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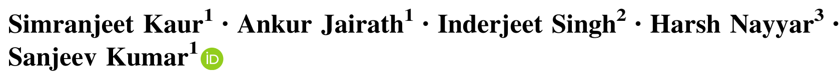
ORIGINAL ARTICLE

Alternate mild drought stress (−0.1 MPa PEG) immunizes sensitive chickpea cultivar against lethal chilling by accentuating the defense mechanisms



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Abstract The changes in climate particularly, the rise in temperature and humidity affect the physiological functions of plants subsequently affecting crop productivity adversely. A strategy is required which can be directly implemented in fields to induce the tolerance in crop plants. In present study, two chickpea varieties with contrasting sensitivity PDG3 (Tolerant) and GPF2 (Sensitive) were raised hydroponically, preconditioned with mild drought stress (0.1 MPa PEG-6000) for 3 days (above 0.1 MPa is lethal) and subsequently recovered for double time (6 days) and finally exposed to lethal cold stress (4 °C) for 3 days. We hypothesize that preconditioning with non-lethal drought stress may immunize the plants to combat lethal cold stress. Membrane integrity improved in root and shoot, lipid peroxidation decreased to control level in preconditioned seedlings. Cellular respiration ability (% TTC reduction) increased in the preconditioned seedlings to almost 90 % in the shoot and 60 % in the root, concurrently it was 45 % in non-preconditioned seedlings. Proline content also increased in preconditioned seedlings, especially roots. Carbohydrate had a shift in terms of a high amount of total, reducing sugars and starch in non-preconditioned seedlings compared to preconditioned. Both PDG3 and GPF2 showed enhanced SOD, CAT and GPOX activity indicating tolerance against cold-induced oxidative stress and preconditioning induced improvement against lethal cold stress.

Keywords Preconditioning · Cold stress · Lethal · Electrolyte leakage · Lipid peroxidation · Cryoprotectant

Introduction

In nature, plants do not always live in their most hostile environment; they are exposed to various biotic and abiotic factors and forced either to adapt to these conditions or perish. There is huge demand for research into the cellular and molecular mechanisms of plant stress responses as understanding these mechanisms can help to improve crop growth in response to global environmental changes (Anand and Gill 2015; Mittler 2006). Chickpea (*Cicer arietinum* L.) is the third most important crop in the world after common bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.). India contributes ca. 70 % of worlds' production. The chief restrictions in chickpea production are biotic stresses like Ascochyta blight, Fusarium wilt, pod borer, and abiotic stresses such as drought, heat, cold and high-salinity. Global annual production losses due to abiotic stresses alone are estimated to be around 3.7 million tons (mt), which amounts to 40–60 % average loss (Singh 1997; Varshney et al. 2009). In fact, the estimated communal yield losses due to abiotic stresses are significantly higher (6.4 mt) than for biotic stresses (4.8 mt) (Ryan 1997; Varshney et al. 2009). Chickpea fulfills a noticeable nutritional requirement of population in developing countries as it is a rich source of carbohydrates (40–59 %), proteins (13.5–31.7 %), vitamins, minerals, etc. This crop

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comes across cold stress, terminal drought and heat during its reproductive stage which poses major constraints to chickpea production. It often experiences abnormally low temperature (−1.5 to 15 °C) during reproductive phase, which severely affects seed germination, photosynthesis, respiration, membrane stability, fertilization, fruit maturation, quality of seeds, and yield (Kaur et al. 2011). Cold stress cause chilling injury that results into reduced growth of plant, discoloration and lesions of leaves, vitrified foliage and several dysfunctions at cellular level like damage to membrane, production of ROS and protein denaturation (Leport et al. 2006).

In northern India, chickpea is grown as a winter-season crop; ideal temperature for growth of chickpea seedling is 23–30 °C. During cold stress, formation of reactive oxygen species causes the oxidative damage which moderates the rate of metabolic activities in plants. The plants protect itself from oxidative stress through adaptation, avoidance and tolerance mechanisms. Consequently, plants stimulate their antioxidative defense system by stimulating endogenous antioxidants, which are the main constituents of antioxidative defense system. The signaling pathways for cold stress and drought stress have many similarities because both share many common features. Cold and drought stress affect the water relations of the plant causing specific and unspecific reactions. Damage or adaptation reactions have common mechanisms in their physical responses and tolerance against drought and cold stresses. There may be positive correlation which can provide tolerance against one of stress. Preconditioning of the plants with mild drought stress at vegetative stage might triggers mechanisms that will prepare the plants to combat lethal cold stress at later stage. It can help in developing strategies to improve cold stress resistance in plants (Beck et al. 2007; Liu et al. 1998). Preconditioning of chickpea with non-lethal drought stress may stimulate the defense mechanism and may prepare the crop for lethal cold temperature stress. The basis of the present study is quite simple in terms of the feasibility and cost effectiveness under field conditions.

Materials and methods

Chickpea (*Cicer arietinum* L.), released varieties GPF2 (cold sensitive) and PDG3 (cold tolerant) were employed to study the preconditioning response on membrane damage, antioxidative enzyme activity, cryoprotective solute accumulation and carbohydrates distribution. Healthy chickpea seeds of chickpea varieties PDG3 and GPF2 were selected and raised hydroponically for 10 days in a completely randomized block design with three replications and ten seeds per replication at 27 °C and 16/8 h light/dark photoperiod in a climate controlled walk-in growth chamber. Sufficient replications were maintained keeping in view the sampling at various treatment stages. On 11th day, plant samples were harvested and tested for various cellular responses; on the same day, remaining plants were subjected to mild drought stress for 3 days. On 15th day, samples from control and plant subjected to mild drought stress were harvested and processed for various parameters. The remaining samples were kept for recovery by shifting them to normal conditions for double the time of stress (6 days). After recovery on 19th day, samples were collected from control and recovery for assays and remaining plants; control (non-preconditioned) and treated + recovered (preconditioned) were subjected to lethal cold stress phase at 4 °C for 3 days. On 23rd day, the experiment was terminated and same parameters were reanalyzed in root and shoots.

Membrane damage analysis

Membrane damage at various stages was analysed by electrolyte leakage index (% EL), lipid peroxidation (MDA content) and lipoxygenase (LOX) enzyme activity. Percent ELI was done by the method given by Lutts et al. (1996). The MDA content was measured by the method given by Heath and Packer (1968). The final MDA content formed was calculated using extinction co-efficient 155 mM^{-1} -cm^{-1}. A modified protocol (Palma et al. 2013) was used for measuring LOX activity, 50 mg of fresh shoot and root samples were homogenized in pre-chilled 50 mM potassium phosphate buffer (pH 6.5) containing 0.5 % (v/v) Triton X-100, 1 % (v/v) Tween-20 and 20 % (w/v) PVP. The homogenate was centrifuged at 15,000×*g* for 15 min at 4 °C and supernatant was collected for analysis. The assay mixture contained 200 mL of 0.2 mM linoleic acid, 790 mL of 100 mM potassium phosphate buffer (pH 6.5) and 10 Ml of plant extract. For blank, 800 Ml of 100 mM potassium phosphate buffer (pH 6.5) and 200 Ml of 1 mM linoleic acid (final concentration of linoleic acid in 1 mL reaction mixture was 0.2 mM) was used. Lipoxygenase activity (LOX, EC 1.13.11.12) was determined spectrophotometrically at 234 nm.

Cellular respiratory efficiency (TTC reduction assay)

The root and shoot samples collected from each stage (six replicates) were washed three times with sterile distilled water, air dried and segregated into normal and heated samples. The samples were incubated in 3 mL of 0.4 % (w/v) TTC (50 mM phosphate buffer pH 7.4) for 20 h at

28 °C in dark. The formazan formed was extracted in 3 mL of ethanol by incubating at 80 °C for 20 min. The absorbance was recorded at 530 nm (Nayyar et al. 2005b). TTC reduction was expressed in terms of percentage activity, based upon the following equation: TTC reduction (%) = [(T1 − T2)/(C1 − C2)] × 100, where T1 stands for treated samples, T2 for 'heated' treated samples, C1 for control samples and C2 for 'heated' control samples.

Antioxidative enzymes assay

50 milligrams fresh matter was grounded to powder by liquid nitrogen and homogenized in 2 mL of plant extraction buffer (50 mM phosphate buffer pH 7.8, 0.1 mM EDTA, 1 mM PMSF and 2 % PVP). For APX activity, 5 mM ascorbic acid was added extra in the extraction buffer. The samples were homogenized and centrifuged at 14,000×*g* for 30 min at 4 °C, supernatant was used for the determination of protein content and enzyme activities (Ceylan et al. 2013). Total protein content was measured according to Bradford using Bovine Serum Albumin as standard (Bradford 1976).

Superoxide dismutase (SOD; 1.15.1.1), catalase (CAT; 1.11.1.6), were measured as described by (Chakrabarty et al. 2009). Ascorbate peroxidase (APX; 1.11.1.11) and guaiacol peroxidase (GPOX; 1.11.1.7) were measured by (Verma and Dubey 2003).

Carbohydrates status

50 milligrams of oven dried plant material was homogenized in 5 mL 80 % hot ethanol, centrifuged at 5000 rpm for 5 min. The extract was heated completely in a china dish at 80 °C. 5 mL of 20 % ethanol was added to the residue and this ethanol extract was used further for the estimation of total sugars (Yemm and Willis 1954) and reducing sugars (Odunfa 1985) and starch content was measured by acid hydrolysis (McCready et al. 1950).

Proline estimation

Proline was estimated using the method of (Bates et al. 1973). Fresh material of plant was homogenized in 3 % aqueous sulfosalicylic acid, centrifuged to 10,000 rpm. The reaction mixture consisting of 2 mL supernatant, 2 mL acid ninhydrin (1.2 g ninhydrin in 30 mL glacial acetic acid), 20 mL 6 M orthophosphoric acid and 2 mL of glacial acetic acid was boiled at 100 °C for 1 h. After termination of the reaction in ice bath, the reaction mixture was extracted with 4 mL toluene and the absorbance was read at 520 nm. The amount of proline was calculated from the standard curve plotted with known concentrations of proline (10 mg/100 mL).

Data analysis

The data obtained in triplicates was subjected to all pair wise multiple comparison analysis (varieties × tissue) using two-way ANOVA with Tukey's test using Sigma plot 11.0 software. The level of significance was measured at $p < 0.05$.

Results

Membrane damage

Membrane damage is a primary indicator of stress injury. The membrane damage was measured in terms of Percent Electrolyte Leakage Index, lipid peroxidation in the form of TBARS (malondialdehyde formed) and lipoxygenase activity.

Percent Electrolyte Leakage Index (% EL)

Both PDG3 and GPF2 were significantly different ($p < 0.05$) from each other in terms of sensitivity to the stress as the percent EL was more in GPF2 than PDG3. But within tissue, both root and shoot were equally sensitive (Fig. 1).

In PDG3 significant differences were observed, i.e., before exposure and after exposure to lethal stress treatment. It can be inferred from comparison that on the 11th day control (before exposure) damage to tissues was negligible (20 %) as compared to the highest degree of damage (50 %) caused by water deficit induced by PEG-6000 (−0.1 MPa). The preconditioning improved the membrane integrity as the percent EL significantly decreased when exposed to 4 °C, whereas non-preconditioned seedlings showed much higher damage (45 %) in both root and shoot ($p < 0.05$) (Fig. 1).

Similar observations were recorded in GPF2 (root and shoot). Initially on 11th day (before exposure), percent EL was minimum, which increased to the highest value of 50 % with −0.1 MPa treatment. When preconditioned and non-preconditioned seedlings were exposed to lethal low temperature stress (4 °C), the percent EL was negligible in preconditioned or equal to the subsequent control but the non-preconditioned seedlings showed higher degree of damage (46 %) elucidating the positive effect of preconditioning in improving the membrane integrity.

Lipid peroxidation

Thiobarbaturic acid reactive substances (TBARS) are the indicator of the membrane damage. In present study, two-way ANOVA did not show significant interaction in

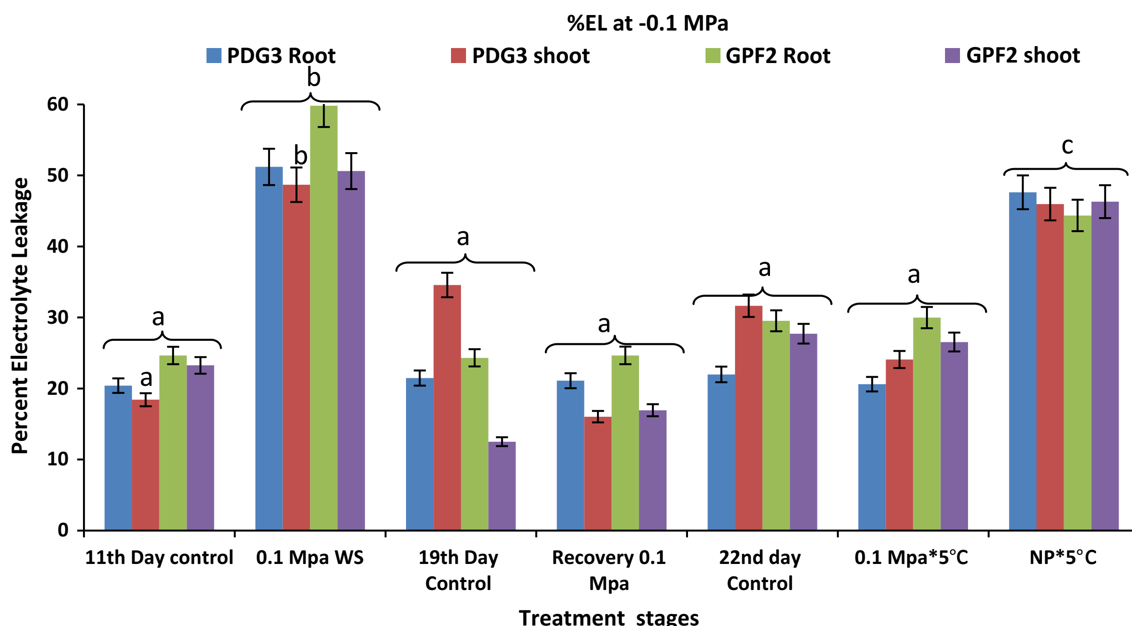

Fig. 1 PEG (−0.1 MPa) induced membrane damage in terms of Percent Electrolyte Leakage Index in Chickpea seedlings. % ELI in PDG3 (root, shoot), % ELI in GPF2 (root, shoot). *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

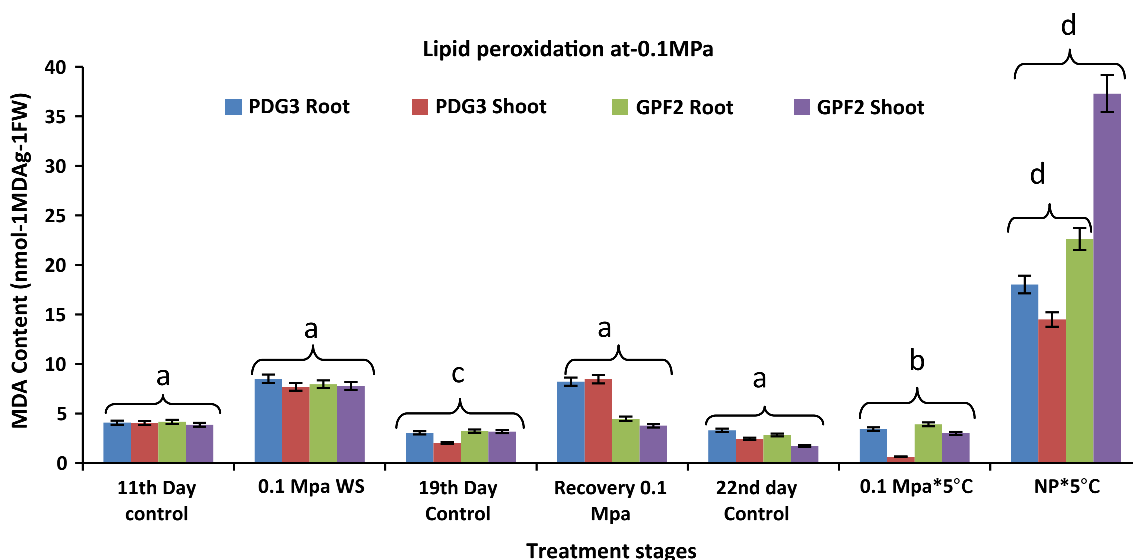

Fig. 2 Membrane damage in terms of Lipid peroxidation (MDA content nmol^{-1} MDA g^{-1} FW) after preconditioning in PDG3 and GPF2 seedlings. MDA content in PDG3 (root, shoot) and in GPF2 (root, shoot). *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

genotypes and tissues. This indicates the equal sensitivity of varieties and their tissues. But individual analysis showed significant variations in both varieties. In PDG3, there was significant increase in the MDA content in non-preconditioned roots (18 nmol^{-1} MDA g^{-1} FW) shoot samples (14.5 nmol^{-1} MDA g^{-1} FW), which strongly signifies the extent of damage in the non-preconditioned chickpea seedlings (Fig. 2). Less MDA content after preconditioning clearly indicate the tolerance to the lethal low temperature stress. In GPF2, almost similar trend was observed indicating no significant change in MDA content in preconditioned root and shoot. Here also non-preconditioned seedlings exposed to lethal low temperature stress showed exceptionally higher MDA content in root and shoots (22.6 and 37.3 nmol^{-1} MDA g^{-1} FW) than the preconditioned seedlings (Fig. 2).

Lipoxygenase activity

There was drastic increase in PDG3 LOX activity in both root and shoot which further decreased significantly after preconditioning the plants, whereas no change was observed in GPF2 statistically. This clearly indicates the association of preconditioning mechanism with the LOX enzyme activity or oxidation of poly unsaturated fatty acids (Fig. 3).

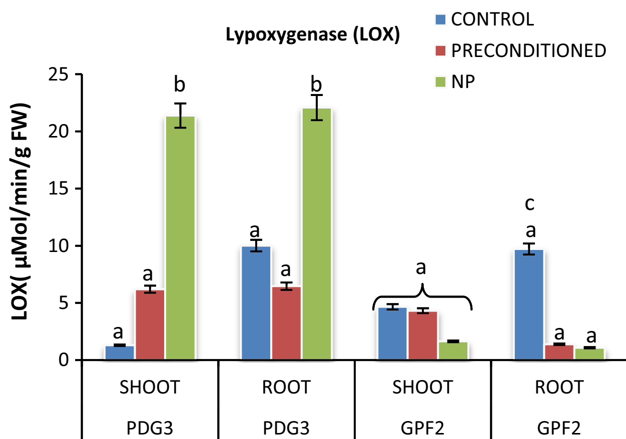

Fig. 3 LOX activity after preconditioning in PDG3 and GPF2 seedlings. LOX activity in PDG3 and in GPF2 roots and shoots. *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

Cellular respiration (% TTC reduction)

Percent TTC revealed no significant interaction between genotypes and genotypes × tissue, but there are significant interactions within the tissues. Both root and shoot parts showed differential response to the preconditioning. In PDG3, both shoot and root did not show any significant difference before preconditioning but after recovery, the TTC reduction increased significantly in root and shoot, (58, 89 %), respectively (Fig. 4). Whereas in GPF2, cellular respiratory capacity decreased significantly (30 %) in shoot, while it increased in the root (81 %). Recovery improved the cellular respiratory capability (75 %). Overall, preconditioning enhanced the mitochondrial efficiency measured in terms of TTC reduction in both the plant parts i.e. root and shoot, as well as in both the varieties (Fig. 4).

Carbohydrates status

Carbohydrates distribution varied in the forms of total Sugars, reducing sugars and starch. Both genotypes showed differential response in context to the amount of total sugars present. In PDG3, there was no significant difference in total sugars content in both tissues till 19th day. But preconditioning improved the total sugars in root (66 mg), whereas in shoot it was significantly less (78 mg) than non-preconditioned (84 mg). In GPF2, there was significant increase in the amount of total sugars in root (140 mg) as well as shoot (94 mg) in non-preconditioned seedlings as compared to preconditioned root and shoot which was 76

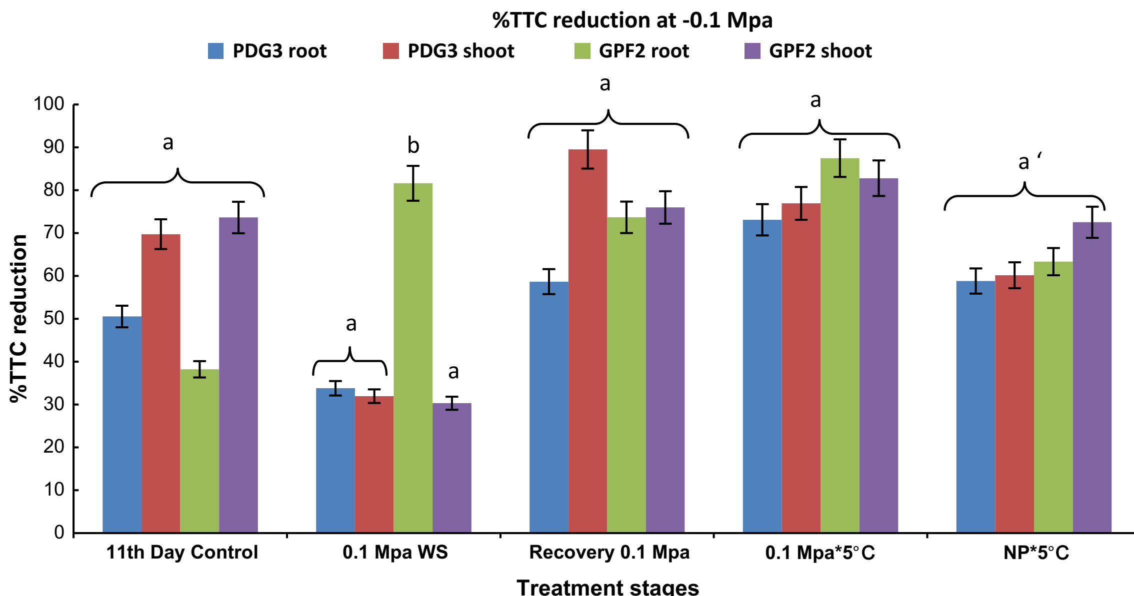

Fig. 4 Cellular respiration in terms of %TTC reduction in Chickpea seedlings. Percent TTC reduction in PDG3, and in GPF2. *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

and 59 mg (Fig. 5a). In both the varieties, non-preconditioned plants showed higher levels of total sugars as compared to preconditioned and control. Total sugars content was more in GPF2 root as well as shoot compared to PDG3 during preconditioning. This depicts that compartmentalization of sugars in GPF2 was more affected than PDG3 by lethal low temperatures. This implies that preconditioning mobilized the total sugars more to roots than shoot. In PDG3, preconditioning did not influence the reducing sugars content in roots as well as shoots (Fig. 5b).

Whereas in GPF2, when non-preconditioned and preconditioned seedlings exposed to lethal low temperature, there was exceptionally high reducing sugars accumulation in non-preconditioned shoot (84 mg) as well as root (71 mg) than preconditioned root (47.1 mg) and shoot (31.6 mg) (Fig. 4d). Interestingly, sensitive genotype GPF2 showed more reducing sugars content than the tolerant PDG3 (Fig. 5b).

Starch content was also not influenced by preconditioning. After exposing to lethal stress, preconditioning did not influence starch content. Whereas, significant increase was noticed in non-preconditioned root and shoot seedlings. In PDG3 preconditioned seedlings, it was 14.3 and 15 mg in root and shoot, respectively. The content was significantly higher in non-preconditioned root (Fig. 5c). In GPF2, when preconditioned seedlings exposed to lethal low temperature were compared, a significant decrease was noticed in preconditioned shoot (11 mg).

No significant difference was noticed in GPF2 preconditioned and non pre-conditioned seedlings (Fig. 5c). Overall, it can be concluded that sugars and starch were influenced by lethal cold stress whereas preconditioning managed to control the carbohydrates breakdown/mobilization.

Antioxidants

Preconditioning improved the SOD activity in both PDG3 and GPF2 shoots. In case of roots, both PDG3 and GPF2 varieties did not show any significant difference among control, pre-conditioned and non-preconditioned (Fig. 6a). SOD activity was more in preconditioned seedlings indicating greater dismutation of $O2^{\cdot -}$radicals than the non-preconditioned ones.

Both PDG3 and GPF2 were equally sensitive for CAT activity. In both the varieties preconditioning played its role and enhanced the CAT activity in both tissues (root and shoot) when compared with the control and non-preconditioned seedlings (Fig. 6b). GPOX showed greater activity in roots of both the genotypes with preconditioning treatment. PDG3 showed greater GPOX activity in preconditioned shoots stage over control and non-preconditioned seedlings whereas, no significant difference was observed in case of GPF2 (Fig. 6c). For APX activity, no significant difference was found when all the three treatments were compared with each other, but overall greater activity of APX was found in GPF2 as compared to PDG3 variety (Fig. 6d), which means that GPF2 is responding as better scavenger of H_2O_2 and helps in preventing the accumulation of toxic levels of H_2O_2 when compared with the PDG3 variety.

In tolerant genotype GPOX responded better whereas in sensitive APX is simulated due to preconditioning.

Accumulation of proline

PDG3 accumulated higher concentration of proline as compared to GPF2. When different tissues were compared, roots accumulated more proline than the shoots (Fig. 7). In PDG3, exposure to lethal low temperature stress gave an additive boost to the proline content (73 mM) in the roots, while non-preconditioned seedlings had significantly low content (22 mM) than the preconditioned ones. It can be elucidated that preconditioning influenced the proline content in the roots than the shoot part (Fig. 7). In GPF2, when pre and non-preconditioned seedlings were exposed to lethal low temperature stress, significant change in proline content was observed. In root, it increased whereas in shoot it decreased (Fig. 7). Accumulation of proline in roots can be considered as a potential marker of tolerance.

Discussion

The present study is aiming at improving the low temperature tolerance by inducing the plant's defense system by pre exposing it with mild drought stress. Improved membrane integrity due to preconditioning is a sign of tolerance, concurrently the damage was more in non-preconditioned seedlings. Preconditioning helped the plants to acclimatize to low temperature, similar response were observed in cold acclimatized seedlings which showed less electrolyte leakage than non-acclimated seedlings (Nayyar et al. 2005a; Shahandashti et al. 2013). Less damage due to chilling injury was observed in preconditioned zucchini fruit (Carvajal et al. 2015a) and Santira var. (Carvajal et al. 2015b). We also measured the membrane damage in the form of lipid peroxidation by measuring MDA content during different stages in both varieties and tissues (Kumar et al. 2011). Both varieties showed equal sensitivity in terms of formation of TBARS (MDA). There was no change in the MDA content in the seedlings after preconditioning as compared to control. We observed that seedlings became more tolerant to incoming chilling stress when preconditioned with -0.1 MPa stress in both varieties (GPF2 and PDG3). Contrastingly, there was huge difference in the MDA content of non-

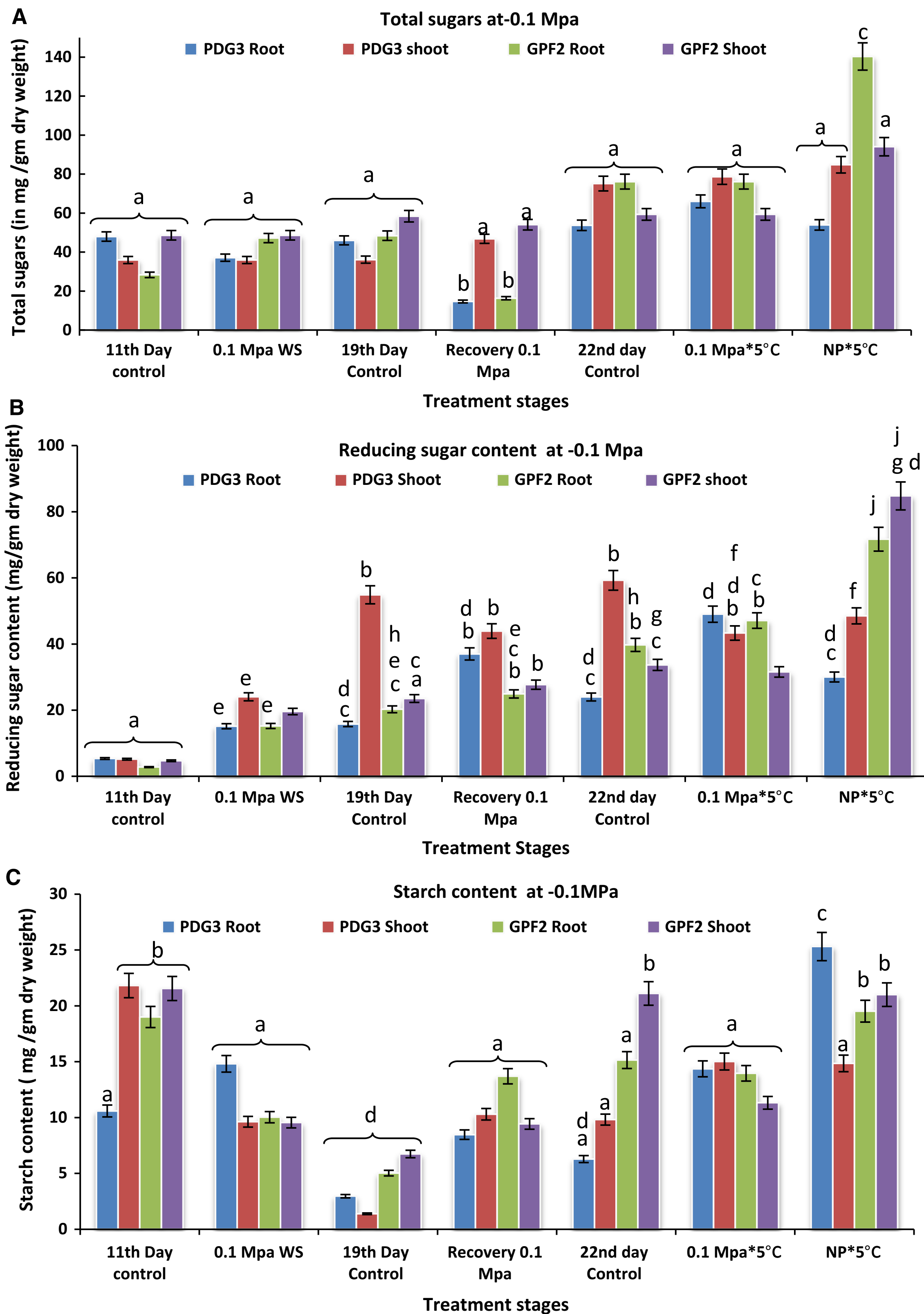

Fig. 5 Total sugars, reducing sugars and starch content (mg/mL/g DW) in Chickpea seedlings, **a** TS in PDG3 and GPF2 (root, shoot), **b** RS content in PDG3 and GPF2 (root, shoot), **c** Starch content in PDG3 and GPF2 (root, shoot), *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

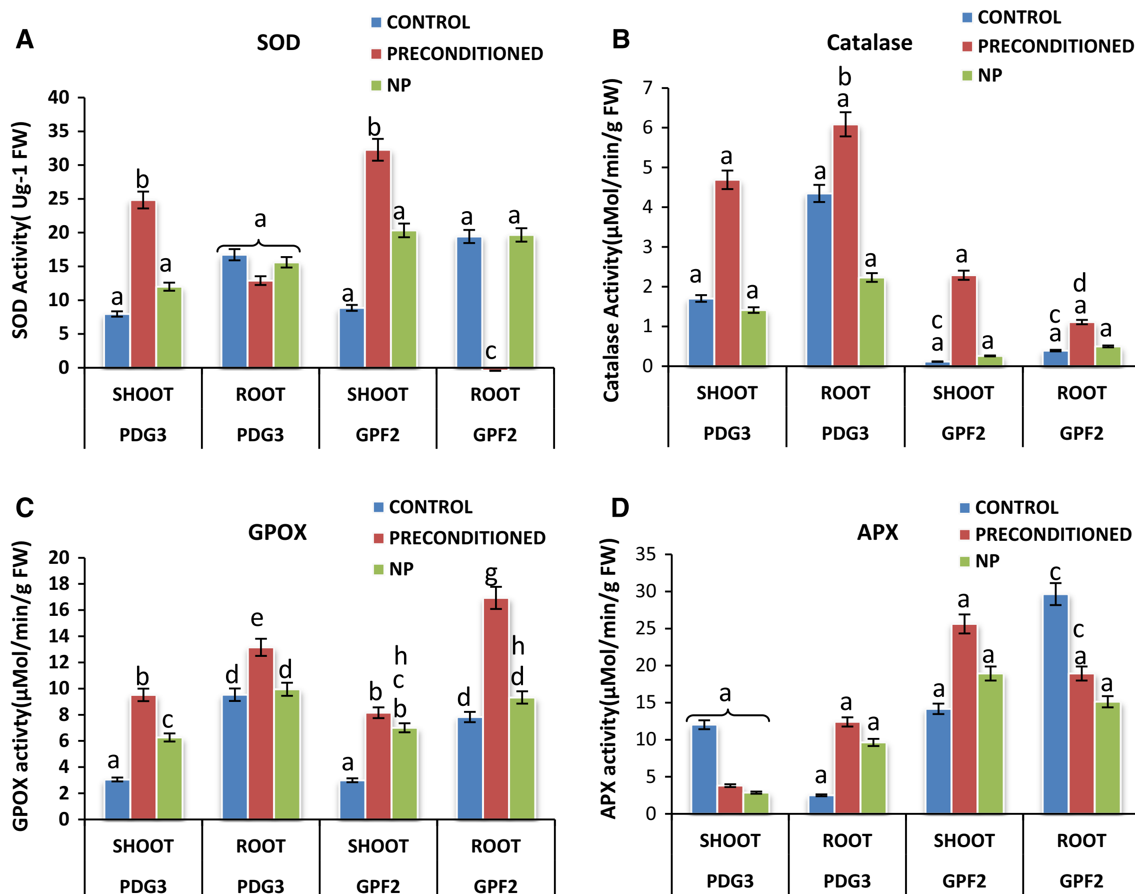

Fig. 6 Antioxidative enzymes activity after preconditioning PDG3 and GPF2 in root and shoot tissue. **a** SOD activity in PDG3 and GPF2 root and shoot). **b** CAT activity in PDG3 and GPF2 root and shoot. **c** GPOX activity in PDG3 and GPF2 root and shoot. **d** APX activity in PDG3 and GPF2 root and shoot. *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

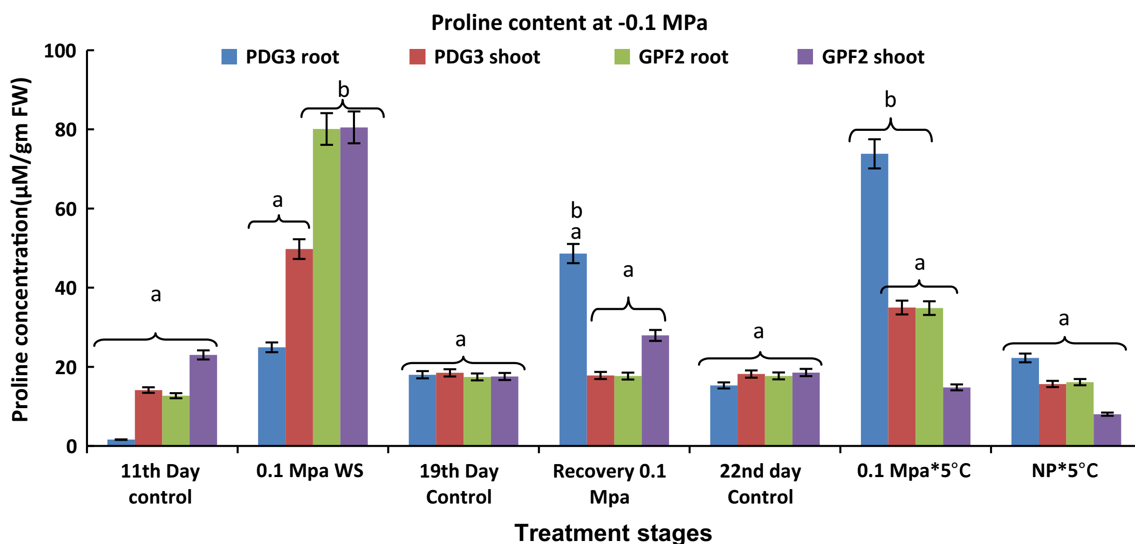

Fig. 7 Proline accumulation (μM^{-1} mL^{-1} g FW) in Chickpea seedlings after preconditioning. Proline content in PDG3 and GPF2 root and shoot after treatment. *Alphabets* depicts the level of significance measured at $p < 0.05$ using ANOVA with multiple comparison using Tukey's test

preconditioned seedlings. Similar kind of pattern of lipid peroxidation has been observed in cold acclimated and non-acclimated chickpea seedlings (Shahandashti et al. 2013), cucumber seedlings when pretreated with methyl jasmonate (Li et al. 2012), cucumber leaves when preconditioned with silicon (Liu et al. 2009) and in zucchini on preconditioning with cold stress (Carvajal et al. 2015b). LOX directly attacks unsaturated fatty acids and increase ROS production in a variety of plant tissues. In present studies, less membrane damage was observed in preconditioned seedlings, similar membrane response has been observed in cold acclimated and non-acclimated chickpea seedlings. LOX activity has also been shown to have close relation with membrane damage in cold acclimated plants (Shahandashti et al. 2013). There are recent reports which describes that during cold treatments, MDA is produced due to oxidative stress. LOX activity is unrelated with MDA content which plays a double role in cold tolerance mechanism in wheat. Lox activity is inversely proportional to MDA content in acclimated vice versa in non-acclimated plants (Nejadsadeghi et al. 2015). High MDA content and parallel low LOX activity in cold stressed GPF2 corroborates with current literature. Similarly low MDA and high LOX in PDG3 can also be correlated with this.

Generally, TTC reduction is directly related to activity of dehydrogenases which are hampered by cold stress (Steponkus and Lanphear 1967). Exposure to lethal low temperature (4 °C) leads to decrease in TTC reduction (Nayyar et al. 2005a) which was found true for non-preconditioned seedlings in our study. Improvement in TTC reduction in preconditioned GPF2 and PDG3 clearly signifies improved cellular respiratory ability with preconditioning.

Soluble sugars play vital role in plant processes during cold acclimation. They act as cryoprotective solules (Nayyar et al. 2005a), nutrients, and key components which interacts with the lipid bilayer (Yuanyuan et al. 2009). Sucrose has been reported to have both cryoprotective solutes as well as signaling molecules. Apart from this, negative role has also been portrayed in enhancing leaf senescence, etc. In our case, total sugars content was more in GPF2 root as well as shoot compared to PDG3, which depicts that compartmentalization of sugars in GPF2 was more affected than PDG3 by lethal low temperatures. It can be inferred that increased amount of sugars in root conferred chilling tolerance to these genotypes especially PDG3. In PDG3, total sugars decreased in preconditioned seedlings compared to non-preconditioned, may account for high amount of reducing sugars in preconditioned plants. It implicated that during control conditions, reducing sugars are mobilized from source tissue (shoot) to sink (root) but during mild and lethal stress conditions, amount increased more in shoot than root due to faulty sink source relationship. In PDG3, reducing sugars in root indicates the active participation of root in stress tolerance. During low temperature and water stress, activity of sucrose phosphate synthase and sucrose synthase increases to provide tolerance to plants, which accounts for increased accumulation of sucrose, i.e., non-reducing sugar in the plants (Guy et al. 1992; Kerepesi and Galiba 2000).

Preconditioning increased the starch content in PDG3 root as well as shoot whereas in non-preconditioned, it was exceptionally high in shoots. More starch content is a sign of poor degradation. Starch acts as transitory storage compound which is converted to glucose and fructose that can be further used to synthesize sucrose as energy source (Grennan 2006). In GPF2, the starch content reduced significantly in preconditioned seedlings compared to control and non-preconditioned seedlings, which imply that preconditioned plants, can metabolize starch even at lethal cold stress. Since both drought and cold stress share many of its processes, the fact has already been established in a previous study that starch content decreases in water deficit conditions as starch synthesis and degradation is affected more than reducing sugar metabolism (Vinocur and Altman 2005). Mild water stress also affects the decrease in the starch synthesis (Geigenberger et al. 1997). In our present work, oxidative stress was significantly reduced in preconditioned plants as compared to the non-preconditioned ones. Reduced oxidative stress was also observed in zucchini fruit when it was pretreated with cold stress. SOD activity was more in preconditioned seedlings indicating greater dismutation of $O^{2\cdot-}$radicals than the non-preconditioned plants. Chilling stress acclimatized seedlings showed higher SOD activity than non-acclimatized cucumber leaves to low temperature stress (Lee and Lee 2000). Catalase activity is another marker to check the oxidative damage caused by any stress in plants. CAT activity also showed similar results as that of the SOD. Therefore, it can be inferred that direct exposure to cold stress without giving any preconditioning decreased the activity of CAT because low temperature stress impaired the repair (Gechev et al. 2003). Similar results were obtained when tobacco was exposed to chilling stress (Gechev et al. 2003). Our results for SOD and CAT are similar with the results obtained when tobacco plants were pretreated and they showed improved tolerance against high temperature as SOD and CAT activities were enhanced (Ghaffari et al. 2015; Wi et al. 2010). In both PDG3 and GPF2, the GPOX activity was more in roots as compared to shoots this may be due to the fact that roots have stronger capability of scavenging ROS. Similar results were obtained when tobacco seedlings were exposed to chilling stress (Xu et al. 2010). Overall significant increase in GPOX activity was observed in preconditioned seedlings over non-preconditioned ones, which

means preconditioning of seedlings can generate a better defense mechanism to cope with incoming lethal low temperature stress. APX activity showed no significant difference among the three treatments; this may be due to the fact that GPOX and APX are competitors of each other working over the same substrate, i.e., H_2O_2 and at the same site in the cell. As previously discussed, GPOX showed higher activity in both the genotypes; therefore; we can conclude that higher GPOX activity have competitively suppressed the activity of APX enzyme (Cordoba-Pedregosa et al. 1996). Cold stress induces oxidative stress in plants, high membrane damage in terns of MDA content can be correlated with the degree of oxidative stress. (Nejadsadeghi et al. 2015). Our results corroborates with the recent literature and found similar to the results obtained in creeping bentgrass, by Zhang and co-workers. According to them, preconditioning with drought stress causes an increase in SOD, CAT and GPOX activity (Zhang et al. 2015).

Proline has long been known as an osmolyte, which gets accumulated in plants during stressed conditions (Szabados and Savouré 2010; Kaushal et al. 2011; Kaur et al. 2011). In our studies, free proline content in preconditioned chickpea seedlings was significantly higher in root than shoot in both genotypes. Roots accumulated more proline as compared to shoot due to primary target of water stress. Accumulation of proline was more in PDG3 root than GPF2 root indicating higher tolerance in PDG3 than GPF2 (Sharma et al. 2011; Yamada et al. 2005). High proline content in roots may be considered as better indicator of stress tolerance. Overall, better antioxidative system, high accumulation of proline and better mobilization of carbohydrate can be treated as positive response of preconditioning and improved the tolerance against ROS over the non-preconditioned seedlings.

Conclusion

Preconditioning with mild water stress has the ability to improve chilling tolerance as it improved the membrane integrity in terms of reduced % EL and less lipid peroxidation, which further can be correlated with increased accumulation of cryoprotectant, i.e., proline and sugars, increased degradation of starch to sugars and increased activity of different antioxidative enzymes in preconditioned seedlings compared to non-preconditioned ones. Above all, roots are better sensor and can be treated as a better indicator for stress tolerance. This in vitro technique can be easily used in fields by withholding the water before onset of period of chilling injury to chickpea. This study is being further elaborated to molecular level to identify regulation of particular genes during preconditioning.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

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