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Impact of cold acclimation on the oxidative and anti-oxidative system of bitter gourd seedlings exposed to low-temperature stress

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ABSTRACT

Bitter gourd (*Momordica charantia* L.) is a cold-sensitive flowering vine, but it remains in demand around the year due to its medicinal properties. Therefore, a prime need is there to develop a cold resilient bitter gourd genotype. The study was designed to inspect the impact of cold stress and acclimation on oxidative and anti-oxidative parameters in two bitter gourd genotypes *viz.,* PAUBG-56 and Punjab-14. For that fifteen-day-old bitter gourd seedlings were subjected to low-temperature stress (5°C) both directly and after acclimation (gradual decrease in temperature from 25 to 5°C before cold exposure). Leaflets were collected on various days after temperature treatment, and seven days of recovery period and analyzed for the above-mentioned parameters. Results showed a significant increase in oxidative stress markers in non-acclimated Punjab-14 seedlings, while PAUBG-56 demonstrated higher resilience through elevated enzymatic and non-enzymatic antioxidant activity. Acclimation improved stress tolerance in both genotypes, although PAUBG-56 showed superior recovery. Therefore, it is concluded that acclimation is an eminent method, that helps to enhance the tolerance capacity of bitter gourd against low-temperature stress.

Key words: *Momordica Charantia* L., lipid peroxidation, non-enzymatic antioxidants, oxidative stress.

INTRODUCTION

Bitter gourd (*Momordica charantia* L.), an annual flowering vine, belonging to the genus *Momordica* family Cucurbitaceae, is also referred to by names like bitter melon, balsam pear, and karela. It abounds in tropical, subtropical, and temperate regions preferably warm tropical weather thriving from low land areas to altitudes of up to 1,000 m (Behera *et al.,* 3). It is grown throughout the world because of its unripe and ripened fruit parts that have medicinal and dietary value. The main areas of tropical regions cultivating this crop include parts of Asia, East Africa, the Caribbean, and South America. It is a cold-sensitive plant so in Punjab, it survives till mid-October. Low-temperature stress includes chilling (0-15°C) and freezing stress (<0°C). Chilling conditions cause injury to plants without the formation of ice crystals, whereas freezing conditions lead to ice crystal formation. Plants differ in their tolerance toward freezing and chilling stress (Singh *et al.,* 18). Low-temperature exposure to tropical and sub-tropical crops may affect their survival through various processes such as cell division, photosynthesis, plant growth, development, and metabolism, and finally reduce the crop yield (Li *et al.,* 14). Plants generate reactive oxygen species (ROS) as a by-product of aerobic metabolism, especially within the chloroplasts, peroxisomes, and mitochondria. The chloroplast is regarded as the primary ROS producer, while additional contributors include cell wall-bound peroxidases, NADPH oxidases, and amine oxidases. Low-temperature stresscauses excess accumulation of ROS *viz.*, singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), leading to oxidative stress. Oxidative stress is characterized by the peroxidation of membrane lipids and the destruction of carbohydrates, proteins, and DNA (Mo *et al.*, 17). The levels of ROS are kept low through the action of various antioxidants which comprise both non-enzymatic and enzymatic molecules. Former types of antioxidants include ascorbate (AsA) and glutathione (GSH), proline, carotenoids, and tocopherols (Li *et al.,* 14). Whereas enzymatic antioxidants include various enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), peroxidase (POD), glutathione reductase (GR), and ascorbate peroxidase (APX). Winter canola exhibited higher activity of antioxidant enzymes (CAT, APX, and SOD) and lower malondialdehyde (MDA) levels in comparison to spring canola. This suggests a positive relationship between antioxidant enzyme activity

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and cold resilience in the cultivars of winter canola (Fahimirad *et al.,* 9).

Low-temperature tolerance can be achieved through acclimation, which involves exposure to low, non-chilling, and non-damaging temperatures, enhancing subsequent cold injury resistance. Cold acclimation leads to various changes in terms of biochemical and physiological factors, together with stabilization of membrane, improved detoxification of ROS and methylglyoxal (MG), stimulation of cold-sensitive protein kinases, and hormone biosynthesis. It also promotes the accumulation of antioxidants, heat shock proteins (HSPs), cold-regulated proteins (CORs), and dehydrins, thereby protecting plants from freezing injury. Gong *et al.* (10) noted that cold acclimation in maize seedlings led to the amplified activity of catalase, ascorbate peroxidase, and superoxide dismutase, alongside reduced electrolyte leakage. An aforementioned study by Devi *et al.* (7) reported a higher survival percentage, secondary metabolites, and proteins in acclimated seedlings of bitter gourd. Therefore, this study aims to scrutinize the effect of direct exposure to low temperature and after acclimation on the dynamics of oxidative and anti-oxidative systems of bitter gourd.

MATERIALS AND METHODS

Two genotypes of bitter gourd (*Momordica charantia L.*) *viz.,* PAUBG-56 and Punjab-14 were used as the former one was observed to show a prolonged survival and harvesting period than the former genotype. The seeds of both genotypes were procured from the Department of Vegetable Science, PAU, Ludhiana. The seeds were grown in plastic trays, containing a combination of coco peat, perlite, and vermiculite in a ratio of 3:1:1, respectively in the greenhouse. Each tray contained 50 seedlings. Seedlings were watered with tap water on every alternate day. Fifteen days after germination (DAG) the seedlings were subjected to two different types of experimental treatment *i.e.*, (i) acclimation before low temperature stress and (ii) direct exposure of seedlings to the low temperature. For acclimation, seedlings (fifteen days old) of both genotypes were subjected to a progressive decrease of temperature from 20° to 5°C, at the frequency of 2°C/h, then allowed to remain for seven days at 5°C, then after the seedlings were shifted to recovery temperature at 25°C for seven days. Leaves of acclimated seedlings were picked on the 7th day after stress and recovery whereas leave samples of directly exposed seedlings were collected on the 1st, 3rd, and 7th day after stress and 7th day after recovery, and stored at -20°C till further analysis.

Superoxide dismutase (*EC 1.15.1.1*) was assessed by using the protocol of Xing *et al.* (20). Briefly, seedling leaves (0.1 g) were crushed in 50 mM ice-cold sodium phosphate buffer (7.0 pH) inclosing 1 % polyvinylpyrrolidone (PVP) and 5 mM β-mercaptoethanol. The mixture was then centrifuged at 4°C for 15 min at 10,000 g, and the buoyant was utilized as the source of enzyme for measuring its activity. The reaction admixture (3.15 mL) comprised 0.2 mL methionine, 0.1 mL NBT (nitroblue tetrazolium), 0.1 mL EDTA, 0.1 mL Na_2CO_3, 1.5 mL phosphate buffer, 0.95 mL distilled water, 0.1 mL extract. The reaction was underway after adding 0.1 mL riboflavin and tubes were kept in light till a blue color developed. Then the tubes were placed under dark conditions for 10 min and read the optical density at 560 nm. Catalase (*EC 1.11.1.6*) enzyme was assessed by using the protocol of Aebi (1), briefly, the SOD extract was utilized for the estimation of CAT also. The reaction admixture comprised 1.95 mL buffer and 1 mL of 30 mM H_2O_2 and the chemical process was initiated with the addition of extract (50 µL) and change in optical density was monitored at 240 nm up to 3 min at 30-sec intervals. Peroxidase (*EC 1.11.1.7*) was assessed by the protocol of Claiborne and Fridovich (6). The extraction of the enzyme was the same as CAT and the same extract was used to measure the POX activity. The reaction mixture comprised phosphate buffer (3.5 mL, 6.5 pH), enzyme extract (0.1 mL), and O-dianisidine solution (0.1 mL). The solution was warmed to 37°C by using a water bath and then added 6 % H_2O_2 (0.1 mL). The variation in optical density was monitored at 430 nm at 30 sec intervals for up to 3 min. Ascorbic acid was assessed by the protocol of Jagota and Dani (12). Briefly, seedling leaves (0.1 g) were homogenized in 2 mL of TCA (10%) and the homogenate was centrifuged for 10 min at 10,000 g. The chemical mixture comprised 1 mL of each extract and TCA whereas the blank contained 2 mL of TCA only. 0.2 mL of Folin ciocalteu reagent was added in all tubes, incubation of the reaction mixture was done at 37ºC for 20 min and optical density was monitored at 760 nm. The standard of ascorbic acid in the range 10-60 µg was run simultaneously along with the samples and ascorbic acid content was represented as mg g^{-1} FW of leaf tissue. Glutathione was measured by the protocol of Beutler *et al.* (3). Briefly, the seedling leaf sample (0.2 g) was homogenized in 1 mL of 0.4 M tris-HCI buffer (7.0 pH) and the homogenate was centrifuged for 25 min at 13,000 g. To buoyant (0.75 mL), double distilled water (0.25 mL) and precipitation solution (1 mL) were added, and centrifuged for 10 min at 5,000 g. To 1 mL buoyant, phosphate buffer (3 mL) and DTNB (5,5-Dithiobis,2-nitrobenzoic acid) solution (0.5 mL) was added. Further, the reaction mixture was mixed properly, and the optical density of

the solution was taken at 412 nm. The assay of proline was carried out by the protocol of Chinard (5). Briefly, the seedling leaf sample (0.1 g) was homogenized in 1 mL of sulphosalicylic acid (3%) and centrifuged for 10 min at 10,000 g. To 2 mL buoyant, 2 mL each of ninhydrin reagent and glacial acetic acid were added, mixed properly, and boiled at 100ºC for 1 h. The tubes were placed in an ice-bath to terminate the chemical reaction and after cooling, toluene (4 mL) reagent was further added to them. The mixture was mixed thoroughly, the upper layer of toluene including chromophore was pipette out, and optical density was read at 520 nm. A standard curve of proline was run in the range of 10-50 µg and the concentration of proline was expressed as mg g^{-1} FW tissue. TAA (Total antioxidant activity) was evaluated by the protocol of Mensor *et al.* (16). Briefly, seedling leaf tissue (0.1 g) was homogenized in methanol (2 mL) and then centrifuged for 10 min. at 10,000 g. To buoyant (10 µL), methanol (0.9 mL) and DPPH solution (1 mL) were added, and mixed properly, and incubation was done for 30 min in the dark at room temperature. The optical density of blank (methanol), sample, and control (methanol + DPPH) was observed at 517 nm. Hydrogen peroxide was assessed by the protocol of Sinha (19). Briefly, seedling leaf (0.1 g) was homogenized in 10 mM ice-cold potassium phosphate buffer (2 mL, 7.0 pH), then centrifuged for 20 min at 10,000 g, buoyant was collected and further utilized for the assessment. In buoyant (1 mL), potassium dichromate (2 mL of 5%) and glacial acetic acid mixture (1:3) were added, vortexed, and optical density was read at 570 nm in contradiction of reagent blank. The standard of H_2O_2 was run in the range of 20 to 100 µmole and content was presented as µmoles g^{-1} FW tissue. Lipid peroxidation was evaluated in terms of malondialdehyde content by the protocol of Dhindsa and Matowe (8). Briefly, seedling leaf tissue (0.1 g) was homogenized in 0.1 % trichloroacetic acid (TCA, 1 mL) and centrifuged for 5 min at 10,000 g. In buoyant (1 mL), 20% TCA (4 mL) encompassing 0.5 % 5-thiobarbituric acid (TBA) was mixed, incubation was done for 30 min at 95°C and further centrifuged at 10,000 rpm for 5 min. After that absorbance was read at 532 nm and the amount of MDA was presented as nmoles of MDA formed g^{-1} FW tissue.

The experimentation was designed in a CRD (Completely randomized block) way, having two treatments (temperature and days of sampling) and each parameter was evaluated in three biological replicates. Statistical analysis of data was done by using two-way analysis of variance (ANOVA) between different parameters to evaluate the significance difference at $P< 0.05$ and correlation analysis was done to evaluate the connection among the different parameters by using CPCS1 software (designed by Punjab Agricultural University).

RESULTS AND DISCUSSION

Low-temperature stress leads to a significant intensification in MDA content in both genotypes (Table 1) concerning to their respective controls. At 5°C, an 8-fold increase was registered in the Punjab-14 genotype and a 7-fold increase in PAUBG-56 w.r.t controls (Fig. 1a). Higher MDA content in bitter gourd seedlings under cold stress probably suggested more damage to cell membranes. After seven days of recovery, the MDA content was still higher in comparison to control values. The acclimated seedlings of both genotypes maintained their MDA content to control levels (Table 1). Its content was increased by 50 % and 20% in acclimated seedlings of Punjab-14 and PAUBG-56 respectively, but lower in comparison to non-acclimated seedlings (Fig. 2).

Table 1. ANOVA table of (a) lipid peroxidation, (b) hydrogen peroxidase, (c) superoxide dismutase, (d) catalase, (e) peroxidase, (f) proline, (g) glutathione, (h) ascorbic acid, and (i) total antioxidant activity in non-acclimated seedlings of bitter gourd.

Source	A	B	AB	C	AC	BC	ABC
CD (5%) of a	2.43	2.98	NS	3.44	4.86	5.96	8.43
CD (5%) of b	0.04	0.05	0.06	0.05	0.07	0.09	0.13
CD (5%) of c	0.003	0.003	0.005	0.004	0.005	0.007	0.009
CD (5%) of d	NS	22.23	31.44	25.67	NS	44.47	NS
CD (5%) of e	0.02	0.02	0.04	0.03	0.05	0.06	0.08
CD (5%) of f	0.0007	0.0008	0.01	0.0009	0.01	0.02	0.02
CD (5%) of g	NS	0.04	NS	0.04	0.06	0.07	0.10
CD (5%) of h	0.11	0.13	NS	0.16	NS	0.27	0.38
CD (5%) of i	0.72	0.89	NS	1.03	1.45	1.78	2.52

Note: A- Genotype, B-Treatments, C-Days

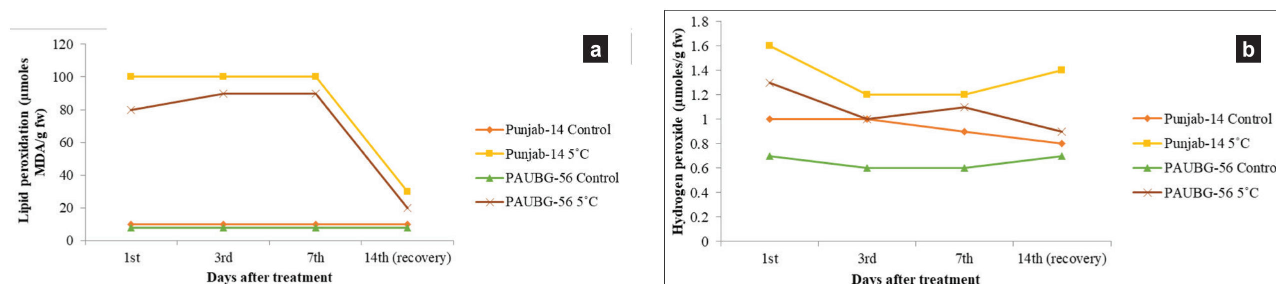

Fig. 1. Effect of low-temperature on (a) lipid peroxidation and (b) hydrogen peroxide in leaves of bitter gourd seedlings. values are mean±SD from 3 replicates.

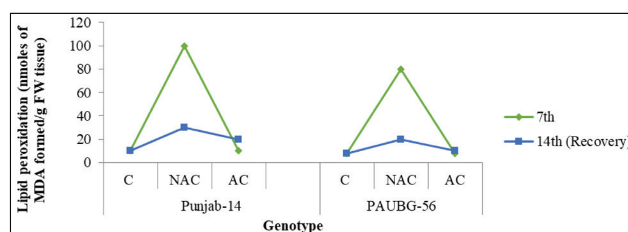

Fig. 2. Effect of cold acclimation on lipid peroxidation in leaves of bitter gourd seedlings. C-control, NAC-non-acclimated at 5°C, AC-acclimated at 5°C, values are mean±SD from 3 replicates.

The H_2O_2 content showed an inclining trend and the increase was significantly higher at 5°C compared to controls. Moreover, the increase was higher in Punjab-14 (Fig. 1b, Table 1). Higher H_2O_2 levels in Punjab-14 probably resulted in higher lipid peroxidation, indicating that cold stress affected this genotype more. Acclimated seedlings of the genotypes showed lower H_2O_2 levels as compared to non-acclimated. Similar types of results were also reported by Jiang *et al.* (13). Acclimation helped to lower the MDA content, and similar findings were reported by Yadegari *et al.* (21), Gong *et al.* (10), and Hajiboland and Habibi (11).

Under LT stress, SOD activity showed an inclining trend in both genotypes (Fig. 3a, Table 1). Activity increased by 62.9 % in Punjab-14 and 15 % in PAUBG-56 and after recovery, the activity was higher in Punjab-14 concerning their controls. SOD activity was higher in non-acclimated than acclimated seedlings of both genotypes. In acclimated seedlings of Punjab-14 and PAUBG-56, SOD activity increased by 50 % and 11 % respectively at 5°C (Fig. 4a). After recovery, activity was slightly higher in acclimated Punjab-14 seedlings but recovered to normal in PAUBG-56. In reaction to LT stress CAT showed a declining trend in both the genotypes at 5°C (Fig. 3b). Acclimated seedlings showed higher CAT than non-acclimated seedlings. Its activity in acclimated seedlings of Punjab-14 and PAUBG-56 genotypes was 354.6 and 388.4 µmoles of H_2O_2 decomposed/min/g FW tissue. CAT activity in acclimated seedlings of PAUBG-56 was higher as compared to Punjab-14. After the recovery period, CAT activity was significantly higher in acclimated seedlings of PAUBG-56 (Fig. 4b, Table 2). Under LT stress, POD activity showed a significant decrease in Punjab-14 but increased in PAUBG-56 (Table 1) concerning their controls. Its activity in the PAUBG-56 genotype increased by 71.4 % (Fig. 3c). Post-recovery, Punjab-14 harbored

Table 2. ANOVA table of (a) lipid peroxidation, (b) superoxide dismutase, (c) catalase, (d) peroxidase, (e) proline, (f) glutathione, (g) ascorbic acid, and (h) total antioxidant activity in acclimated seedlings of bitter gourd.

Source	A	B	AB	C	AC	BC	ABC
CD (5%) of a	NS	3.00	2.45	NS	NS	4.24	6
CD (5%) of b	0.18	0.22	0.18	0.31	NS	0.31	0.44
CD (5%) of c	28.6	35.03	NS	49.54	NS	49.54	70.07
CD (5%) of d	0.02	0.02	0.02	0.03	NS	0.03	0.04
CD (5%) of e	0.002	0.002	0.002	0.004	0.003	0.004	0.005
CD (5%) of f	NS	0.07	0.60	NS	NS	0.10	0.14
CD (5%) of g	0.19	0.23	0.19	NS	0.26	0.32	0.46
CD (5%) of h	1.27	1.56	1.28	2.21	NS	2.20	NS

Note: A- Genotype, B-Treatments, C-Days

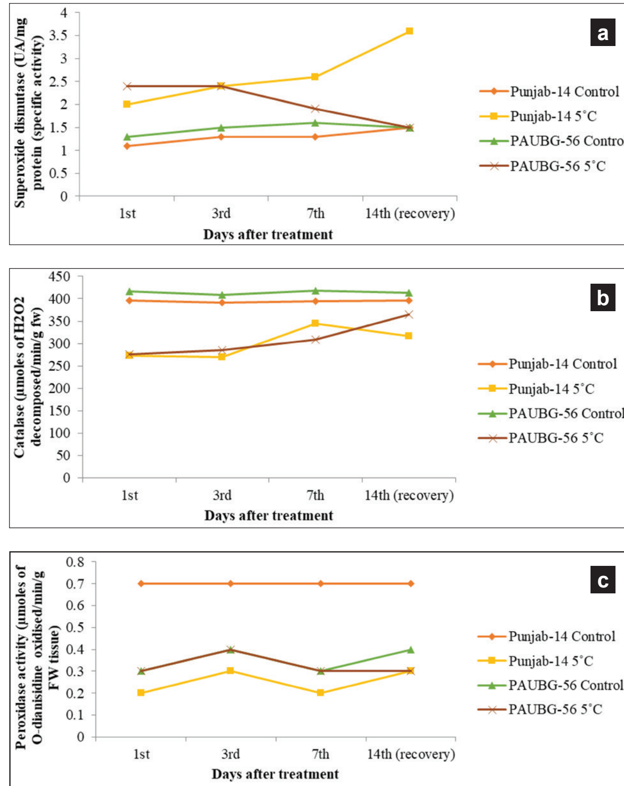

Fig. 3. Effect of low-temperature on (a) superoxide dismutase, (b) catalase, and (c) peroxidase in leaves of bitter gourd seedlings. values are mean ± SD from 3 replicates.

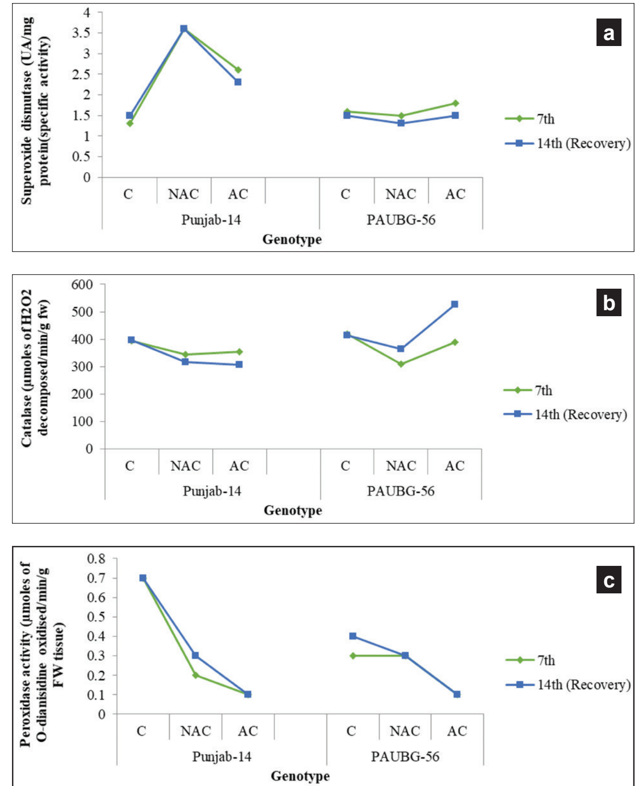

Fig. 4. Effect of cold acclimation on (a) superoxide dismutase, (b) catalase, and (c) peroxidase in leaves of bitter gourd seedlings. values are mean±SD from 3 replicates.

lower POD activity concerning control. In acclimated seedlings of Punjab-14 and PAUBG-56, POD activity decreased by 86 % and 67 % respectively (Fig. 4c) concerning their respective controls.

The higher activity of enzymatic and non-enzymatic antioxidants in PAUBG-56 genotype seedlings suggested that the antioxidant system of this genotype works more efficiently and provides resistance against the cold. Many studies have linked chilling tolerance to antioxidant capacity in chilling-sensitive plants. In seedlings (cold-treated), SOD activity showed a significant increase, while in acclimated seedlings, its activity remained similar to that of the control (Hajiboland and Habibi, 11). POD activity was higher in naked oats (*Avena nuda* L.) underneath low temperatures compared to normal temperatures. However, over time, POD activity decreased significantly, indicating that low temperatures impacted POD enzyme synthesis (Liu *et al.,* 15). The elevated activity of POD and SOD likely indicate their role in reducing environmental stress. Cold acclimation boosted the antioxidant enzymes activities such as APX, SOD, and CAT, with higher levels being maintained after the acclimation period. Acclimated plants subjected to cold stress performed better because of their efficient and active antioxidant system (Gong *et al.,* 10). This suggested that, in the acclimated seedlings, improvement of chilling tolerance was partly due to an enhanced antioxidant enzyme system.

Proline remained unchanged under LT stress in Punjab-14 genotype, but in PAUBG-56 genotype, it increased by 50 % (Fig. 5a) and the content remained higher even post-recovery. The proline content increased in acclimated seedlings of both genotypes after LT exposure and increase was higher in PAUBG-56 genotype. (Fig. 6a). Glutathione content in leaves of both genotypes increased significantly after cold stress (Fig. 5b, Table 1) and content was higher in PAUBG-56. Further, it decreased in Punjab-14 post-recovery period. Acclimated seedlings of both the genotypes showed higher glutathione content concerning to non-acclimated seedlings and even after recovery, content remained higher in acclimated seedlings of Punjab-14 (Fig. 6b). Ascorbic acid increased significantly in both genotypes under low-temperature exposure (Fig. 5c, Table 1). In acclimated seedlings of Punjab-14 and PAUBG-56,

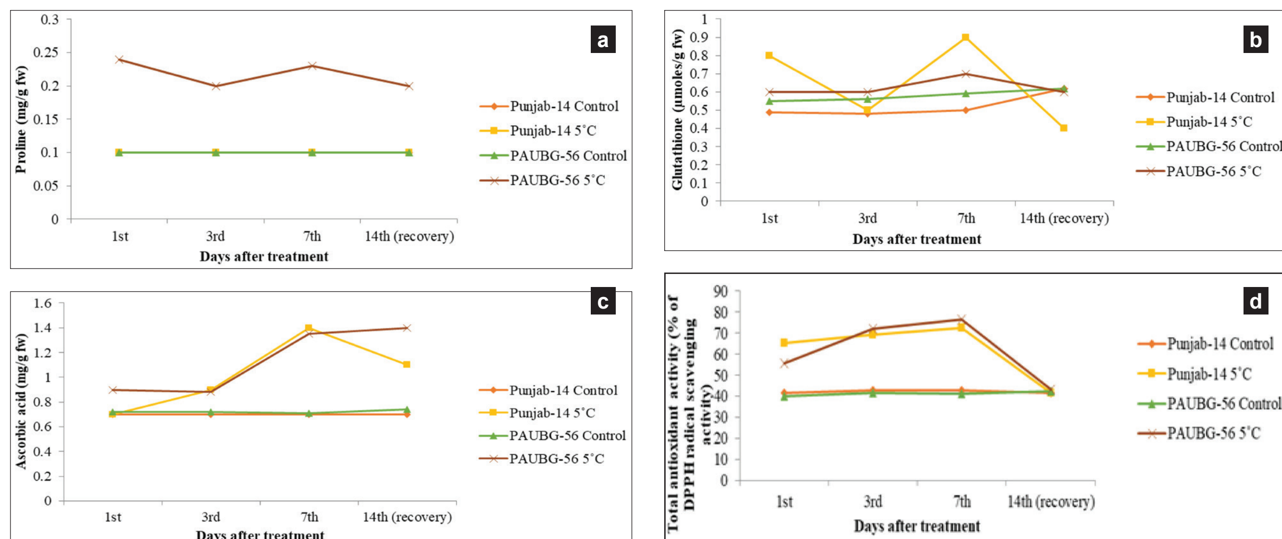

Fig. 5. Effect of low-temperature on (a) proline, (b) glutathione, (c) ascorbic acid, and (d) total antioxidant activity in leaves of bitter gourd seedlings. Values are mean ± SD from 3 replicates.

ascorbic acid content increased by 30 % and 36 % respectively concerning controls (Fig. 6c). After recovery ascorbic acid content remained higher in both genotypes.

Total antioxidant activity significantly ($P \leq 0.05$) increased after LT stress in both the genotypes concerning their controls. It increased in Punjab-14 by 41.2 % whereas it increased by 46.1 % in PAUBG (Fig. 5d, Table 1) compared to their controls. Acclimation enhanced the TAA in Punjab-14 and PAUBG-56 increased by 52 % and 50.1 % respectively (Fig. 6d). ANOVA table of lipid peroxidation, superoxide dismutase, catalase, peroxidase, proline, glutathione, ascorbic acid, and total antioxidant activity in acclimated seedlings are displayed in Table 2.

Proline plays a vital role in a plant's stress tolerance by mediating osmotic adjustment, stabilizing proteins and membranes, inducing genes related to osmotic stress, and scavenging ROS under both normal and stressed conditions. Consequently, when vegetation is exposed to abiotic stresses *viz.,* drought and cold stresses, the accretion of proline helps to control the osmotic pressure of plant cells, maintaining healthy cell structure so plants can withstand environmental stresses (Zuther *et al.,* 22). Yadegari *et al.* (21) found that proline content

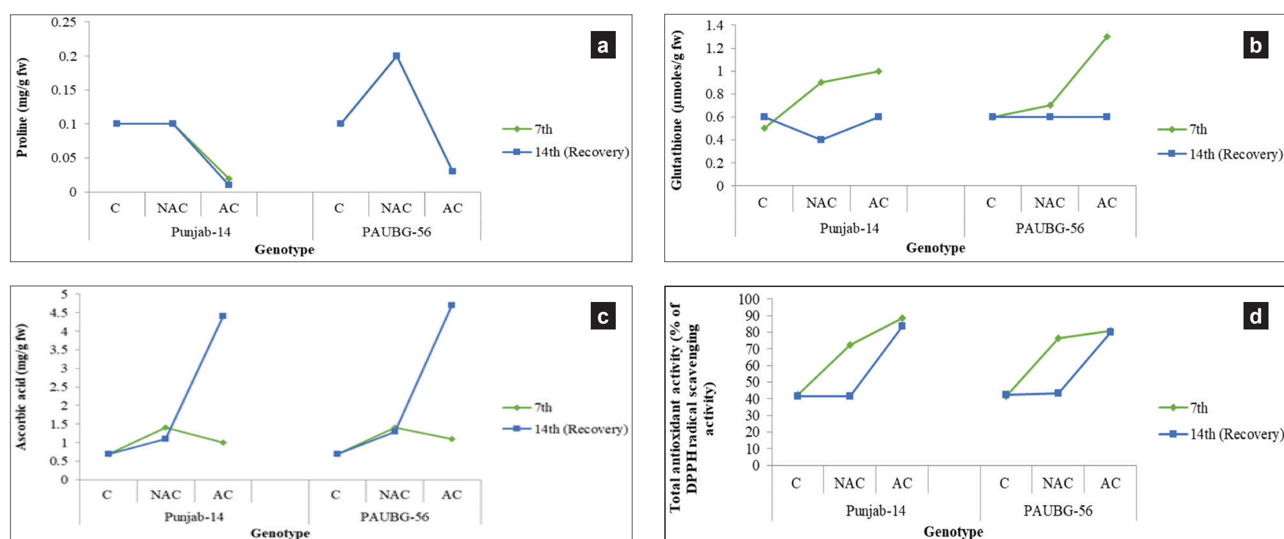

Fig. 6. Effect of cold acclimation on (a) proline, (b) glutathione, (c) ascorbic acid, and (d) total antioxidant activity in leaves of bitter gourd seedlings. Values are mean ± SD from 3 replicates.

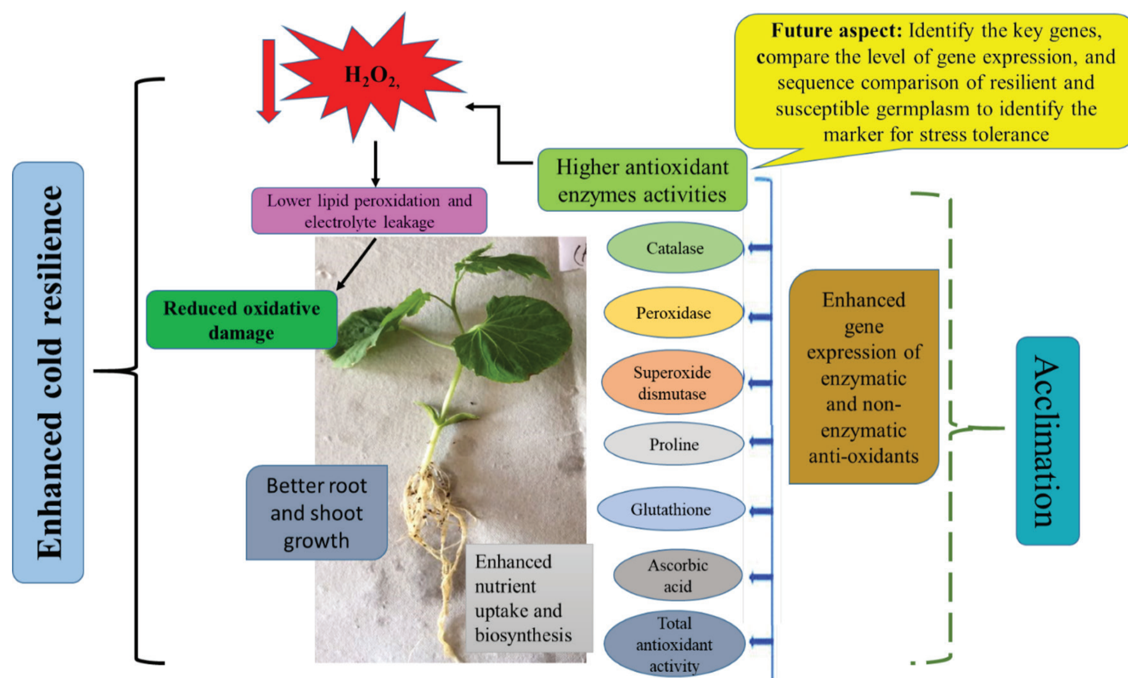

Fig. 7. Overall impact of cold acclimation on oxidative and anti-oxidative parameters in bitter gourd seedlings

augmented more significantly in acclimated soybean seedlings compared to non-acclimated ones, leading to greater resilience. Zuther *et al.* (22) observed that proline levels were greater in the acclimated leaves of Arabidopsis thaliana as compared to non-acclimated leaves, returning to average altitudes afterward de-acclimation. Additionally, Airaki *et al.* (2) described that low-temperature stress significantly elevated the altitudes of soluble non-enzymatic antioxidants, such as ascorbate and glutathione in pepper plants.

In conclusion, seedlings exposed to low-temperature stress showed genotype-specific effects on various biochemical parameters. Both the genotypes showed resilience against cold stress, however in terms of survival percentage, PAUBG-56 was better. Acclimation, in general, improved various estimated parameters in both genotypes which probably led to an increase in survival to 100 % (Devi *et al.,* 7). Therefore, acclimation treatment prior to the sowing of the seeds will improve the survival and yield percentile and thus enhance the economic benefits (Fig. 7). However, the response of these genotypes to cold stress at the maturity stage might be different, which can be ascertained by extending the study to the maturity stage. Further, the genome-wide identification of genes that can enhance acclimation under cold stress conditions can be performed which helps to analyze the variations in the gene structure. This will be a stepping stone for developing molecular markers for assessing cold-resilient varieties.

AUTHORS' CONTRIBUTION

Conceptualization of research (Sangha M.K. and Devi V.); Designing of the experiments (Sangha M.K. and Devi V.); Contribution of experimental materials (Pathak M.); Execution of field/lab experiments and data collection (Devi V. and Kumar P.); Analysis of data and interpretation (Devi V. Devi, S. Ritesh and Kumar P.); Preparation of the manuscript (Devi V. Devi, S. Ritesh and Sangha M.K.)

DECLARATION

The authors declare no conflict of interest.

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