°C and night temperature < 10°C) are detrimental for flowering and pod set in chickpea (Srinivasan et al. 1998; Nayyar et al. 2005). These temperatures are commonly experienced by chickpea in northern India and southern Australia during its reproductive phase (Clarke and Siddique 2004; Nayyar et al. 2005). The damage by chilling stress leads to floral abortion, poor seed filling and hence reduced seed yield (Srinivasan et al. 1998; Nayyar et al. 2005). The cold-induced floral abortion has been attributed to impaired pollen tube growth in the style and consequent failure of fertilization (Clarke and Siddique 2004; Nayyar et al. 2005).

Till now, due to lack of availability of genotypes having contrasting cold sensitivity, it was difficult to elucidate the metabolic basis of cold tolerance in chickpea. We have been screening chickpea genotypes to find out the ones having ability to set pods at chilling temperatures. This

approach has led to the identification of some promising genotypes such as ICC 16348 and ICC 16349 which are able to produce flowers and set pods at very low temperatures (<10°C). With availability of these genotypes, it has become possible for us to compare their functioning at all organizational levels with those genotypes which fail to set pods at stressful chilling temperatures to know the metabolic basis of cold tolerance, hitherto unknown in chickpea.

Previous reports on contrasting genotypes of different crop species indicate variations in their metabolic responses to cold stress. Thus, chilling-tolerant maize genotypes possessed greater glutathione and glutathione reductase activity (Kocsy et al. 1996), high hydraulic conductance (Aroca et al. 2001), low 1-aminocyclopropane-1-carboxylic acid (ACC) (Janowiak and Dorffling 1996) and high ABA levels (Capell and Dörffling 1993), while no difference occurred in antioxidants as compared to chilling-sensitive ones (Janda et al. 2005). Similarly, cold-tolerant rice genotypes experienced low membrane damage and lipid peroxidation, higher levels of antioxidants and carbohydrates (Morsy et al. 2007) as well as low chlorophyll fluorescence (Bertin et al. 1997). In cucumber, the cold-induced inhibition in photosynthesis was lesser in cold-tolerant genotypes (Yu et al. 2002). In chickpea, information on the metabolic status of contrasting chickpea genotypes growing under cold stress conditions is scarce. Hence, in the present study, we compared the components of stress injury, carbohydrate metabolism and oxidative stress in the leaves of ICC 16348, ICC 16349 (cold-tolerant genotypes identified by us) and GPF2 and PBG1 (cold-sensitive commercial cultivars) during the reproductive growth with an aim to probe the mechanisms governing the cold sensitivity.

Materials and methods

The seeds of the genotypes ICC 16348 and ICC 16349 were procured from International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India, while those of GPF2 and PBG1 were obtained from Punjab Agricultural University, Ludhiana, India. The seeds were inoculated with *Rhizobium ciceri* and the plants were raised in pots (12-cm diameter) having soil and farm yard manure. The pots were randomly arranged in nine replications per genotype. The plants were adequately irrigated as and when required.

Temperature profile

The daily temperature was recorded from the day of sowing till the maturity. Average, maximum and minimum weekly temperatures are presented in Fig. 1. From 15 November 2007, the night temperature came down to 10°C and remained so till 22 February 2008. But the day temperature remained 20–25°C till the end of December 2007. It started dipping below 20°C from 14 January 2008 and remained so till 10 February 2008, so we have designated this period as the coldest one when the day/night temperatures were <20°C/<10°C. Observations on flowers, pods and metabolic studies (in leaves) were conducted during this phase (14 January–10 February; average max. 17.6°C and min. 4.9°C).

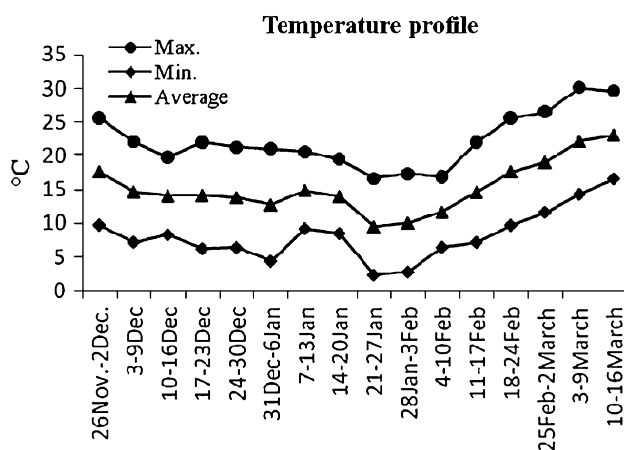

Fig. 1 Temperature profile (weekly) during the vegetative and reproductive growth of chickpea

Phenology

The plants were observed for days to flowering, podding, completion of flowering and maturity. In addition, a set of the plants were tagged for weekly data on flowering and podding (pod set %), especially during the coldest period. The pod set % was calculated by dividing number of pods set by total number of retained flowers multiplied by 100.

Growth and yield parameters

After maturity, a set of ten plants from three replications were uprooted, recorded for plant weight, plant height, number of primary branches, total number of pods, number of seeds per plant and seed weight per plant.

Stress injury

The membrane damage, relative leaf water content and total chlorophyll content were measured as per the method of Premchandra et al. (1990), Barrs and Weatherley (1962) and Arnon (1949), respectively, and have been elaborated elsewhere (Nayyar and Gupta 2006).

Carbohydrate metabolism

Enzymes

The activity of α- and β-amylase was assayed according to the method of Shuster and Gifford (1962). Invertase activity was measured following the procedures of Hawker and Hatch (1965) and Nygaard (1977). For estimation of sucrose synthase activity, the method of Hawker et al. (1976) was followed.

Carbohydrates

The oven-dried plant material was homogenized in hot ethanol (80%) and centrifuged at 2,000 rpm for 10 min. The supernatant was clearly decanted off. Three milliliters of ethanol (80%) was added to the residue and re-centrifuged. The extraction was repeated twice to ensure the complete recovery of sugars. The residue was kept for further analysis of starch. The supernatant was pooled and evaporated to dryness in china-dish in boiling water both. The residue was eluted with 5 ml of 20% ethanol and subjected to analysis for total sugars (Yemm and Willis 1954) and reducing sugars (Sumner 1935). Starch content was measured by acid hydrolysis method given by McReedy et al. (1950).

Oxidative stress

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation by the method described by Heath and Packer (1968) and hydrogen peroxide was estimated by the method of Mukherjee and Choudhuri (1983), elaborated earlier (Nayyar and Gupta 2006).

Antioxidants

The estimation of ascorbic acid was done according to the method of Mukherjee and Choudhuri (1983), while proline content was measured using the method of Bates et al. (1973). The activity of superoxide dismutase (SOD) was assayed following the method of Dhindsa et al. (1981). For the assay of ascorbate peroxidase (APX) and catalase (CAT), the methods of Nakano and Asada (1981) and Teranishi et al. (1974), respectively, were followed as described previously (Nayyar and Gupta 2006).

Statistical analysis

The observations were replicated thrice and subjected to analysis of variance using AGRISTAT software to calculate LSD ($P < 0.05$).

Results

Phenology, growth and yield

ICC 16349 and ICC 16348 genotypes showed flowering in about 46–50 days after sowing (DAS; average maximum and minimum temperatures 22.1 and 7.2°C; Table 1), while GPF2 and PBG1 genotypes took 80 and 86 DAS, respectively (average maximum and minimum temperatures 20.6 and 11.2°C). ICC 16349 and ICC 16348 genotypes took considerably shorter time to show podding compared to GPF2 and PBG1 genotypes. No variation was observed in number of days to end flowering (Table 1). Genotypes ICC 16348 and ICC 16349 achieved maturity slightly earlier than GPF2 and PBG1.

The number of flowers produced (Fig. 2) during the coldest period was highest in ICC 16348 followed by ICC 16349, while GPF2 and PBG1 genotypes produced only few flowers during the stress period. The pod set % (Fig. 2) during this period was zero in GPF2 and PBG1 compared to 44–45% in ICC 16348 and ICC 16349 genotypes.

The dry plant weight (Table 2) was recorded to be the highest in PBG1 followed by ICC 16348. The other two genotypes possessed relatively lower dry weight. Maximum plant height (Table 2) was observed in ICC 16349 followed by ICC 16348, while PBG1 and GPF2 were relatively shorter at maturity. The number of branches was maximum in ICC 16348 followed by PBG1. Minimum number of branches (Table 2) was recorded in genotypes GPF2 and ICC 16349 that explain their less dry weight per plant compared to PBG1 and ICC 16348 genotypes.

The yield components such as pods per plant, seed number per plant and seed yield per plant were significantly

Table 1 Phenology of chickpea genotypes experiencing chilling stress during reproductive phase

Genotypes	Days to flowering	Days to podding	Days to end flowering	Days to pod maturity
ICC 16348	50	61.3	143	148.3
ICC 16349	45.7	56.7	144.3	150
GPF2	80	135.0	144	153.7
PBG1	86	134.3	147.3	158
LSD (<0.05)	3.7	13.3	5.2	11.2

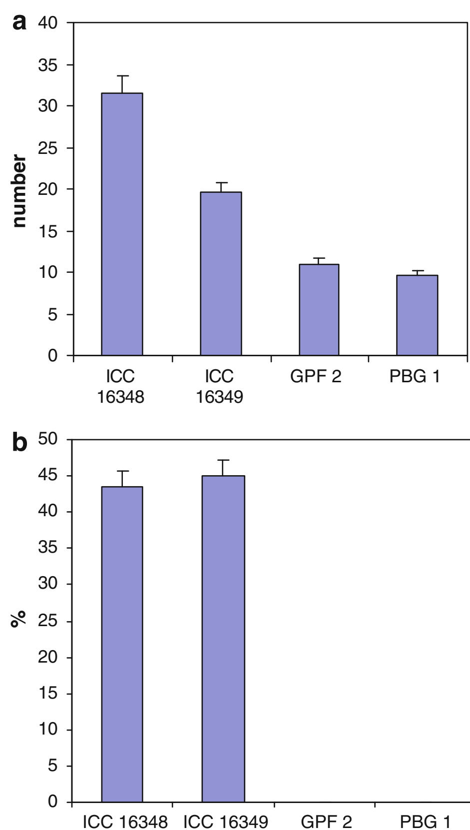

Fig. 2 Number of flowers (**a**) and pod set (**b**) during the coldest period in chickpea genotypes. *Bars* represent standard errors (LSD < 0.05: for number of flowers, 3.8; pod set %, 3.4)

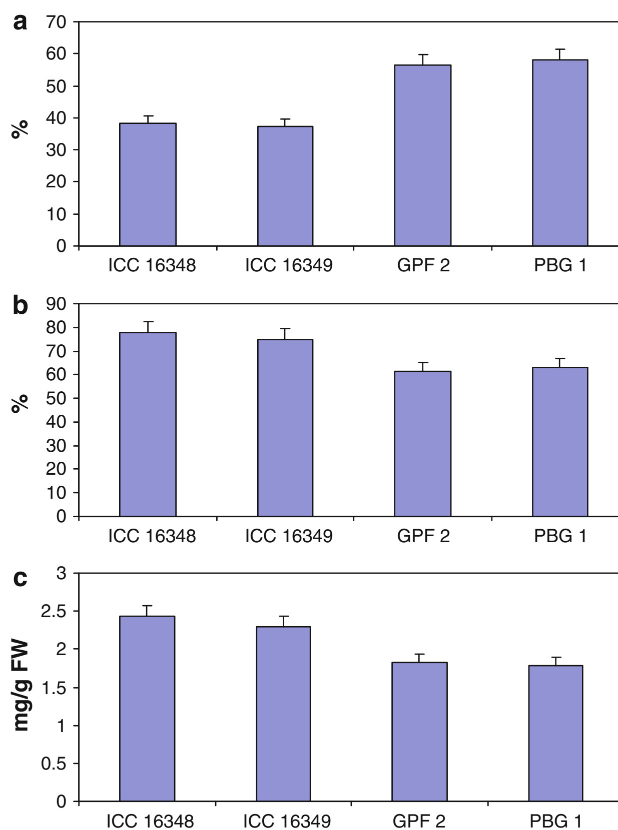

Fig. 3 Electrolyte leakage (**a**), relative leaf water content (**b**) and chlorophyll content (**c**) in leaves of the chickpea genotypes during the coldest period. *Bars* represent standard errors (LSD < 0.05: for electrolyte leakage, 1.7; relative leaf water content, 2.3; chlorophyll content, 0.86)

higher in ICC 16348 and ICC 16349 compared to GPF2 and PBG1 genotypes (Table 2).

Stress injury

The membrane damage (Fig. 3a) in the leaves during cold stress period was found to be significantly higher (~20%) in PBG1 and GPF2 compared to ICC 16349 and ICC 16348 genotypes. ICC 16348 and ICC 16349 genotypes possessed ~16% higher leaf water content compared to other genotypes (Fig. 3b). The total chlorophyll content (Fig. 3c) in ICC 16348 and ICC 16349 was 28–34% higher than PBG1 and GPF2 genotypes.

Carbohydrate metabolism

Enzymes

The activity of α-amylase (Fig. 4a) was significantly higher in ICC 16348 as well as in GPF2 than ICC 16349 and PBG1 genotypes. The activities of β-amylase (Fig. 4b), invertase (Fig. 4c) and sucrose synthase (Fig. 4d) were also appreciably higher in ICC 16348 and ICC 16349 as compared to other two genotypes. Relatively, the extent of increase in enzyme activity in ICC 16348 and ICC 16349

Table 2 Growth and yield of chickpea genotypes experiencing chilling stress during reproductive phase

Genotypes	Plant weight (g)	Plant height (cm)	Number of primary branches	Number of pods	Number of seeds/plant	Seed weight/plant
ICC 16348	6.8	73.3	14.7	23	32.0	5.5
ICC 16349	5.2	82.3	9.3	21.0	28.5	4.8
GPF2	6.2	57.3	9	11.0	15.0	2.5
PBG1	7.5	65.7	12.3	13.0	19.0	3.1
LSD (<0.05)	1.3	3.5	2.1	1.3	2.6	0.8

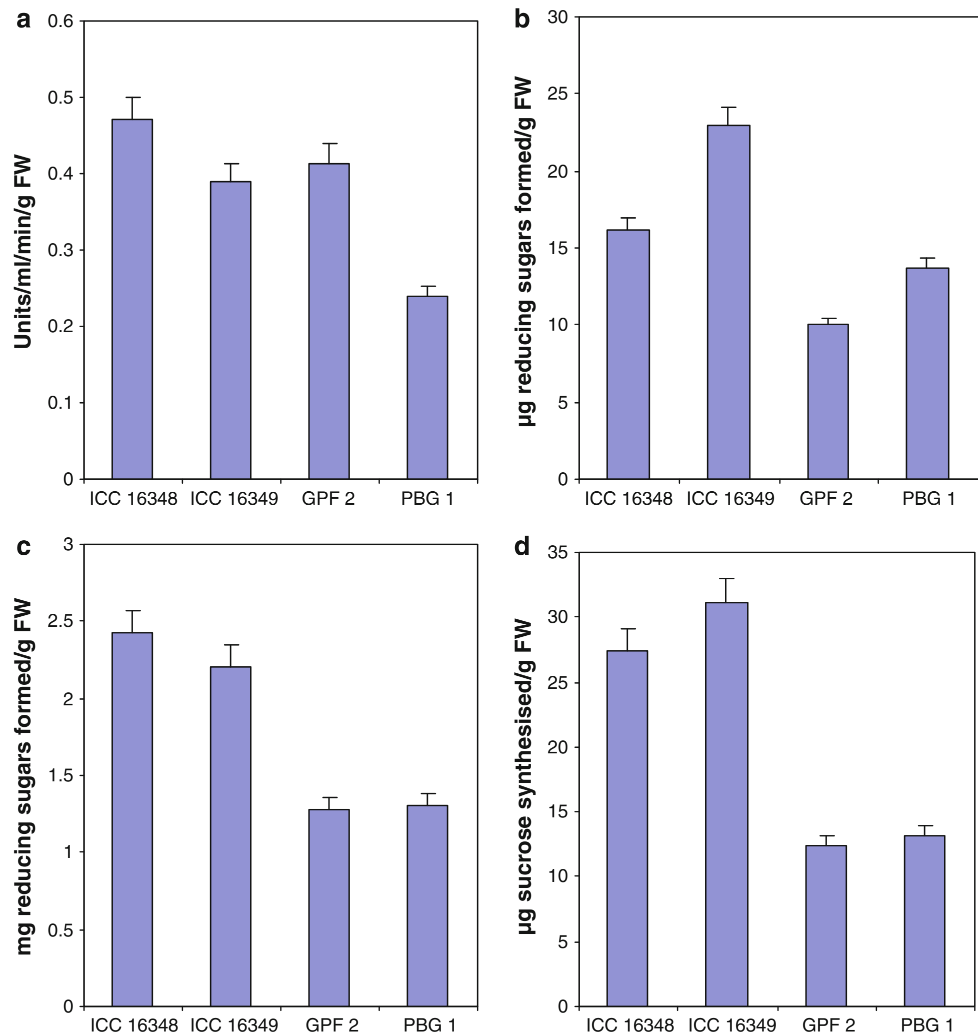

Fig. 4 α-Amylase (**a**), β-amylase (**b**), invertase (**c**) and sucrose synthase (**d**) in leaves of the chickpea genotypes during the coldest period. *Bars* represent standard errors (LSD < 0.05: for α-amylase, 0.02; β-amylase, 2.1; invertase, 0.24; sucrose synthase, 1.3)

was highest for β-amylase followed by invertase and sucrose synthase enzymes.

Carbohydrates

ICC 16348 and ICC 16349 had considerably greater content of total sugars (1.6–1.9 folds) (Fig. 5a) and starch (2.2–2.6 folds) (Fig. 5c) than GPF2 and PBG1 genotypes. On the other hand, the content of reducing sugars was significantly less in ICC 16348 and ICC 16349 genotypes than other two genotypes (Fig. 5b).

Oxidative stress and antioxidants

It was measured as elevation in levels of MDA (Fig. 6a) and hydrogen peroxide (Fig. 6b). Both these molecules were significantly lower in ICC 16348 and ICC 16349 compared to GPF2 and PBG1 genotypes. The extent of oxidative damage was greater with MDA compared to hydrogen peroxide in PBG1 and GPF2 genotypes.

The activities of antioxidants such as SOD (Fig. 7a), CAT (Fig. 7b) and APX (Fig. 7c) were appreciably higher in ICC 16348 and in ICC 16349 compared to GPF2 and PBG1 genotypes. Of all the enzymatic antioxidants, the extent of increase was highest for APX, followed by CAT and SOD enzymes.

Pertinently, there was a large variation in the levels of ascorbic acid and proline between the contrasting genotypes. Thus, ICC 16348 and ICC 16349 possessed 2.5–3 folds higher ascorbic acid than PBG1 and GPF2 genotypes. Similarly, the proline content (Fig. 8b) was 3–3.6 times more in ICC 16348 and ICC 16349 genotypes compared to PBG1 and GPF2 genotypes.

Discussion

Genotypes ICC 16348 and ICC 16349 can be termed as cold tolerant since they were able to set the pods during the coldest phase compared to GPF2 and PBG1 which could

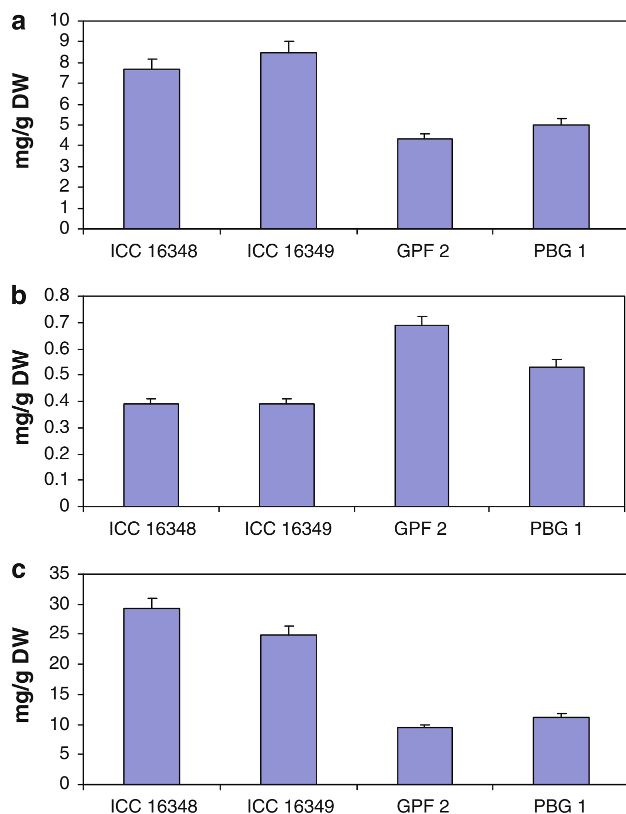

Fig. 5 Total sugars (**a**), reducing sugars (**b**) and starch (**c**) in leaves of the chickpea genotypes during the coldest period. *Bars* represent standard errors (LSD < 0.05: for total sugars, 1.1; reducing sugars, 0.03; starch, 1.6)

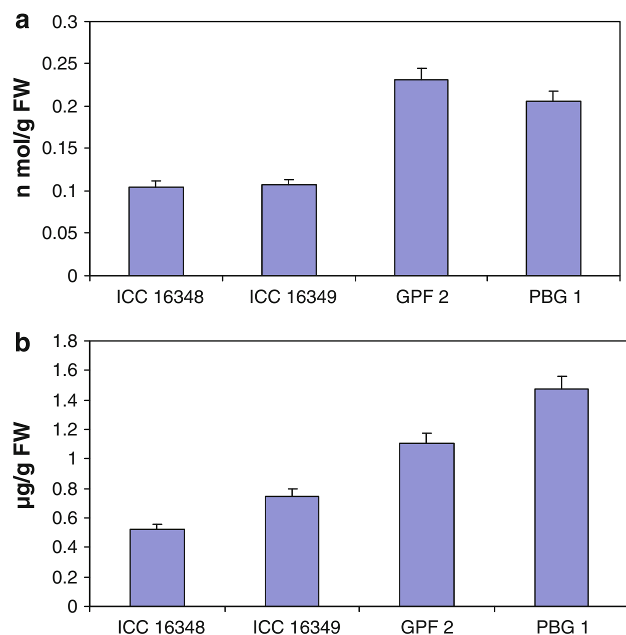

Fig. 6 Malondialdehyde (**a**) and hydrogen peroxide (**b**) content in leaves of the chickpea genotypes during the coldest period. *Bars* represent standard errors (LSD < 0.05: for malondialdehyde, 0.007; hydrogen peroxide, 0.14)

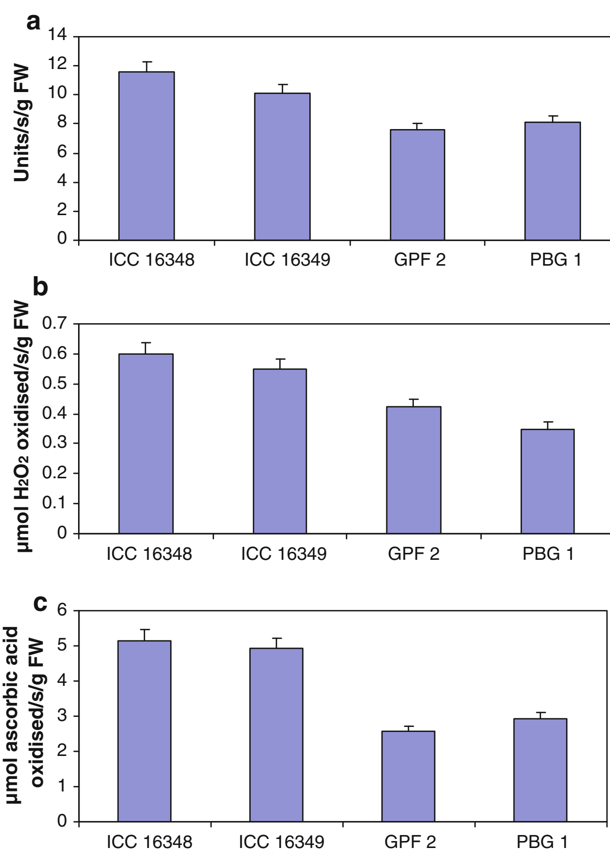

Fig. 7 Superoxide dismutase (**a**), catalase (**b**) and ascorbate peroxidase (**c**) in leaves of the chickpea genotypes during the coldest period. *Bars* represent standard errors (LSD < 0.05: for SOD, 0.62; CAT, 0.07; APX, 0.23)

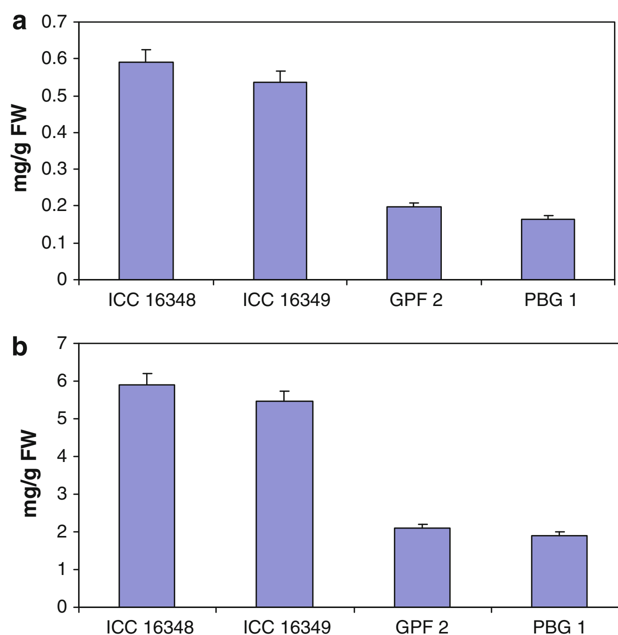

Fig. 8 Proline (**a**) and ascorbic acid (**b**) content in leaves of the chickpea genotypes during the coldest period. *Bars* represent standard errors (LSD < 0.05: for proline, 0.04; ascorbic acid, 0.83)

not set any pods at the same time. GPF2 and PBG1 also delayed their podding and maturity significantly due to cold stress and attained these stages only when temperatures became warm (average maximum and minimum temperatures as 26.5 and 13.6°C, respectively) corroborating their cold sensitivity. During the coldest phase, the number of flowers produced by ICC 16348 and ICC 16349 was substantially higher, while PBG1 and GPF2 produced relatively small number of flowers during coldest period and also failed to convert them into pods leading to loss of potential yield. Consequently, seed yield/plant was also greater in ICC 16348 and ICC 16349. Genotypes PBG1 and GPF2 were partially able to compensate themselves after the stressful temperatures were over during the warmer conditions of late February and March months and produced flowers and set pods.

We worked on the possible metabolic reasons associated with contrasting cold sensitivity of these differentially sensitive genotypes. The stress injury was evaluated as damage to membranes, chlorophyll and leaf water status. The membrane damage was much lower in ICC 16348 and ICC 16349 genotypes relative to GPF2 and PBG1. These findings are in accordance with the earlier ones on cowpea reporting better membrane integrity in tolerant genotypes during stress (Thiaw and Hall 2004). One of the reasons causing membrane damage might be due to chilling-induced oxidative stress that was found to be higher (measured as MDA and hydrogen peroxide levels) in GPF2 and PBG1 than in ICC 16348 and ICC 16349 genotypes. The relative leaf water content decreased to a greater extent in cold-stressed plants of GPF2 and PBG1 than in ICC 16348 and ICC 16349. These findings are similar to observation in maize where a decrease in water content was observed in cold-stressed plants (Janowiak and Markowski 2008).The chlorophyll content was significantly higher in cold-stressed plants of ICC 16348 and ICC 16349 genotypes than in GPF2 and PBG1 genotypes. Low temperature is known to cause damage to chlorophyll due to photo-oxidation that consequently inhibits the photosynthesis (Ying et al. 2000). Earlier reports also indicate that the stress-tolerant genotypes of a crop species maintain higher chlorophyll content during stress conditions suggesting better stability of the photosynthetic apparatus than their sensitive counterparts (Sairam and Saxena 2000).

During cold stress, the starch content was higher in ICC 16348 and ICC 16349 genotypes relative to others. Starch is an end product of photosynthesis; its greater content in stressed plants signifies the stability of this process under stress. In this context, ICC 16348 and ICC 16349 genotypes appeared to be superior to GPF2 and PBG1. These observations are in accordance with the findings of Du and Nose (2002) who observed higher and stable starch levels in cold-tolerant sugarcane genotype as compared to its cold-sensitive counterpart. At the same time, the activity of amylases especially that of β-amylase was higher in ICC 16348 and ICC 16349 compared to GPF2 and PBG1 genotypes. Previous studies have correlated the activity of amylases with stress tolerance in rice plants exposed to flooding conditions (Ismail et al. 2009). Amylases break down the starch into reducing sugars. In our studies, the content of reducing sugars was higher in GPF2 and PBG1 and thus did not correlate with the activity of amylases in these genotypes. The higher amount of reducing sugars might possibly be related to restriction in their conversion into sucrose since the activity of sucrose synthase enzyme was lower in GPF2 and PBG1, compared to ICC 16348 and ICC 16349 genotypes. The elevated activity of sucrose synthase during cold stress in latter genotypes implies higher capacity for sucrose generation and possibly its availability for the developing pods, which in turn might improve the pod set in these genotypes. It was noticed that the activity of invertase (cleaves sucrose into reducing sugars) was significantly higher in ICC 16348 and ICC 16349 than in GPF2 and PBG1 genotypes. Its greater activity coupled with higher sucrose synthase activity indicates better ability of these genotypes to synthesize and hydrolyze sucrose. The coupled functioning of invertase and sucrose synthase has earlier been reported to have a major role in sucrose metabolism in cold tolerance (Santoiani et al. 2006). In this regard, our findings are similar to Santoiani et al. (2006) who reported greater sucrose synthase activity in cold-tolerant wheat genotypes. Previously, the seeds of cold-tolerant chickpea genotypes were reported to possess higher expression of sucrose synthase than their sensitive counterparts (Kaur et al. 2009).

The oxidative damage evaluated as MDA and hydrogen peroxide (H_2O_2) levels was higher in GPF2 and PBG1 relative to ICC 16348 and ICC 16349 genotypes. The accumulation of MDA is used as an indicator of lipid peroxidation (Smirnoff 1995), which occurs due to cold-induced oxidative stress where several types of reactive oxygen species attack membrane lipids causing their oxidation. Our findings are in accordance with a previous study reporting low MDA content in cold-tolerant genotype of cucumber (Shen et al. 1999). Hydrogen peroxide is one of the most reactive oxygen species produced during the cold-induced oxidative stress due to dismutation of superoxide radicals by enzyme SOD. Thus, it reflects the degree of oxidative stress in different genotypes and has been inversely correlated with the extent of cold tolerance in studies involving differentially cold-sensitive crop species (Saruyama and Tanida 1995). Thus, ICC 16348 and ICC 16349 genotypes had lower content of both MDA and hydrogen peroxide indicating less oxidative damage than in PBG1 and GPF2 genotypes.

The enzyme SOD catalyses the dismutation of superoxides and this enzyme functions in coordination with CAT, which detoxifies the hydrogen peroxide; thus, these two enzymes collectively act as antioxidants (Bowler et al. 1992; Mitter 2002). The activities of SOD and CAT were greater in ICC 16348 and ICC 16349 genotypes implying their superior antioxidative ability. Some studies show elevation of SOD in chilling-stressed plants (Kuk et al. 2003), while the others report decrease in its activity (Zhang et al. 1995). It has been reported that genotypes expressing higher SOD activity possess superior cold tolerance (Huang and Guo 2005; Wang et al. 2009). Our findings on CAT and APX activity are in agreement with observations on rice (Guo et al. 2006) and alfalfa (Wang et al. 2009), where cold-tolerant genotypes had greater activity of these enzymes.

Ascorbic acid was significantly higher in ICC 16348 and ICC 16349 compared to other genotypes. Ascorbic acid that acts as a substrate for APX is a very strong antioxidant and has several other functions in plant growth and development (Smirnoff 2000). It protects the chloroplasts against oxidative damage and its level gets elevated in response to stress conditions (Kuk et al. 2003). The degree of stress tolerance has been positively correlated with ascorbic acid content (Shalata and Neumann 2001). Proline accumulation was markedly higher in ICC 16348 and ICC 16349 genotypes. Our findings are similar to the previous ones where tolerant genotypes of *Zoysia* spp. were also found to possess greater content of proline compared to cold-sensitive ones (Patton et al. 2007). Earlier studies indicate the involvement of proline in cold tolerance (Duncan and Widholm 1987; Chen and Li 2002). Proline has several diverse functions in stressed cells that include protection of membranes from stress and as hydroxyl radical scavenger (Hare and Cress 1997).

Thus, the present study which probed the metabolic reasons associated with cold sensitivity indicated that of all the molecules examined, ascorbic acid and proline showed the largest variations between the cold-tolerant and cold-sensitive genotypes implicating their vital involvement in governing the cold tolerance. Genetic manipulation of these two molecules in chickpea for their higher expression in the leaves may increase the cold tolerance in this crop.

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