



OPEN Deciphering the dynamics of enzymes associated with the synthesis of cryoprotectants during cold acclimation in contrasting chickpea genotypes

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Chickpea, a vital legume crop, is highly susceptible to cold stress, especially during its reproductive phase, resulting in significant flower and pod abortions and reduced seed yield. Our previous study demonstrated that cold acclimation is effective in enhancing cold tolerance but benefits only cold-tolerant (CT) genotypes, while cold-sensitive (CS) genotypes remain unaffected. In this extended study aimed at probing the detailed mechanisms of this differential response, we further examined the expression profiles of enzymes involved in the synthesis and breakdown of osmolytes (pyrroline-5-carboxylate synthase, proline dehydrogenase (PDH), betaine aldehyde dehydrogenase) and sugars (sucrose synthase, acid invertase, trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase, and trehalase activity), along with the expression of various antioxidants (superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase) in both CT and CS genotypes. Seeds of two contrasting chickpea genotypes, cold-tolerant ICC 17258 and cold-sensitive ICC 15567, were planted in pots during the first week of November in an outdoor field environment. After 40 days, the plants were transferred to walk-in growth chambers for cold acclimation at specific temperatures. Initially, the plants were exposed to 25/18°C (pre-acclimation stage; PAS) for 2 days, followed by a 21-day cold acclimation period with progressively decreasing temperatures over seven days for each cold acclimation stage (CAS): CAS1 (21/13°C), CAS2 (18/10°C), and CAS3 (15/8°C). Subsequently, the plants were subjected to cold stress at 13/7°C for 15 days and then exposed to 30/23°C (12 h day/night) until maturity. Our findings demonstrated that the expression of various enzymes involved in the synthesis of osmolytes and sugars in leaves, anthers, and ovules was significantly upregulated during the cold acclimation process in the CT chickpea genotypes but not in the CS genotypes. This enhanced metabolic activity, coupled with elevated levels of enzymatic antioxidants during the acclimation process, contributed to improved leaf water status, photosynthetic efficiency, and ultimately, superior reproductive performance (pollen germination, pollen viability, stigma receptivity, and ovule viability) under cold stress conditions compared to CS genotypes. The enhanced cold tolerance observed in the CT genotypes is likely attributable to their genetic predisposition and efficient stress defense mechanisms facilitated by the upregulated expression of cold-responsive enzymes.

Keywords Chickpea, Chilling; stress, Proline, Sugar dynamics, Osmolytes, Antioxidant metabolism

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Chickpea (*Cicer arietinum* L.), an essential legume crop, encounters significant growing challenges due to its vulnerability to cold stress, especially during its reproductive stages^{1–4}. This sensitivity leads to the abortion of flowers and pods, substantially decreasing seed production⁵. Overcoming this limitation is vital to ensure stable chickpea yields in areas prone to cold spells. Temperatures below 15 °C during the flowering period pose a major concern for chickpea production in regions such as northern India and southern Australia^{1,6,7}. The susceptibility of chickpeas to cold has been associated with damage to cell membranes, loss of chlorophyll, impaired photosynthesis and respiration, altered root physiology and hydraulic conductivity, reduced leaf water content, and decreased stomatal conductance^{5,8–10}. Cold stress severely impacts chickpea reproduction, resulting in flower abortion, sterility of pollen and ovules, fertilization problems, reduced pod formation, impaired seed development, and smaller seed size, ultimately leading to a significant reduction in crop yield^{1,5,9,11,12}.

The ability of plants to withstand cold conditions relies on their capacity to increase tolerance through exposure to low, non-fatal temperatures, a process called cold acclimation¹³. Cold acclimation triggers extensive modifications in physiological, biochemical, and molecular characteristics, potentially leading to changes in gene expression that enhance cold resistance^{13,14}. Cold acclimation has been well-documented in several legumes, including *Trifolium pratense* (Nordic red clover)¹⁵, *Vicia sativa* (common vetch)¹⁶, and *Medicago* species¹⁷ illustrate biochemical, transcriptomic, and physiological responses to cold acclimation in legumes. Additionally, we have clarified the novelty of our study by emphasizing that it investigates the differential accumulation patterns of osmolytes, sugars, and antioxidant defense mechanisms in chickpea genotypes with contrasting cold sensitivity. Unlike previous studies, our research examines how these metabolic traits correlate with cold tolerance, particularly during acclimation at the reproductive stage, which is critical for yield stability in chickpea under cold stress conditions. However, the specific metabolic adjustments underlying cold acclimation in chickpea, particularly at the reproductive stage, remain largely unexplored¹⁰. This study addresses this gap by examining the differential accumulation of osmolytes, sugars, and antioxidant defense mechanisms in chickpea genotypes with contrasting cold sensitivity, providing insights into key metabolic traits that influence cold tolerance. The process of cold acclimation encompasses intricate physiological and biochemical responses that allow plants to withstand cold stress¹⁸. This study seeks to elucidate the complex molecular mechanisms of cold acclimation in chickpea, with a particular emphasis on specific metabolic pathways and physiological reactions that differentiate cold-tolerant (CT) and cold-sensitive (CS) genotypes. We examined the accumulation patterns of osmolytes and sugars, as well as the expression profiles of enzymes involved in their metabolism, during various stages of cold acclimation in both vegetative (leaves) and reproductive (anthers, ovules) components of CT and CS chickpea genotypes. Osmolytes, a varied group of low-molecular-weight compounds, are crucial for plant stress tolerance, as they accumulate in cells, preserve cell turgor, stabilize cellular structures, and potentially neutralize free radicals¹⁸. Sugars, which are essential to plant metabolism, function as cryoprotectants, shielding cellular membranes from freezing damage, and act as signaling molecules that trigger stress tolerance responses¹⁹. Reactive oxygen species (ROS), unavoidable byproducts of cellular metabolism, serve dual purposes in signaling and oxidative stress; however, excessive ROS buildup can result in cellular damage²⁰.

By delving into the dynamics of osmolytes and sugar metabolism, this study elucidated differences in their accumulation patterns and their respective contributions to cold tolerance. This study also examined the relationship between reactive oxygen species (ROS) production, scavenging mechanisms, and antioxidant systems in cold-tolerant (CT) and cold-sensitive (CS) chickpea genotypes during cold acclimation.

By investigating these aspects, this study aimed to uncover the metabolic and physiological processes underlying cold acclimation in chickpeas, potentially leading to strategies for improving overall cold resistance. Comprehending the distinctions between the CT and CS genotypes is expected to assist in the development of cold-tolerant chickpea varieties, ensuring consistent and high yields in areas prone to unexpected cold periods. It was hypothesized that cold-tolerant (CT) and cold-sensitive (CS) chickpea genotypes might exhibit distinct physiological, biochemical, and genetic responses during cold acclimation, influencing their ability to tolerate cold stress. Physiological differences may include variations in water status, photosynthetic efficiency, and reproductive success. Biochemically, genotypes may differ in the accumulation patterns of osmolytes, sugars, and related metabolic enzymes, affecting cold tolerance. Genetically, variations in the regulation of cold-responsive genes could influence ROS generation, scavenging mechanisms, and antioxidant defense systems, contributing to differential stress responses.

Materials and methods

Raising of plants

Chickpea seeds of two contrasting genotypes (cold-tolerant ICC 17258 and cold-sensitive ICC 15567) were selected from an exploratory screening of 40 cold-tolerant genotypes. Response: In this study, we selected two contrasting chickpea genotypes, ICC 17258 (cold-tolerant) and ICC 15567 (cold-sensitive), based on a broader initial screening. This selection, consistent with our previous study²¹, ensures continuity and comparability of findings. Focusing on these two genotypes allowed for a comprehensive investigation of cold acclimation mechanisms through detailed biochemical, physiological, and molecular analyses without introducing excessive analytical complexity. While broader screenings can provide insights into cold tolerance variation across multiple genotypes, an in-depth characterization of this contrasting pair establishes a strong foundation for future studies, including large-scale screenings and field-based validations to refine breeding strategies for cold tolerance in chickpea.

The seeds of these genotypes were immersed in water for 12 h and inoculated with *Rhizobium ciceri* culture. Five treated seeds were planted in pots containing a mixture of farmyard manure and sandy loam soil in a 1:3 ratio supplemented with tricalcium phosphate fertilizer. Seeds were sown in outdoor enclosures in the first week of November to protect from birds and animals. Seedlings were thinned to two per pot 15 days after sowing.

Weather data, shown in Fig. 1 indicate mean day and night temperatures of 24.9 and 15.9 °C, respectively, with light intensity of 1300–1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity of 60–70%.

The plants were transferred to walk-in growth chambers 40 days after sowing for the cold acclimation treatment. The initial treatment involved exposing the plants to 25/18 °C (12 h day/night) (pre-acclimation stage; PAS) with a light intensity of 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity of 65–70% for two days, followed by a 21-day cold acclimation period, with progressively decreasing temperatures over 7 days for each cold acclimation stage (CAS): CAS1 (21/13 °C), CAS2 (18/10 °C), and CAS3 (15/8 °C). Following this, the plants were exposed to cold stress at 13/7 °C (12 h day/night) with a light intensity of 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity of 65–70%. The temperature remained at 13/7 °C until the completion of podding (15 days) and then gradually increased by 2 °C per day to reach 30/23 °C (12 h day/night) until maturity. The selected temperature regimes were designed to simulate the natural decline in temperatures during winter, ensuring a gradual acclimation process that mimics field conditions. The acclimation phase (progressively decreasing from 25/18 °C to 15/8 °C) allowed plants to develop adaptive responses before exposure to the stress phase (13/7 °C), a critical threshold known to affect chickpea reproduction. This temperature (13/7 °C) was also used in our previous study²¹, reinforcing its relevance for assessing cold tolerance in chickpea under temperate conditions.

The plants were examined for the following traits; each assay was performed with three biological replicates, and each measurement was taken in triplicate (technical replicates) to ensure accuracy.

Assessment of stress injury

The plants were evaluated for stress injury to the leaves during the reproductive stage after exposure to low temperatures during each 7-day acclimation stage (CAS1, CAS2, and CAS3), followed by 15 days of cold stress. Young leaves located beneath the flowers were collected from the second and third nodes and the flowers were sampled simultaneously. Leaf traits, including electrolyte leakage (EL), relative leaf water content (RLWC), stomatal conductance, photosystem II function, and cellular viability, were analyzed from three young leaves. Biochemical traits were assayed using the same leaves, anthers, and ovules collected from three flowers across three separate plants (three replicates). The data were combined and mean values with standard errors (SE) were computed.

Membrane damage (as electrolyte leakage)

Leaf tissues were rinsed with deionized water, cut into small pieces, and submerged in 10 mL deionized water at 25 °C for 12 h. The initial electrical conductivity (C1) was then measured after 12 h. The tissue segments were then heated to 80 °C for 10–15 min in a water bath. After equilibration, the final electrical conductivity (C2) was measured. Membrane injury was determined as EL percentage: $(C1/C2) \cdot 100$ ²².

Cellular viability

Fresh tissue samples (leaves, anthers, or ovules) were placed in a solution containing 50 mM sodium phosphate (pH 7.4) and 500 mg of 2,3,5-triphenyl tetrazolium chloride (TTC) per 100 mL and then incubated in the dark at 25 °C for 1 h without agitation. The tissue samples were extracted twice with 95% ethanol and combined to a total volume of 10 mL²³.

Leaf water status (relative leaf water content)

Fresh leaf samples (500 mg) were placed in Petri dishes filled with distilled water for 2 h before surface drying with filter paper and weighed to obtain the turgid weight (TW), representing the weight of fully hydrated leaves. After oven drying at 110 °C for 24 h, the leaves were weighed again to determine the dry weight (DW). Relative leaf water content (RLWC) was calculated as $[(FW - DW)/(TW - DW)] \times 100$ ²⁴.

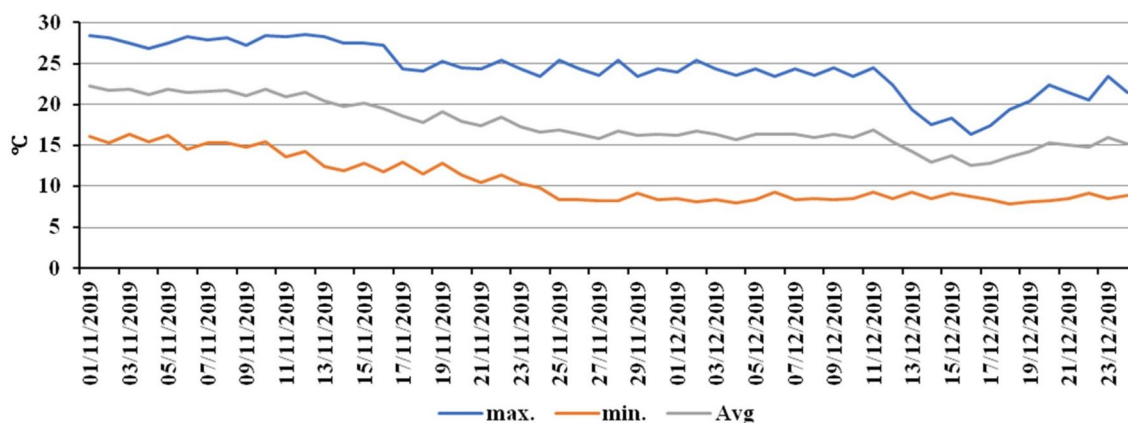


Fig. 1. Weather data (maximum, minimum, and average temperatures) from sowing to 40 days after sowing.

Stomatal conductance

Stomatal conductance was measured using a portable leaf porometer (Decagon Devices, Pullman, WA, USA) and expressed as millimoles per square meter per second ($\text{mmol m}^{-2} \text{s}^{-1}$), as described in Kaushal et al.²².

Chlorophyll fluorescence

Leaf chlorophyll fluorescence was measured using a chlorophyll fluorometer (OS1-FL model, Opti-Sciences, Hudson, New Hampshire, USA). The Fv/Fm ratio was recorded to assess the photosynthetic efficiency, as described in Kaushal et al.²².

Photosynthetic pigments

Chlorophyll and carotenoids were extracted from fresh leaf samples (500 mg) using 80% acetone. The mixture was centrifuged at $5702 \times g$ for 15 min and the resulting supernatant was collected. The absorbance of the extract was measured at 666, 653, and 470 nm using a spectrophotometer. Pigment concentrations were calculated according to the method described by Lichtenthaler and Wellburn²⁵.

Reproductive function

Pollen grains gathered from the flowers of treated plants were subjected to a germination test in a growth medium containing sucrose (10%), 990 mM nitrate (pH 6.5), 1640 mM boric acid, 812 mM magnesium sulfate, and 1,269 mM calcium nitrate^{22,26,27}.

Approximately 200 pollen grains were collected from the flowers on the day of anthesis and stained with 0.5% acetocarmine to evaluate their percentage viability based on color intensity, size, and shape using a microscope²².

Stigma receptivity was assessed using the esterase test²⁸. Stigmas were taken from the flowers one day before anthesis and immersed in a solution of fast blue B (dissolved in phosphate buffer) and α -NAA (naphthaleneacetic acid) at 37 °C for 15 min. Stigma receptivity was evaluated based on the intensity of the brown color with a rating scale of 1 (minimum receptivity) to 5 (maximum receptivity).

Ovule viability was assessed using the TTC reduction assay. Ovules were gathered from flowers one day prior to anthesis, placed on a glass slide, and exposed to 0.5% TTC prepared in a 1% solution. After moving the ovules to a petri dish containing two filter papers dampened with distilled water, they were incubated at 25 °C for 15 min in a growth chamber. The intensity of the resulting red color was assessed on a scale ranging from 1 (least intense) to 5 (most intense)²².

Oxidative stress and antioxidants

Malondialdehyde (MDA) concentration

Fresh tissue (anthers and ovules) was homogenized in 0.1% trichloroacetic acid (TCA) and centrifuged at $3360 \times g$ for 5 min. The resulting supernatant was combined with 4 mL 0.5% thiobarbituric acid dissolved in 20% TCA. The mixture was heated to 95 °C for 30 min, cooled in an ice bath, and centrifuged at $3360 \times g$ for 10 min at 4 °C. The optical density of the supernatant was measured at a wavelength of 532 nm. MDA concentration was calculated using an extinction coefficient of 155 mM cm^{-1} and expressed in nanomoles per gram of DW²⁹.

Hydrogen peroxide (H_2O_2) concentration

Fresh tissue (anthers and ovules) was extracted in 5 mL chilled 80% acetone and filtered using Whatman filter paper. The filtrate was mixed with 4 mL titanium reagent and 5 mL of 25% ammonia solution and centrifuged at $3360 \times g$ for 10 min. The residue was dissolved in 1 M H_2SO_4 and the absorbance of the solution was read at 410 nm. Hydrogen peroxide concentration was determined using an extinction coefficient of $0.28 \text{ mmol cm}^{-1}$ and recorded as nanomoles $\text{g}^{-1} \text{ DW}^{30}$.

Superoxide dismutase (SOD) activity

Fresh plant tissue was homogenized in a pre-chilled 50 mM phosphate buffer solution (pH 7.0) and centrifuged at $3360 \times g$ for 5 min at 4 °C. SOD activity was determined by creating a reaction mixture containing 0.1 mL enzyme extract, 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 25 mM nitro blue tetrazolium chloride, and 0.1 mM ethylene diamine tetra acetic acid (EDTA), with a total volume of 3 mL. Riboflavin (2 mM) was added before exposing the mixture to 15 W fluorescent light for 10 min. The absorbance was measured at 560 nm and SOD activity was calculated according to Dhindsa and Matowe³¹ and expressed as units per milligram of protein.

Catalase (CAT) activity

The reaction mixture included 0.1 mL enzyme extract, 50 mM phosphate buffer (pH 7.0), and 200 mM H_2O_2 . The absorbance of the mixture was monitored at 410 nm wavelength for 3 min. The activity was calculated using an extinction coefficient of 40 mM cm^{-1} and expressed as millimoles of H_2O_2 decomposed per milligram of protein³².

Ascorbate peroxidase (APX) activity

0.1 mL enzyme extract was added to a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM EDTA, with H_2O_2 added as a substrate. The activity was calculated using an extinction coefficient of 2.8 mM cm^{-1} and expressed as millimoles of oxidized donor decomposed per minute per milligram of protein³³.

Glutathione reductase (GR) activity

The reaction mixture contained 0.1 mL enzyme extract, 1.5 mL of 100 mM phosphate buffer (pH 7.6), 0.2 mL bovine serum albumin (BSA), 0.35 mL nicotinamide adenine dinucleotide phosphate (NADP), and 0.1 mL

oxidized glutathione. The enzyme activity was measured by monitoring the reduction in absorbance at 340 nm over 3 min. The activity was expressed as millimoles of oxidized donor decomposed per minute per milligram of protein³⁴.

Ascorbic acid (AsA) concentration

Fresh plant tissue was homogenized in a 6% TCA solution and centrifuged at $3,649.15 \times g$ for 15 min. A 4 mL aliquot of the supernatant was combined with 2 mL 2% dinitrophenylhydrazine (DNPH) solution and a drop of 10% thiourea. The reaction mixture was heated in a water bath for 15 min and allowed to cool to room temperature. Subsequently, 5 mL of pre-chilled sulfuric acid was added and the absorbance was measured at 530 nm. AsA concentration was determined by referencing a standard curve and expressed as milligrams per gram of DW³⁰.

Reduced glutathione (GSH) concentration

Fresh plant tissue was blended with 2 mL metaphosphoric acid and centrifuged at $3650 \times g$ for 15 min. A 0.9 mL aliquot of the supernatant was combined with 0.6 mL of a 10% sodium citrate solution. The assay mixture was prepared by combining 100 μ L of the extract, 100 μ L distilled water, 100 μ L of a 6 mM 5,5-dithio-bis-(2)-nitrobenzoic acid (DTNB) solution, and 700 μ L of a 0.3 mM NADPH solution. To this mixture, 10 μ L glutathione reductase (Sigma-Aldrich, Burlington, MO, USA) was added, and the optical density was measured at 412 nm. GSH concentration was measured using a standard graph and reported as nanomoles per gram of DW³⁵.

Soluble protein measurements

Oven-dried plant tissue was extracted with 0.1 M phosphate buffer (pH 7.0) and centrifuged at $545 \times g$ for 15 min. The protein concentration was determined using the method described by Lowry et al.³⁶.

Osmolytes and enzymes

Proline

Proline in the plant tissue was extracted with 3% sulfosalicylic acid and centrifuged at $2150 \times g$ for 20 min at 4 °C. The supernatant was treated with an acidic ninhydrin reagent and the resulting color was measured at 520 nm using toluene as a control. Proline concentration was expressed as $\text{nmol g}^{-1} \text{DW}^{37}$.

Proline-metabolizing enzymes

Plant tissue samples were collected and immediately frozen before homogenization with a pre-chilled pestle and mortar containing a buffer solution (potassium phosphate, polyvinylpyrrolidone, potassium chloride, magnesium chloride, mercaptoethanol, EDTA) and centrifuged at $2150 \times g$ for 30 min at 4 °C. The supernatant was used to assess proline-metabolizing enzymes. Pyrroline-5-carboxylate synthase (P5CS) activity was determined using a previously described method³⁸. Proline dehydrogenase (PDH) activity was measured by monitoring NADP reduction at 340 nm in a buffer solution containing sodium carbonate, NADP, and proline³⁹.

Glycine betaine concentration

Leaf tissue was oven-dried and homogenized into a fine powder. The powdered samples were combined with 20 mL of deionized water and shaken at 25 °C for 24 h. The resulting extracts were diluted (1:1) with 2 N sulfuric acid (H_2SO_4) before measuring glycine betaine concentration⁴⁰.

Betaine aldehyde dehydrogenase (BADH) activity

Leaf tissue was extracted in a medium containing 50 mM HEPES–KOH buffer (pH 8.0), 2% polyvinylpyrrolidone (PVP), 20 mM sodium metabisulfite, 10 mM sodium borate, 5 mM ascorbic acid, 5 mM dithiothreitol, and 1 mM EDTA. The extract was centrifuged at $3360 \times g$ for 15 min at 4 °C. BADH activity was measured in the desalted extract at 340 nm⁴¹ and expressed as units per milligram of protein.

Trehalose concentration

Trehalose was measured using the method described by (Trevelyan and Harrison, 1952). Plant tissues were extracted in 80% hot ethanol and centrifuged at $3510 \times g$ for 15 min. The supernatant (0.1 mL) was mixed with 2 mL TCA and analyzed according to the method described by Kumar et al.⁴².

Trehalose-metabolizing enzymes

Enzymes involved in trehalose metabolism were analyzed according to the protocols outlined by Habibur Rahman Pramanik and Imai⁴³ with minor modifications. Trehalose-6-phosphate synthase (TPS) activity was measured using the method described by Hottiger et al.⁴⁴. Trehalose-6-phosphate phosphatase (TPP) activity was analyzed according to the method described by Klutts et al.⁴⁵. Neutral trehalase activity was evaluated by activating the enzyme through phosphorylation via cyclic adenosine monophosphate (cAMP) and determining glucose release⁴⁶.

Sucrose concentration

Sucrose concentration was analyzed using the method described by Jones et al.⁴⁷, elaborated earlier²². Reducing sugars were determined using the DNSA method outlined by Sumner and Howell⁴⁸, elaborated previously²².

Sucrose synthase and acid invertase activities

Fresh samples (~500 mg) were snap-frozen and extracted in ice-cold 200 mM HEPES/KOH buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone (PVP), 10 mM dithiothreitol (DTT), 3 mM magnesium acetate, and

3 mM EDTA Na₂·2H₂O. The homogenate was centrifuged at 3360 × *g* for 20 min at 4 °C, and the supernatant was desalted by passing it through 4 mL Sephadex G-25 columns (Sigma, St Louis, MO, USA) pre-equilibrated with a buffer solution containing 20 mM HEPES–NaOH (pH 7.5), 0.05% BSA, 1 mM EDTA, 0.01% mercaptoethanol, and 0.25 mM MgCl₂. The desalted extract was assayed for sucrose synthase^{49,50} and vacuolar acid invertase⁵¹ activities.

Experimental design and statistical analysis

The experiment had a three-factor randomized block design, with CT and CS chickpea genotypes, five treatments, and three organs. Each genotype had ten pots, with two plants per pot and three replications per treatment. For yield trait measurements, 15 pots (five pots in triplicate) were maintained separately for each treatment, resulting in 30 plants per treatment. The pots were moved periodically to avoid positional effects. Analysis of variance (ANOVA) for genotypes × treatments × organs interactions was performed using the Agristat software (India), and the least significant difference (LSD) values were calculated at a significance level of 0.05. Tukey's post-hoc test was used to evaluate means. Principal component analysis (PCA) was conducted using OriginPro 2024 graphing and analysis software to investigate the relationships between the different measurements.

Results

Figure 2 illustrates the contrasting effects of cold acclimation on cold-tolerant and cold-sensitive chickpea genotypes following exposure to cold stress.

Stress injury

Stress injury to leaves was evident through various physiological changes observed in CT and CS genotypes during cold acclimation and subsequent cold stress conditions (Table 1).

Electrolyte leakage

Cold acclimation led to an increase in electrolyte leakage in both CT and CS genotypes, indicating membrane damage. However, this increase was more pronounced in the CS genotype. Upon subsequent exposure to cold stress, the CT genotype exhibited relatively lower electrolyte leakage compared to the CS genotype (Table 1).

Chlorophyll content

Cold acclimation resulted in a decline in chlorophyll levels in both genotypes, with a more substantial reduction observed in the CS genotype, compared to pre-acclimation stage (PAS). When subjected to cold stress conditions, the CT genotype demonstrated remarkably higher chlorophyll content than the CS genotype (Table 1).

Chlorophyll fluorescence (CF)

The cold-sensitive (CS) genotype showed a decline in chlorophyll fluorescence (CF) during the initial cold acclimation stage (CAS1), while the cold-tolerant (CT) genotype exhibited this decrease in the subsequent cold acclimation stage (CAS2) (Table 1). The reduction in CF was more substantial in the CS genotype compared to the CT genotype. When exposed to cold stress, the CS genotype displayed a notably lower CF value than the CT genotype.

Carotenoids

During cold acclimation, the CT genotype maintained relatively consistent carotenoid levels with only a slight decrease under cold stress. In contrast, the CS genotype exhibited a substantial reduction in carotenoid levels during both the cold acclimation and cold stress periods (Table 1). The CT genotype demonstrated a significantly higher retention of carotenoid levels than the CS genotype during cold stress.

Relative leaf water content (RLWC)

During cold acclimation, the CT genotype maintained a relatively stable Relative Leaf Water Content (RLWC), whereas the CS genotype exhibited a significant decrease in RLWC (Table 1). The CS genotype demonstrated a substantial reduction in RLWC under cold stress conditions, in contrast to the CT genotype, which maintained a significantly higher RLWC.

Stomatal conductance (gS)

The CT genotype displayed a minor increase in stomatal conductance (gS) at CAS1 compared with PAS, followed by a slight decline, but maintained a relatively stable gS under cold stress conditions. In contrast, the CS genotype exhibited a substantial decrease in gS during CAS2 and CAS3 (Table 1). Under exposure to cold stress, the CS genotype showed significantly lower gS levels than the CT genotype.

Cellular viability (CV)

During cold acclimation, the CT genotype exhibited a notable increase in cellular viability within leaves, anthers, and ovules. In contrast, the CS genotype showed only a slight increase at CAS2 and a significant decrease during cold stress (Fig. 3). Under cold stress conditions, the CT genotype maintained superior cellular viability compared with the CS genotype, with the most pronounced difference observed in leaves, followed by ovules and anthers.

Osmolytes and enzymes

Proline and glycine betaine metabolism

Proline levels (Fig. 4a) steadily increased during acclimation in the CT genotype but only showed a transient rise in the CS genotype before declining at cold acclimation stage 3 (CAS3) in all organs. This trend correlated with



Fig. 2. Effect of cold acclimation followed by cold stress (13/7 °C) on plants of cold tolerant (CT) (a), Cold Sensitive (CS) genotypes (b), CT flowers (c), CS flowers (d), CT anthers (e), CS anthers (f), CT pollen viability (g), CS pollen viability (h), CT pollen germination (i), CS pollen germination (j), CT stigma receptivity (k), CS stigma receptivity (l), CT ovule viability (m), CS ovule viability (n), CT ovary (o), CS ovary (p). Note marked damage to flowers, anthers, reduction in pollen viability, pollen germination, stigma receptivity, ovule viability, and ovary in CS genotypes.

P5CS ($\Delta 1$ -pyrroline-5-carboxylate synthase activity) (Fig. 4b), which increased significantly in the CT genotype but remained unchanged in the CS genotype. Conversely, PDH (Proline dehydrogenase) activity (Fig. 4c) decreased in the CT genotype throughout acclimation, whereas the CS genotype exhibited an initial decline followed by a later increase. Under cold stress, the CT genotype consistently maintained higher proline content and P5CS activity across leaves, anthers, and ovules compared to the CS genotype.

During cold acclimation, GB (Glycine betaine) (Fig. 5a) levels steadily increased in all organs of the CT genotype, while the CS genotype showed an initial rise followed by a decline. This pattern mirrored BADH (Betaine aldehyde dehydrogenase) (Fig. 5b) activity, which increased throughout acclimation in the CT genotype but peaked at cold acclimation stage 1 (CAS1) in the CS genotype before decreasing. Under cold stress, both GB levels and BADH activity remained significantly higher in the CT genotype across leaves, anthers, and ovules compared to the CS genotype.

Sugar metabolism

During cold acclimation, sucrose levels (Fig. 6a) initially increased in both genotypes; however, the CT genotype sustained higher levels throughout, whereas the CS genotype exhibited a transient increase followed by a decline. This pattern matched sucrose synthase (Fig. 6b) and acid invertase activity (Fig. 6c), which steadily increased in the CT genotype but showed an early peak (CAS1) before declining in the CS genotype. Consequently, during cold stress, the CT genotype maintained significantly higher sucrose levels, along with enhanced sucrose

Parameters	Genotype	PAS	CAS1	CAS2	CAS3	Stress
Membrane damage (%)	CT	11.5 ± 1.9f	14.3 ± 2.1e	17.9 ± 2.3d	19.3 ± 2.5d	19.9 ± 2.4d
	CS	10.9 ± 1.9f	14.8 ± 2.1e	21.4 ± 2.5c	25.4 ± 2.9b	28.3 ± 2.9a
Chlorophyll content (mg g ⁻¹ DW)	CT	23.1 ± 2.9a	22.4 ± 2.6ab	21.5 ± 3.2a	19.3 ± 2.4ab	20.6 ± 2.9ab
	CS	22.8 ± 2.7ab	18.4 ± 2.3bc	16.4 ± 3.5 cd	13.6 ± 2.9 cd	10.6 ± 3.2d
Chlorophyll fluorescence (Fv/Fm)	CT	0.76 ± 0.081a	0.76 ± 0.084a	0.73 ± 0.079b	0.71 ± 0.081c	0.69 ± 0.076d
	CS	0.76 ± 0.081a	0.71 ± 0.079c	0.61 ± 0.069e	0.58 ± 0.061f	0.49 ± 0.056 g
Relative leaf water content (%)	CT	79.4 ± 4.4a	81.3 ± 4.9a	79.6 ± 4.3a	80.3 ± 4.5a	78.4 ± 3.1a
	CS	78.4 ± 4.4a	79.4 ± 3.5a	76.3 ± 3.1a	72.3 ± 3.2b	69.3 ± 2.8c
Stomatal conductance (mmol m ⁻² s ⁻¹)	CT	314 ± 11.3a	325 ± 17.6a	306 ± 12.4ab	297 ± 12.4b	306 ± 13.4ab
	CS	329 ± 13.6a	331 ± 12.6a	269 ± 19.5b	201 ± 13.5c	187 ± 12.4d
Carotenoids (mg g ⁻¹ DW)	CT	1.34 ± 0.18a	1.31 ± 0.17a	1.29 ± 0.17ab	1.21 ± 0.15b	1.17 ± 0.19c
	CS	1.3 ± 0.18a	1.16 ± 0.16c	0.89 ± 0.09d	0.76 ± 0.081e	0.71 ± 0.079f

Table 1. Effect of cold acclimation succeeding cold stress on leaf injury parameters in contrasting chickpea genotypes. Different small letters with values indicate significant differences ($P < 0.05$; Tukey's test). Treatments: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3). Genotypes: cold tolerant (CT), cold-sensitive (CS).

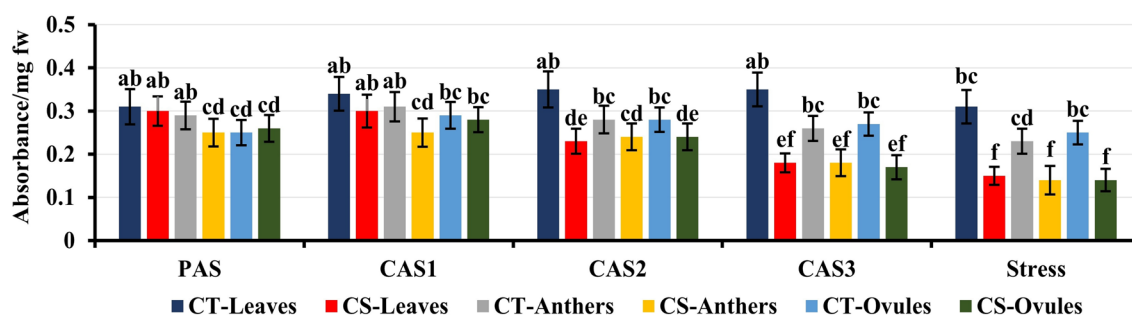


Fig. 3. Cellular viability (CV) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean ± S.E.; $n = 3$). Three-way ANOVA was performed to analyze the interaction between genotypes × treatments × organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

synthase and acid invertase activity, leading to greater reducing sugar accumulation (Fig. 6d) in leaves, anthers, and ovules compared to the CS genotype.

During cold acclimation, the CT genotype maintained elevated trehalose levels (Fig. 7a), whereas the CS genotype exhibited an initial increase at CAS1, followed by a decline. This trend was supported by the activity of TPS (trehalose-6-phosphate synthase) (Fig. 7b) and TPP (trehalose-6-phosphate phosphatase) (Fig. 7c), both of which showed sustained increases in the CT genotype but only transient rises in the CS genotype before declining. In contrast, trehalase activity (Fig. 7d) was consistently lower in the CT genotype but increased in the CS genotype after CAS1. As a result, during cold stress, the CT genotype exhibited significantly higher trehalose levels, along with enhanced TPS and TPP activity, compared to the CS genotype.

ROS metabolism

Malondialdehyde and hydrogen peroxide

During cold acclimation, oxidative stress, as indicated by malondialdehyde (MDA) levels, showed a moderate increase in the CT genotype, mainly at later stages (CAS2 and CAS3), while the CS genotype exhibited a continuous and substantial rise throughout acclimation (Fig. 8a).

Similarly, hydrogen peroxide (H_2O_2) levels increased only slightly in the CT genotype but more prominently in the CS genotype during acclimation (Fig. 8b). As a result, during cold stress, the CT genotype maintained significantly lower MDA and H_2O_2 levels in leaves, anthers, and ovules, suggesting more effective oxidative stress regulation compared to the CS genotype.

Antioxidants

During cold acclimation, the CT genotype exhibited a sustained increase in superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activity across all organs, whereas the CS genotype showed an initial rise at CAS 1 followed by a decline (Fig. 9a, b, c, d). As a result, during

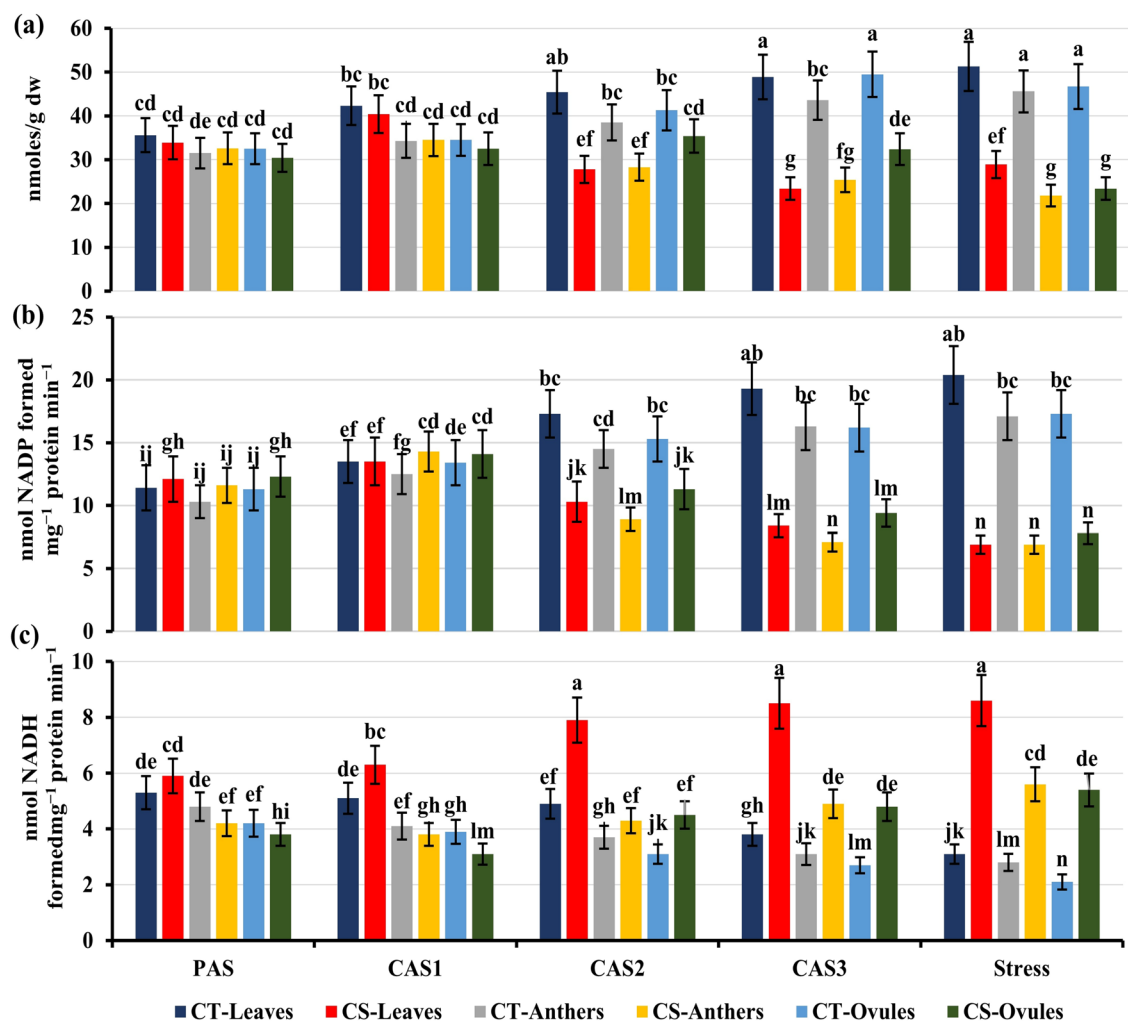


Fig. 4. Proline (a), Pyrroline 5 carboxylate synthase (b), and Proline dehydrogenase (c) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean \pm S.E; $n = 3$). Three-way ANOVA was performed to analyze the interaction between genotypes \times treatments \times organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

subsequent exposure to cold stress, the CT genotype maintained significantly higher antioxidant enzyme activity in leaves, anthers, and ovules compared to the CS genotype.

Similarly, ascorbic acid and glutathione (GSH), the non-enzymatic antioxidants, increased and remained elevated in the CT genotype during acclimation as well as stress conditions, whereas in the CS genotype, both antioxidants initially increased at CAS 1 but subsequently declined (Fig. 10a, b).

Reproductive traits

Under cold stress conditions, the cold-acclimated CT genotype exhibited significantly higher pollen viability and germination rates than those of the CS genotype (Table 2). Furthermore, the CT genotype demonstrated substantially enhanced stigma receptivity and ovule viability under cold-stress conditions.

Yield traits

The cold-acclimated CT genotype exhibited a significant increase in reproductive success, under cold stress conditions (Table 2). In contrast, the CS genotype failed to produce any pods under the same conditions.

Principal component analysis

Principal Component Analysis (PCA) was used to investigate the relationships between stress injury traits (MDA, malonaldehyde; EL, electrolyte leakage; Chl, chlorophyll content; CF, chlorophyll fluorescence; RLWC, relative leaf water content; gS, stomatal conductance; CV, cellular viability; CAR, carotenoids) in leaves and yield traits (PS%, pod set; SW: seed weight plant⁻¹, PN: pod number plant⁻¹) in chickpea genotypes under cold

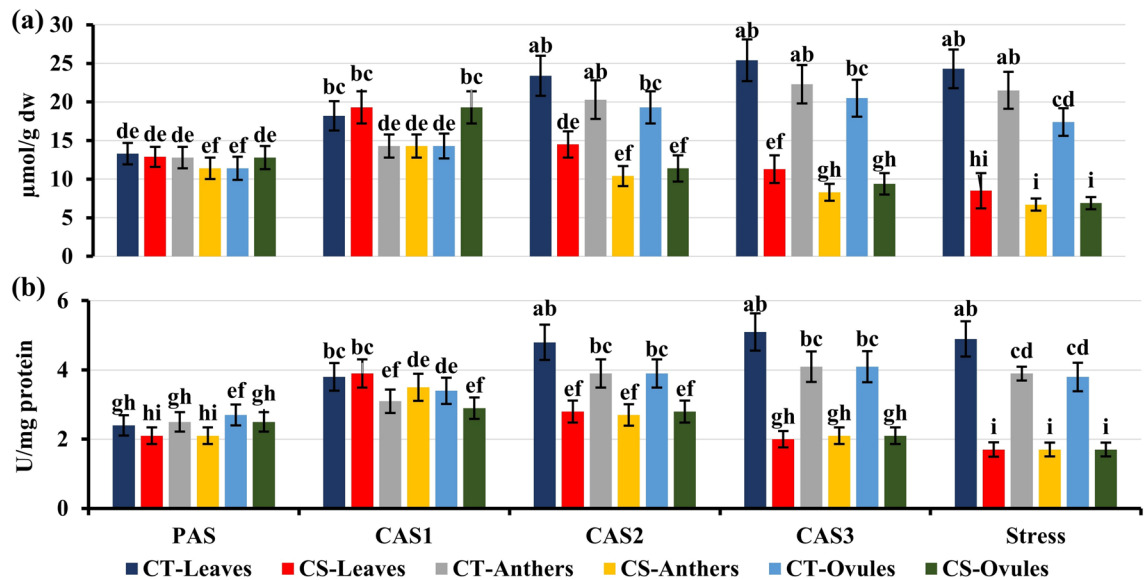


Fig. 5. Glycine betaine (a) and Betaine aldehyde dehydrogenase (b) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean \pm S.E.; $n = 3$). Three-way ANOVA was performed to analyze the interaction between genotypes \times treatments \times organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

stress (Fig. 11). The analysis revealed that the first two principal components (PCs) explained 97.64% of the total variability, with PC1 accounting for 95.27% and PC2 accounting for 2.37%. The yield traits associated with PC1 were positively correlated with leaf injury traits, except for MDA and EL. The major negative contributors to PC1 were EL (-0.301) and MDA (-0.288), while the highest positive contributors were gS (0.307), Car (0.307), PS (0.306), and PN (0.306).

An additional PCA (Fig. 12) investigated the relationships between yield traits and all biochemical traits (oxidative and antioxidative traits (H_2O_2 , hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; AsA, ascorbic acid; GSH, reduced glutathione); carbohydrate metabolism traits (SUC, sucrose; SSY, sucrose synthase; RS, reducing sugars; AI, acid invertase; TRE, trehalase; TREH, trehalase; TPS, trehalose phosphate synthase; TPP, trehalose phosphate phosphatase), osmolytes (Pro, proline; GB, glycine betaine; PDH, proline dehydrogenase; P5CS, pyrroline-5-carboxylate synthase; BADH, betaine aldehyde dehydrogenase) in the leaves, anthers, and ovules under cold stress. The analysis revealed that the first two PCs explained 99.02% of the total variability, with PC1 accounting for 95.69% and PC2 accounting for 3.33%. In PC1, the yield and most biochemical parameters pointed in the same direction, indicating that they are positively connected except H_2O_2 , PDH, and TREH showed a negative correlation. This suggests that plants with higher levels of these beneficial biochemical compounds tend to perform better in terms of yield. PC1 primarily explained the variability in antioxidants, carbohydrates, and osmolytes, except PDH, across different plant tissues (leaves, anthers, and ovules), as well as yield traits, whereas PC2 primarily explained the variability in H_2O_2 , PDH, and TREH in the same plant tissues. This indicates that antioxidants (e.g., SOD, CAT, etc.), sugars (e.g., RS, SSy, TPS, TPP), and osmolytes contribute positively to plant health and productivity, likely by enhancing stress tolerance and energy balance. Their significant association with yield suggests that these traits may assist in maintaining or boosting yield under stressful circumstances (e.g., cold). H_2O_2 , a sign of oxidative stress, frequently accumulates during stress and is typically negatively connected with plant performance if not adequately scavenged. Similarly, PDH and TREH are involved in proline and trehalose metabolism, and their activity might reflect stress severity or metabolic imbalance, hence their lack of positive correlation with yield. The major positive contributors to PC1 were RS (anthers, 0.128), SSy (leaves and anthers, each 0.128), TPS (anthers, 0.129), TPP (leaves, 0.128), and ASC (anthers and ovules, each 0.128). The most negative contributors to PC1 were H_2O_2 (anthers, -0.119), PDH (leaves, -0.125 ; anthers, -0.122 ; ovules, -0.124), and TREH (leaves, -0.118).

Discussion

Expanding on our previous research²¹, which demonstrated that cold acclimation enhances the performance of vegetative and reproductive structures in the CT genotype under cold stress, but not in the CS genotype, this study aimed to elucidate the molecular mechanisms underlying these physiological changes. Specifically, we sought to understand why CT genotypes benefit from cold acclimation, whereas CS genotypes do not exhibit such advantages. To achieve this, we examined the expression patterns of key enzymes involved in the

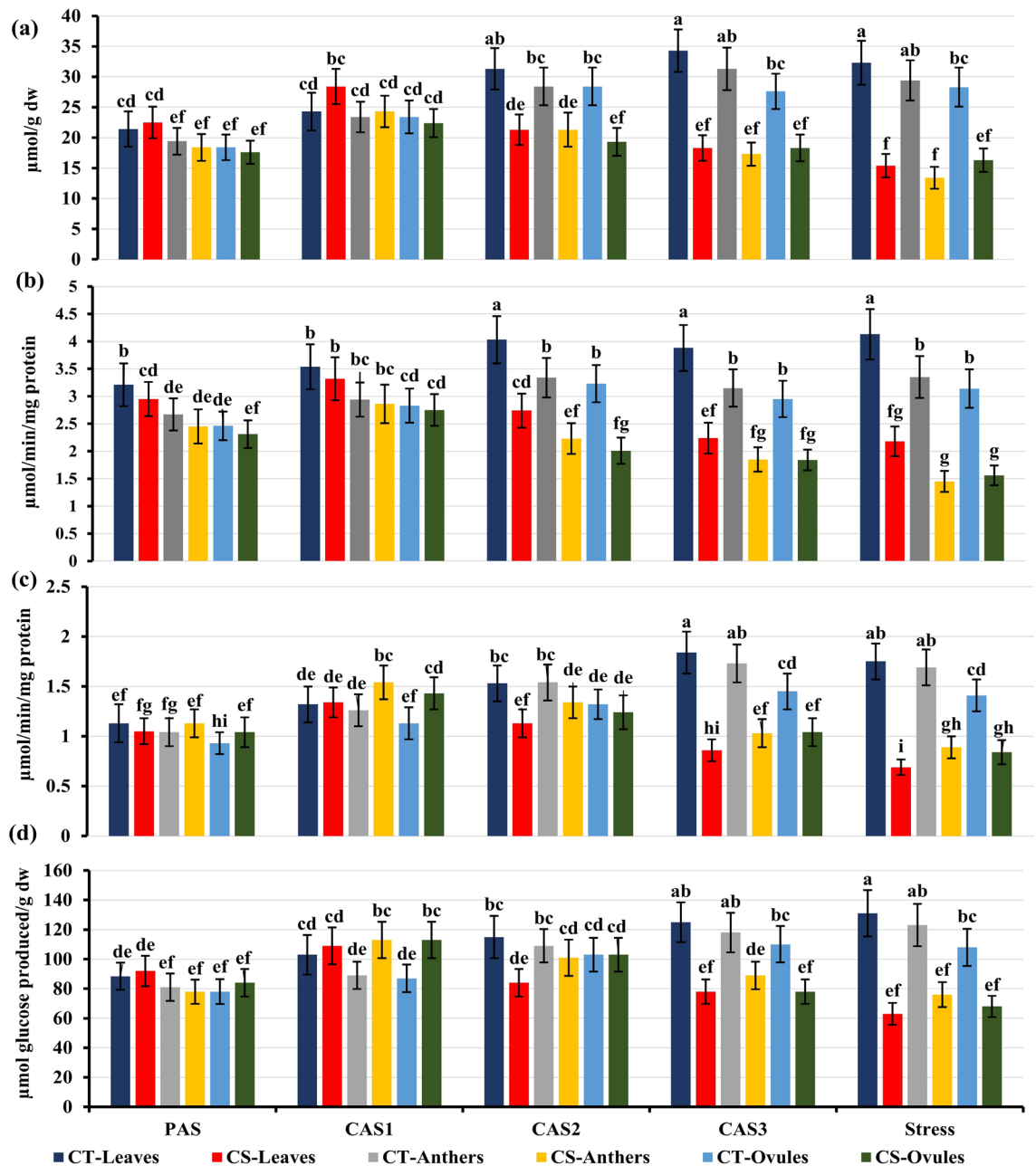


Fig. 6. Sucrose (a), sucrose synthase (b), acid invertase (c), and reducing sugars (d) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean \pm S.E; $n = 3$). Three-way ANOVA was performed to analyze the interaction between genotypes \times treatments \times organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

synthesis and catabolism of osmolytes (proline and glycine betaine), carbohydrates (trehalose and sucrose), and reactive oxygen species (ROS) metabolism during various stages of cold acclimation in the leaves, anthers, and ovules of CT and CS chickpea genotypes. Through a comparative analysis of the physiological responses to cold acclimation and stress in these tissues, we obtained valuable insights into the underlying mechanisms of plant adaptation to low-temperature environments. Our findings elucidate the distinct strategies employed by CT and CS genotypes to mitigate stress-induced damage and maintain cellular homeostasis under cold conditions.

Cold acclimation and stress injury to leaves

Cold acclimation can enhance resilience to cold stress through various mechanisms⁵². To assess the effect of cold acclimation on leaves, we examined specific traits indicative of stress injury to leaves. The maintenance of RLWC

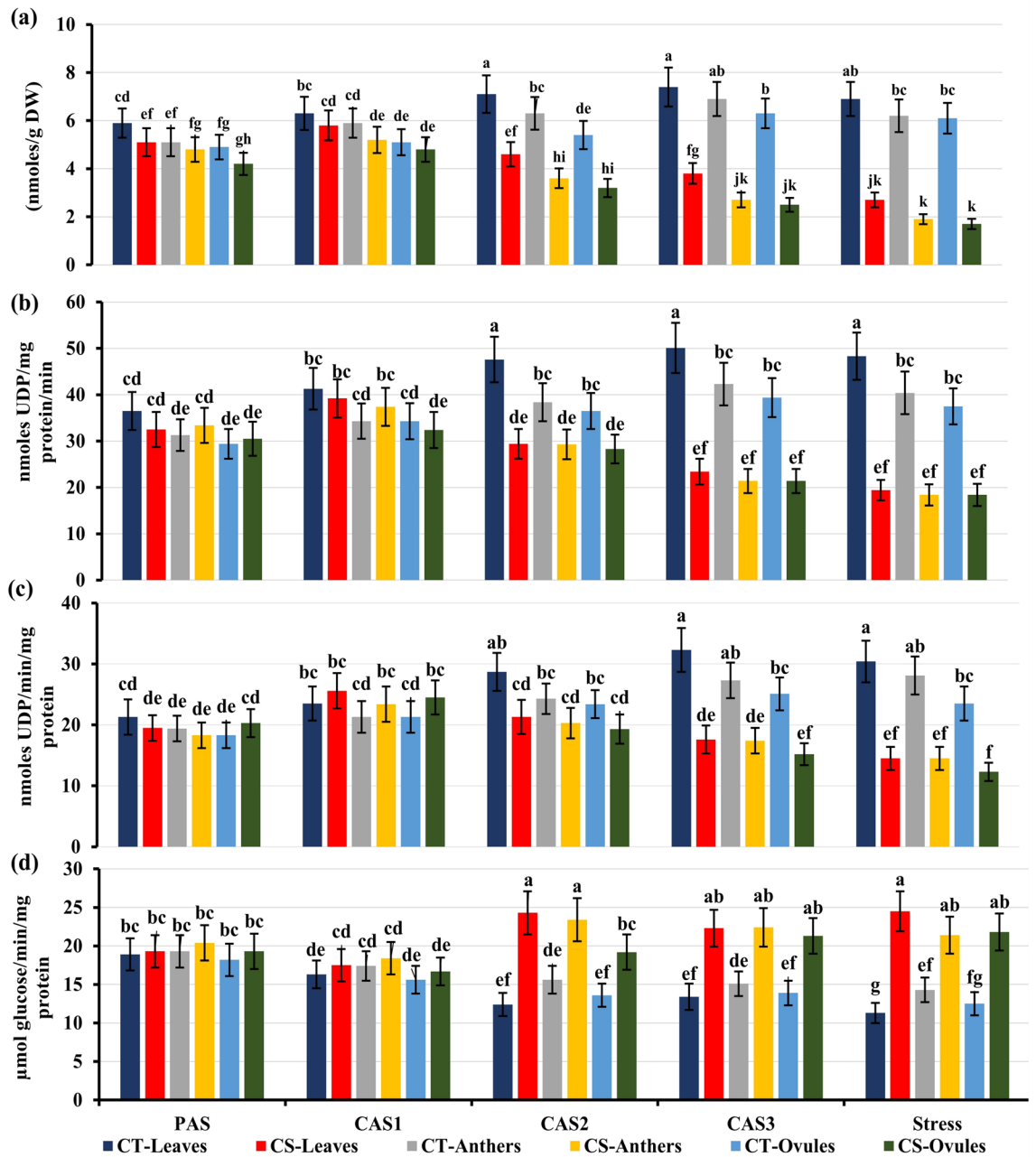


Fig. 7. Trehalose (a), Trehalose phosphate synthase (b), Trehalose phosphate phosphatase (c), and Trehalase (d) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean ± S.E; n = 3). Three-way ANOVA was performed to analyze the interaction between genotypes × treatments × organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

and gS during acclimation in the CT genotype indicated efficient water uptake and stomatal regulation. In contrast, the CS genotype exhibited reduced RLWC and gS during acclimation, suggesting a greater susceptibility to water stress under cold conditions. The ability of the CT genotype to sustain water balance and stomatal function contributes to enhanced tolerance to cold-induced water deficits. Previous studies corroborate that the leaves of cold-acclimated white clover plants subjected to frost maintained a higher relative water content than the non-acclimated plants that exhibited significant dehydration⁵³. Similarly, enhanced leaf water status has been reported in cold-acclimated barley⁵⁴, potentially resulting from improved water transport within the root system and osmolyte accumulation⁵⁵. The observed increase in EL in leaves during cold acclimation emphasizes the vulnerability of cellular membranes to cold-induced stress¹⁰. Simultaneously, cold acclimation in chickpea seedlings improves cold tolerance by altering the fatty acid composition of their cell membranes,

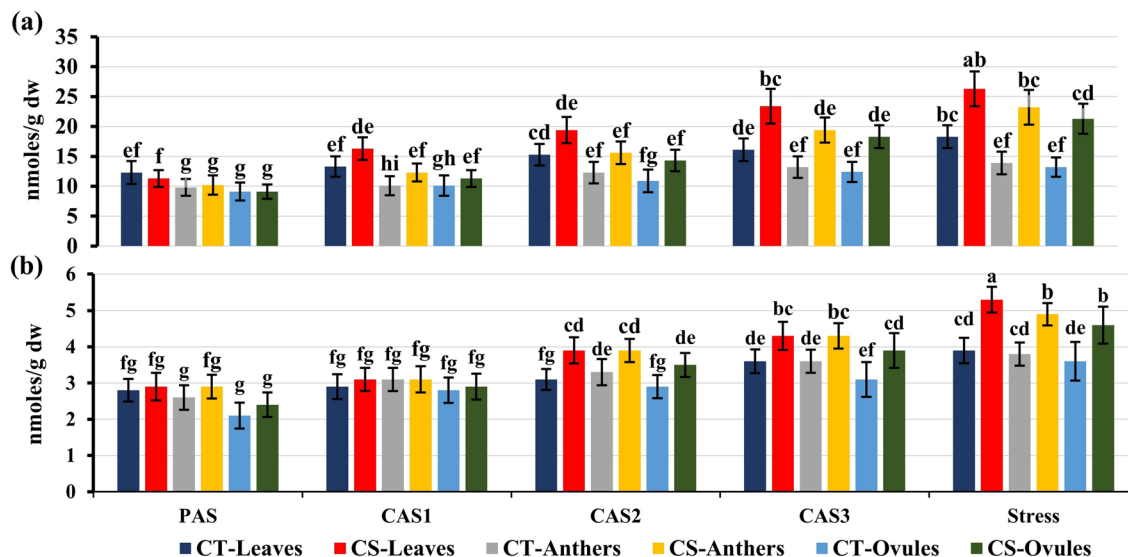


Fig. 8. Malondialdehyde (a) and hydrogen peroxide (b) contents in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean \pm S.E; n = 3). Three-way ANOVA was performed to analyze the interaction between genotypes \times treatments \times organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

increasing the ratio of unsaturated to saturated fatty acids, and enhancing membrane fluidity and resilience during cold stress⁵⁶. Cold acclimation is known to stabilize plant membranes by integrating lipid remodeling, cryoprotectant accumulation, gene expression changes, enzyme regulation, and membrane-based sensing, collectively preserving membrane structure and function to enhance cold tolerance in various legumes and model plants^{15–17,57}. While these mechanisms are well-established, this study primarily focused on enzymes related to Cryoprotectants (osmolytes, sugars, antioxidants), further research is needed to probe the lipid dynamics and gene expression in the studied genotypes. The CT chickpea genotype exhibited superior membrane integrity under cold acclimation, characterized by significantly lower EL than the CS genotype, suggesting more effective mechanisms for preserving membrane integrity and mitigating cellular damage during cold exposure.

Both the CT and CS genotypes exhibited chlorophyll degradation during cold acclimation and stress; however, the CT genotype demonstrated superior chlorophyll retention under cold stress conditions. The reduced chlorophyll loss observed in cold-acclimated chickpea plants may be attributed to improved leaf water status and decreased oxidative damage⁵⁸, which is consistent with previous studies on cold-acclimated chickpea seedlings⁵⁹ and *Arabidopsis thaliana* (C24 accession)⁶⁰ under cold stress. The decrease in chlorophyll fluorescence (CF) during acclimation, particularly in the CS genotype under cold stress, suggests compromised photosynthetic efficiency and potential photoinhibition. Higher CF values in the CT genotype indicated superior photosynthetic performance despite cold stress. Cold acclimation protects CF by enhancing photosynthetic pigments, enzymes, and proteins while reducing photoinhibition and maintaining the redox state of the electron transport chain⁶¹. Carotenoids play a crucial role in maintaining the redox status of leaves and in protecting against photoinhibition under low-temperature stress⁶². Enhanced retention of carotenoids in the CT genotype likely contributes to improved antioxidant defense and protection against oxidative stress during cold exposure⁶³. Thus, cold acclimation plays a critical role in protecting chlorophyll in plants under cold stress by regulating chlorophyll biosynthesis, alleviating photosynthetic inhibition, maintaining chlorophyll and carotenoid concentrations, influencing CF parameters, and modulating translational regulation of plastid-encoded genes. These mechanisms collectively contribute to preserving chlorophyll content and photosynthetic function, ensuring the plant's ability to withstand cold stress⁶¹.

The principal component analysis (PCA) elucidated the correlation between pod set and leaf injury characteristics, including elevated electrolyte leakage, diminished water status, and reduced photosynthetic function. Cold acclimation can reduce these effects by improving the leaf water status, reducing electrolyte leakage, and increasing carotenoid concentrations to improve the performance of cold-stressed plants. These findings corroborated our previous observations regarding the effects of cold acclimation in attenuating leaf damage in cold-tolerant (CT) genotypes, whereas cold-sensitive (CS) genotypes exhibited no response²¹.

Physiological responses to cold acclimation and stress in leaves, anthers, and ovules

Cellular viability

Cellular viability, indicative of cellular respiration, significantly increased across the leaves, anthers, and ovules in the CT genotype during cold acclimation. This increase indicated enhanced metabolic activity and respiratory

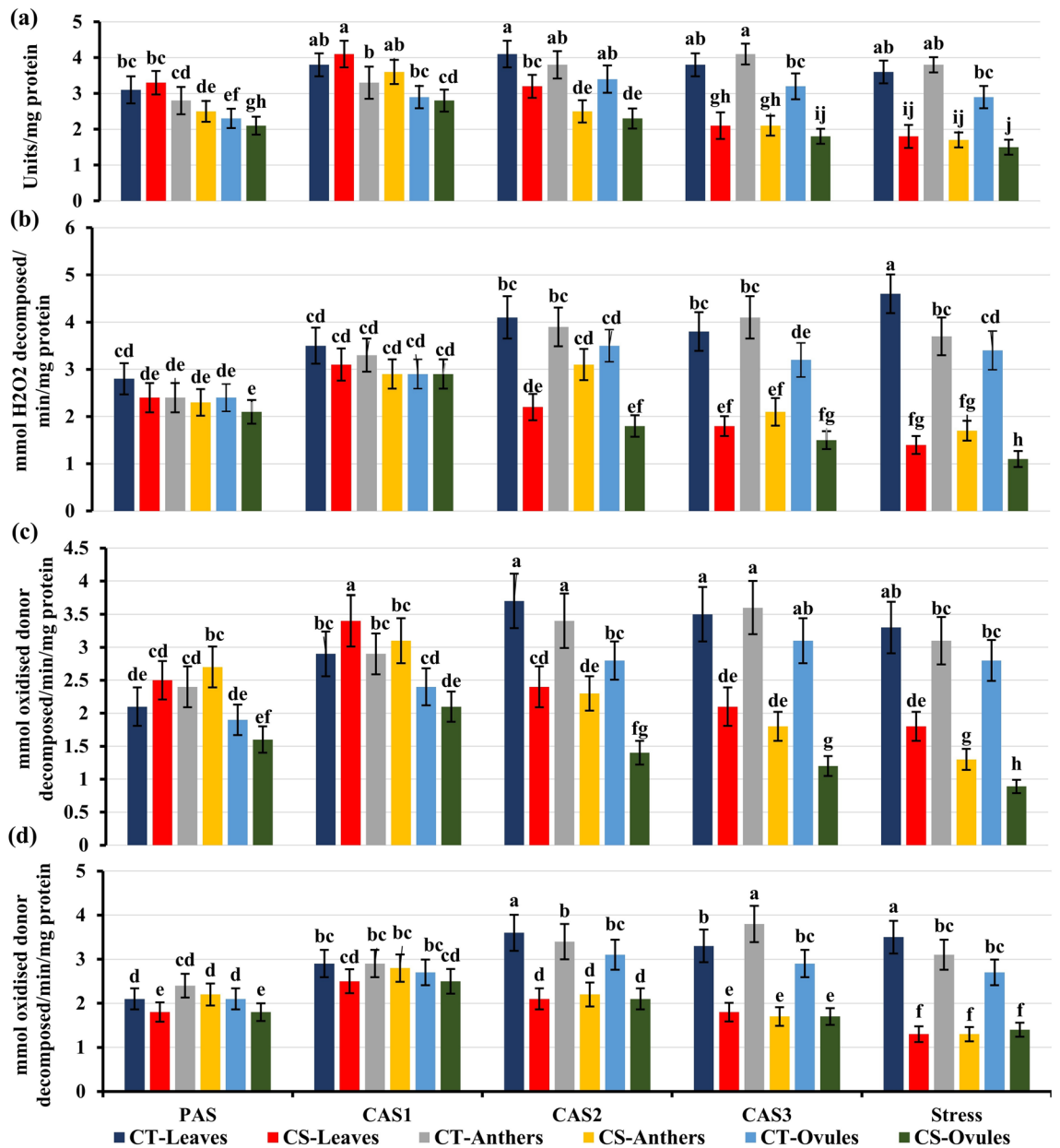


Fig. 9. Superoxide dismutase (a), Catalase (b), Ascorbate peroxidase (c), and Glutathione reductase (d) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean \pm S.E; $n = 3$). Three-way ANOVA was performed to analyze the interaction between genotypes \times treatments \times organs followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages ($P < 0.05$; Tukey test).

capacity in response to cold stress. The upregulation of dehydrogenase activity likely supports the metabolic adjustments necessary to maintain essential cellular functions under low-temperature conditions. In contrast, the CS genotype exhibited only a short-term increase in cellular respiration, suggesting impaired metabolic activity and compromised cellular functions. Cold stress can inhibit cellular respiration by causing structural damage to the mitochondria, disrupting energy flow and enzymatic activity, and ultimately reducing the respiration rate⁶⁴. Cold-tolerant genotypes demonstrate superior temperature homeostasis in respiration and photosynthesis, highlighting their capacity to maintain metabolic activities, even under cold stress⁶⁵.

Osmolytes' metabolism

Osmolytes play a vital role in plant responses to cold stress, functioning as compatible solutes that facilitate the regulation of osmotic balance and protect cells from damage¹⁸. The observed elevation of proline levels

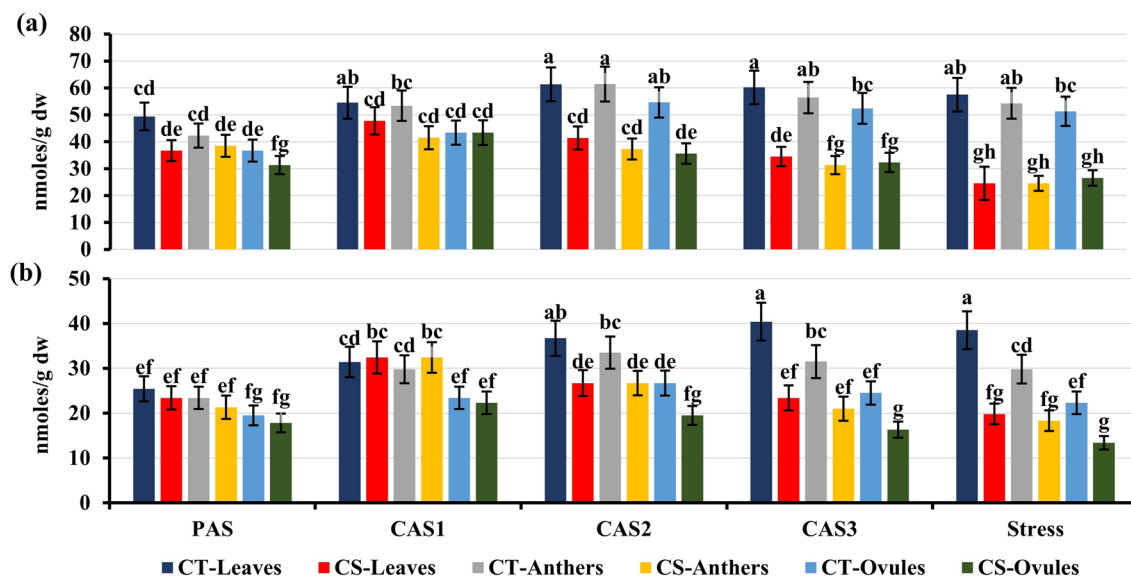


Fig. 10. Ascorbic acid (a) and Reduced glutathione (b) in leaves, anthers, and ovules across different acclimation stages and under cold stress. Stages: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3), and following CS (cold stress) in CT (cold-tolerant) and CS (cold-sensitive) chickpea genotypes. Vertical bars show standard errors (Mean ± S.E; n = 3). Three-way ANOVA was performed to analyze the interaction between genotypes × treatments × organs, followed by the LSD test. Symbols on bars represent significant differences among genotypes and stages (P < 0.05; Tukey test).

Parameters	Genotype	PAS	CAS1	CAS2	CAS3	Stress
Pollen germination	CT	87.3 ± 9.3a	76.5 ± 8.2a	79.5 ± 8.4a	82.4 ± 8.9a	80.6 ± 8.5a
	CS	83.4 ± 8.9a	81.3 ± 8.6a	61.3 ± 5.8b	46.2 ± 5.2c	38.5 ± 5.1d
Pollen viability	CT	82.3 ± 8.6a	81.3 ± 8.5a	79.4 ± 8.3a	76.3 ± 8.2a	72.4 ± 7.6a
	CS	83.4 ± 8.6a	76.3 ± 8.1a	70.3 ± 7.6a	51.3 ± 5.9b	45.7 ± 5.1b
Stigma receptivity	CT	4.1 ± 0.48a	4.3 ± 0.47a	3.9 ± 0.31b	3.5 ± 0.24b	3.1 ± 0.21c
	CS	3.8 ± 0.24b	3.1 ± 0.21c	2.6 ± 0.14d	1.8 ± 0.13e	1.1 ± 0.09f
Ovule viability	CT	4.5 ± 0.48a	4.1 ± 0.43a	3.8 ± 0.21a	3.5 ± 0.21a	3.2 ± 0.26a
	CS	4.1 ± 0.22a	3.8 ± 0.21a	3.2 ± 0.23a	2.6 ± 0.19b	2.1 ± 0.14c
Pod set%	CT	-	-	-	-	61.3 ± 7.8
	CS	-	-	-	-	0
Pod number plant ⁻¹	CT	-	-	-	-	8.4 ± 0.91
	CS	-	-	-	-	0
Seed weight plant ⁻¹	CT	-	-	-	-	2.45 ± 0.28
	CS	-	-	-	-	0

Table 2. Effect of cold acclimation succeeding cold stress on yield and reproductive parameters in contrasting chickpea genotypes. Different small letters with values indicate significant differences from each other (P < 0.05; Tukey’s test). Treatments: PAS (pre-acclimation stage), CAS1 (cold acclimation stage 1), CAS2 (cold acclimation stage 2), CAS3 (cold acclimation stage 3). Genotypes: cold tolerant (CT), cold sensitive (CS).

in cold-stressed CT organs highlights its function as a compatible solute, contributing to osmotic adjustment and cellular protection under cold stress conditions^{66,67}. The differential regulation of proline metabolism enzymes further elucidates the genotype-specific responses to cold stress. The increased activity of P5CS, which is responsible for proline synthesis, in the CT genotype indicates enhanced proline biosynthesis as an adaptive strategy⁶⁸. Conversely, the CS genotype exhibited limited P5CS activity, which correlated with reduced proline accumulation and compromised stress tolerance. Additionally, the decreased PDH activity in organs of CT genotype suggests a mechanism for proline retention, contributing to enhanced stress resilience⁶⁹.

During acclimation, GB levels increased significantly in all organs of the CT chickpea genotype, emphasizing the role of GB as a compatible solute for osmotic adjustment and protection against cold stress⁷⁰. In contrast, the CS chickpea genotype exhibited a short-term increase, followed by a reduction in GB levels, indicating

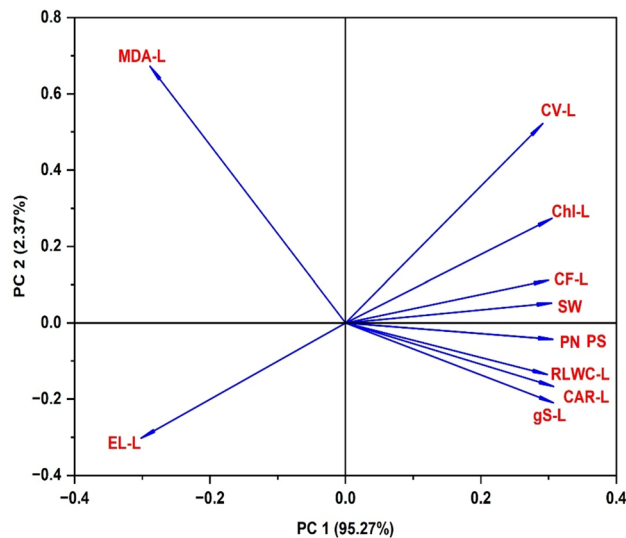


Fig. 11. Principal component analysis of leaf injury and yield traits in chickpea genotypes under cold stress. *L* leaves, *MDA* malonaldehyde, *EL* electrolyte leakage, *Chl* chlorophyll content, *CF* chlorophyll fluorescence, *RLWC* relative leaf water content, *gS* stomatal conductance, *CV* cellular viability, *CAR* carotenoids, *PS* pod set%, *PN* pod number plant⁻¹, *SW* seed weight plant⁻¹.

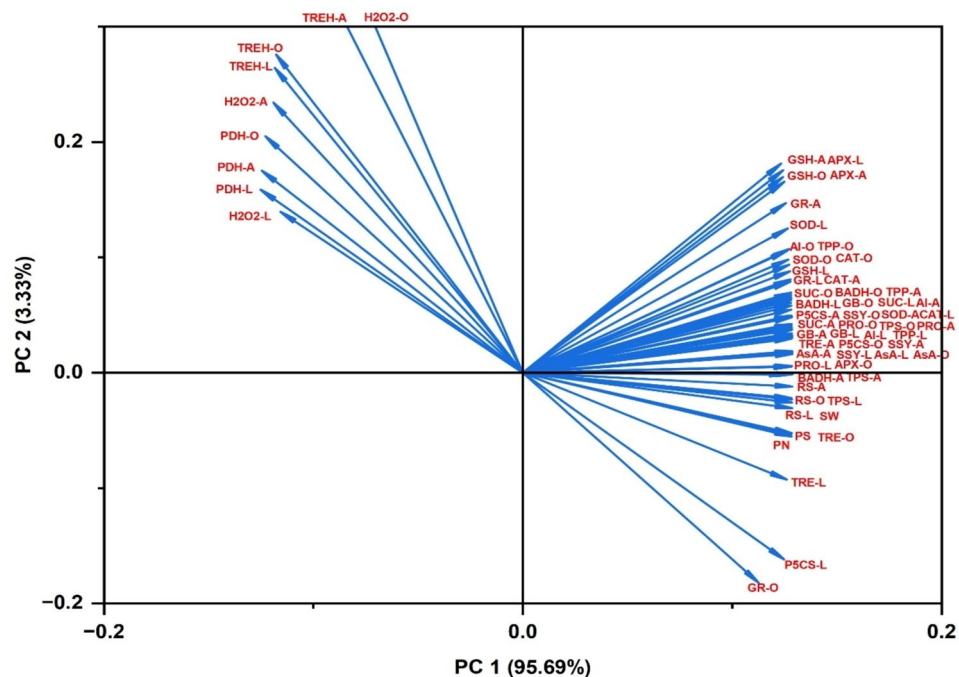


Fig. 12. Principal component analysis of biochemical and yield traits in chickpea genotypes under cold stress conditions. *L* leaves, *A* anthers, *O* ovules, H_2O_2 hydrogen peroxide, *TREH* trehalase, *PDH* proline dehydrogenase, *GSH* reduced glutathione, *APX* ascorbate peroxidase, *GR* glutathione reductase, *SOD* superoxide dismutase, *TPP* trehalose phosphate phosphatase, *AI* acid invertase, *CAT* catalase, *SUC* sucrose, *BADH* betaine aldehyde dehydrogenase, *GB* glycine betaine, *SSY* sucrose synthase, *P5CS* pyrroline-5-carboxylate synthase, *Pro* proline, *TPS* trehalose phosphate synthase, *RS* reducing sugars, *AsA* ascorbic acid, *TRE* trehalose, *SW* seed weight plant⁻¹, *PN* pod numberplant⁻¹, *PS* pod set%.

impaired synthesis or utilization pathways under prolonged cold exposure. During acclimation, the *BADH* enzyme, which is responsible for *GB* synthesis, demonstrated high activity in all organs of the *CT* genotype, indicating efficient *GB* accumulation. Conversely, the *CS* genotype exhibited decreased *BADH* activity, aligning with reduced *GB* levels, and compromised stress tolerance. Increased *BADH* activity in the cold-stressed *CT*

genotype sustains GB synthesis, contributing to enhanced osmotic regulation and stress resilience. Proline and glycine betaine enhance cold stress tolerance in plants through osmotic regulation and membrane stabilization. These compounds function as non-enzymatic antioxidants, scavenge ROS, and prevent oxidative damage under cold stress^{71,72}. Furthermore, they can act as signaling molecules, triggering the activation of stress response pathways and the expression of genes involved in cold tolerance^{71,72}. The superior performance of the CT genotype in proline and glycine betaine metabolism reflects its adaptive strategies for coping with cold stress.

Sugar metabolism

Cold stress significantly influences sugar metabolism and substantially impacts plant stress tolerance and adaptation¹⁹. During cold acclimation, the elevated trehalose content in the CT genotype indicates its function as a compatible solute that contributes to osmotic regulation and stress resilience⁷³. Furthermore, TPS, which is responsible for trehalose synthesis, exhibited increased activity in the CT genotype during acclimation, thereby facilitating enhanced trehalose production and cold tolerance⁷⁴. In contrast, the CS genotype demonstrated decreased TPS activity, corresponding with diminished trehalose accumulation and compromised stress tolerance. Similarly, TPP (trehalose 6-phosphate phosphatase) activity increased in the CT genotype during cold acclimation, suggesting efficient trehalose metabolism, whereas the decrease in CS genotype organs indicated impaired trehalose utilization, further compromising stress adaptation. The CT genotype exhibited reduced trehalase activity during cold acclimation, supporting trehalose retention, whereas the CS genotype demonstrated high trehalase activity, which correlated with reduced trehalose levels and compromised stress tolerance. Trehalose functions as a signaling molecule that regulates gene expression under cold conditions; it initiates the upregulation of specific stress-responsive genes that play a role in cold tolerance, such as those associated with antioxidant defense, membrane stability, and osmoprotectant synthesis to enhance the capacity of plants to endure low temperatures. Often, this regulation occurs through interactions with signaling pathways that involve hormones and transcription factors, resulting in alterations in gene expression at the transcriptional level⁷³.

Sucrose accumulation increased during cold acclimation in both genotypes; however, the CT genotype maintained higher sucrose levels, indicating enhanced sucrose metabolism. Sucrose synthase activity increased significantly in CT organs, facilitating sucrose production and contributing to stress tolerance. In contrast, the CS genotype exhibited a brief increase in sucrose synthase activity, suggesting impaired sucrose metabolism and compromised adaptation to stress. The significant increase in AI activity in CT organs during cold acclimation supports an enhanced sucrose breakdown for energy production and stress responses. Conversely, the CS genotype exhibited decreased AI activity, correlating with reduced sucrose utilization and compromised stress resilience. Reducing sugars increased markedly during cold acclimation in the CT genotype, signifying enhanced carbohydrate mobilization under stress conditions. In contrast, the CS genotype exhibited a short elevation, followed by a significant reduction, indicating impaired sugar utilization and compromised stress adaptation. Sucrose accumulation confers cold tolerance in plants by acting as an osmoprotectant, regulating metabolism and signaling, facilitating transport and accumulation, and interacting with hormones^{19,75}. Overexpression of sucrose synthase genes, such as *SUS3*, enhances cold tolerance in tomatoes⁷⁶. Sucrose metabolism is also involved in the regulation of carbon partitioning during cold acclimation, with limitations in sucrose biosynthesis shaping carbon partitioning and influencing cold tolerance^{77,78}. In the perennial ryegrass, sucrose metabolism is crucial for winter hardiness⁷⁹. Additionally, cold acclimation affects sugar metabolism and sugar-related gene expression in tea plants during winter, highlighting the importance of sucrose metabolism in plant cold tolerance⁸⁰. Overall, the integrative analysis of sugar metabolism and enzymatic activities elucidates genotype-specific adaptations to cold stress in chickpea. The CT genotype's enhanced ability to metabolize trehalose and sucrose contributes to its improved osmotic regulation and resistance to stress.

PCA revealed the significance of biochemical traits, encompassing enzymatic activities, sugars, and osmolytes, in preserving cellular function and diminishing cold-induced damage. Furthermore, yield-related characteristics such as pod formation, pod quantity, and seed mass were strongly associated with these biochemical attributes. Thus, cold acclimation can augment both biochemical and yield traits by alleviating cold-induced damage and enhancing cellular function in chickpea.

ROS metabolism

Oxidative stress and antioxidants are critical components of the plant response to cold stress. Cold stress can disrupt mitochondrial structure and function, leading to increased ROS accumulation and photosynthetic inhibition, which reduce energy flow and trigger ROS formation^{64,81}. Cold-stressed plants manage ROS by activating diverse enzymatic and non-enzymatic antioxidants⁸². The observed increases in enzymatic antioxidants, including SOD, CAT, APX, and GR, reflect essential strategies employed by the CT genotype to counteract oxidative stress during cold acclimation and stress. The ability of the CT genotype to maintain high SOD activity under cold stress indicates its superior ROS-scavenging capacity compared to the CS genotype. Similarly, CAT and APX activities were significantly elevated in the CT genotype across all organs during cold acclimation and stress, demonstrating efficient H₂O₂ detoxification. In contrast, fluctuating CAT and APX activity patterns in the CS genotype suggest compromised antioxidant defense mechanisms under prolonged cold stress. Glutathione reductase activity remained consistently high in the CT genotype, emphasizing its role in maintaining reduced glutathione levels and redox homeostasis during cold acclimation and stress.

Non-enzymatic antioxidants such as ascorbic acid and reduced glutathione (GSH) play crucial roles in scavenging ROS and protecting cellular components from oxidative damage. The CT chickpea genotype exhibited a marked increase in ascorbic acid levels, which remained elevated throughout the acclimation period, demonstrating its potent antioxidant capacity. Conversely, the CS chickpea genotype displayed transient increases in ascorbic acid, followed by reductions, indicative of impaired antioxidant defenses. Furthermore,

GSH levels were significantly elevated in the CT genotype during acclimation, in contrast to the inability of the CS genotype to maintain elevated GSH levels under cold stress. The observed differences in GSH content indicate the capacity of the CT genotype to maintain cellular redox balance and mitigate oxidative stress during prolonged cold exposure. Cold acclimation plays a crucial role in preventing oxidative effects in plants by increasing antioxidant enzyme activities, reducing ROS accumulation, maintaining membrane integrity, upregulating antioxidant enzyme genes, and increasing the levels of antioxidant compounds⁸³. Previous studies have reported that the cold-tolerant genotypes of various crops exhibit increased antioxidant levels, which can effectively prevent oxidative damage⁸⁴.

Increased antioxidative capacity in CT genotypes during cold acclimation is a multifaceted process involving the accumulation of osmoprotectants, maintenance of enzymatic activity, differential gene expression, membrane restructuring, and accumulation of protective proteins⁸⁴, which together contribute to improved cold tolerance and stress resilience. Cold-sensitive genotypes may not undergo the same changes in gene expression, protein activity, and metabolic pathways as CT genotypes during cold acclimation, limiting their ability to enhance their antioxidative capacity⁸⁵.

The interplay between antioxidants and osmolytes is crucial for cold adaptation, involving reciprocal regulation mechanisms. For instance, Glutathione enhances osmolyte synthesis by activating betaine aldehyde dehydrogenase (BADH) via redox-sensitive thiol groups, while ascorbic acid stabilizes osmolyte precursors through redox balance^{86–88}. Conversely, osmolytes like glycine betaine and proline protect antioxidant enzymes (e.g., superoxide dismutase, catalase) and cellular structures, limiting ROS propagation⁸⁹ (Sharma et al., 2019). This bidirectional relationship ensures stress resilience through feedback regulation (GSH activating BADH and betaine inducing glutathione peroxidase), structural stabilization (osmolytes preserving enzyme integrity), and redox buffering (ascorbic acid-glutathione cycling). These interconnected pathways enable plants to maintain cellular homeostasis under cold stress stress.

Reproductive and yield traits

The impact of cold stress on reproductive characteristics is crucial for plant survival and successful reproduction. Under cold stress conditions, the CT chickpea genotype demonstrated notably higher pollen viability and germination rates than the CS genotype, which are vital for fertilization and reproductive success. Several physiological factors, including membrane stability, enzyme activity, and carbohydrate metabolism²¹, likely contribute to these differences between the genotypes. Stigma receptivity, which is essential for pollen recognition and adhesion during pollination, was more favorable in the CT genotype under cold stress, facilitating successful pollination. Additionally, the CT genotype exhibited higher ovule viability under cold stress, indicating an enhanced reproductive potential and seed set capacity compared to the CS genotype. Cold tolerance in plants is closely linked to their ability to maintain reproductive function under cold stress, which is determined by factors such as the capacity of reproductive organs to withstand cold-induced damage through antioxidant and cryoprotective mechanisms^{21,90}. The superior performance of the CT genotype in these reproductive traits demonstrates its adaptive strategies for ensuring reproductive success under cold stress conditions. These adaptive strategies likely involve enhanced antioxidant defense, membrane stability, and metabolic adjustments that support pollen and ovule viability, contributing to a higher seed set and overall yield in cold environments.

Summary and conclusions

The study investigated the distinct physiological and biochemical changes that occur during cold acclimation in CT and CS chickpea genotypes, highlighting how these changes enable CT genotypes to withstand cold stress more effectively than CS genotypes. This study demonstrated that cold acclimation resulted in the upregulation of enzymes involved in the biosynthesis of osmolytes (such as proline and glycine betaine) and sugars (including trehalose, sucrose, and reducing sugars) in cold-tolerant chickpea genotypes, while reducing their catabolism, in contrast to cold-sensitive genotypes. Consequently, the leaves, anthers, and ovules of CT genotypes accumulated these molecules to a significantly greater extent than those of cold-sensitive (CS) genotypes, potentially contributing to reduced damage to various physiological functions. Furthermore, antioxidant defenses were more strongly upregulated in the CT genotypes than in the CS genotypes, which contributed to minimizing cellular damage in CT organs during acclimation and cold stress. The enhanced reproductive efficiency observed in the CT genotype may also be associated with its superior acclimation capacity, providing protection for the development and function of reproductive organs under cold stress. The greater cold tolerance of the CT genotype can be attributed to its genetic predisposition and effective stress defense mechanisms, in contrast to the CS genotypes. Variations in acclimation responses highlight the critical role of synchronized physiological and metabolic adjustments in conferring cold tolerance in chickpea plants. The acclimation capability of the CT genotype emphasizes the importance of understanding and utilizing genetic and physiological processes that contribute to cold tolerance. Although controlled environments cannot fully replicate field conditions, the gradual temperature reduction strategy employed in this study offers a valuable framework for elucidating the fundamental mechanisms of cold acclimation in chickpeas. Insights gained from these metabolic mechanisms involving several enzymes and cryoprotectants can assist the breeding strategies aimed at developing cold-tolerant chickpea varieties with enhanced reproductive resilience and improved yield potential in low-temperature environments. Future research involving transcriptomic analyses and field-based validation will be essential to further refine breeding and genetic engineering strategies for improving cold tolerance in chickpea.

Data availability

All data generated or analyzed during this study are included in this published article. The raw data supporting the conclusions of this article will be made available by the corresponding authors HN and UCJ.

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Author contributions

DP, SK, AR, UCJ: experimental work, UCJ and HN: supervision and original draft, SK, KDS: Statistical analysis, PV, SKP, KHMS: Review and Editing.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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