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Assessing cold stress resilience in wild chickpea accessions using physiological, biochemical, and reproductive traits

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Domesticated chickpea *(Cicer arietinum* L.) exhibits high sensitivity to temperatures below 20/10 °C during its reproductive phase resulting in substantial loss of flowers, pods and crop yields. With the aim to add new sources of cold tolerance and elucidate mechanism of cold-tolerance in wild species of chickpea, the present study evaluated 36 wild accessions of three *Cicer* species *(Cicer judaicum*, *Cicer pinnatifidum*, *Cicer reticulatum*) at the reproductive stage for yield, and reproductive, physiological and biochemical traits under cold stress (15/7 °C) for two consecutive years. Cluster analysis based on yield-related traits such as pod number, seed weight, and total seed count categorized these accessions as cold-tolerant and cold-sensitive. Six *C. judaicum* accessions (ILWC 256, ICC 13852, ILWC 263, ILWC 20, ILWC 223, and ILWC 30) were tolerant to cold whereas the remaining ones were cold-sensitive. Under cold stress, cold-tolerant accessions exhibited lower impairment of physiological processes as compared to the cold-sensitive accessions e.g. lower tissue damage and electrolyte leakage, and higher chlorophyll content, carotenoid content, chlorophyll fluorescence, and leaf water content, thereby resulting in higher photosynthetic efficiency and carbohydrate accumulation in cold-tolerant accessions. At the biochemical level, the tolerant accessions demonstrated significantly higher amounts of cryoprotectants and enhanced activities of enzymatic and non-enzymatic antioxidants resulting in substantially lower levels of reactive oxygen species. Cold-tolerant accessions also accumulated more proline and trehalose compared to their sensitive counterparts. Slight disruptions in physiological processes, low oxidative stress and accumulation of cryoprotectants under cold stress were associated with higher pollen viability, pollen germination, pollen load, ovule receptivity, pod set, number of pods and seed yield in cold-tolerant accessions while opposite was true for cold-sensitive accessions. The wild chickpea accessions exhibiting high seed yield under cold stress are promising candidates for breeding programs aimed at cold tolerance.

Keywords Wild chickpea, Cold stress, Low temperature, Screening, Tolerance, Reproductive success

Chickpea (*Cicer arietinum* L.), a crucial cool-season legume crop, plays a significant role in global food security, particularly in developing nations, owing to its high protein and dietary fiber content[1]. However, its global production faces substantial challenges owing to abiotic stresses, with cold stress emerging as a major constraint[2,3]. Exposure to low temperatures during critical growth stages, such as germination, seedling establishment, flowering, and pod set, can significantly impact chickpea by impeding these processes, ultimately leading to yield reduction[4]. This situation is particularly pronounced in winter-sown chickpeas in regions experiencing unpredictable spring frosts or those with cooler growing seasons, such as the Mediterranean and parts of South Asia[5].

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Cold stress in chickpea causes aberrations at different organizational levels, such as decreased growth at vegetative and reproductive stages, delayed phenology, increased chlorosis and necrosis in the leaves, altered hydration status, abnormalities in the flowers, and harm to the reproductive structures and ultimately lowered yield[5,6]. Cold stress causes flower abortion in chickpea accessions that are susceptible to cold at all anther developmental stages, including gametogenesis, micro-or mega-sporogenesis, and the mature pollen stage[5]. The flower abortion in chickpea is caused either by disruption of gametogenesis or abnormal pollen/ovule development that leads to sterility[5]. Cold stress also reduces the capacity of pollen grains to germinate in flowers and slows the formation of pollen tubes, which can result in failure or lack of fertilization, leading to reduced pod set and fewer seeds per pod[5,6].

Given these challenges, the development of chickpea cultivars with enhanced cold tolerance is crucial to ensure global food security and expand chickpea production into new areas. Although breeding efforts have focused on improving cultivated chickpea varieties, these cultivars often exhibit limited cold tolerance[7]. This limitation necessitates the exploration of alternative genetic sources for cold tolerance genes. Wild *Cicer* species offer a promising solution due to their broader genetic diversity compared to domesticated chickpea[8–10].This genetic richness translates into a wider range of adaptations, including greater tolerance to harsh environmental conditions such as cold stress[11].Berger et al.[2] demonstrated that cultivated chickpea lacks meaningful chilling tolerance, even among genotypes specifically selected for cold resilience (e.g., ICCVs 88501 and 88503, CTS 60543, Rupali, and Sonali). This vulnerability is likely the result of domestication-driven selection, where *C. arietinum* adapted to avoid winter conditions, while its wild relatives evolved under colder environments[12]. As a consequence, modern chickpea has lost much of its vegetative and reproductive cold tolerance[2,7].

Recent studies further show that some wild *Cicer* species can withstand freezing temperatures that would be lethal to cultivated types[9]. Among them, *C. pinnatifidum*, *C. judaicum*, and *C. reticulatum* are particularly promising due to their natural distribution in colder, high-altitude regions[13,14]. These environments likely imposed selection pressures favouring cold-adaptive traits, making these species ideal candidates for investigating cold tolerance mechanisms. Understanding their adaptive responses could provide valuable insights for breeding programs targeting improved cold resilience in chickpea.

In this study, we assessed cold stress responses in 36 wild chickpea accessions representing *C. reticulatum*, *C. pinnatifidum*, and *C. judaicum*. We employed a multi-trait approach under controlled cold stress conditions (15/7 °C), integrating physiological, biochemical, and reproductive responses. Specifically, we evaluated physiological traits (photosynthetic efficiency, membrane stability, chlorophyll retention), biochemical traits (antioxidant enzyme activities—superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase), osmolyte accumulation (proline, trehalose), and carbohydrate content. Reproductive traits included pollen viability, ovule receptivity, pod set, and seed number. These measurements enabled the development of comprehensive trait profiles to dissect cold tolerance variation across species and accessions. Unlike earlier studies focused primarily on field-based phenological screening[2] or transcriptomic and QTL analyses under freezing stress[9]our study provides detailed trait-level characterization of cold stress responses during the reproductive phase. This phenotypic approach fills a key gap in the literature by elucidating physiological and biochemical mechanisms linked to cold resilience.

We hypothesized that accessions of *C. reticulatum*, *C. pinnatifidum*, and *C. judaicum* would exhibit significant variation in cold tolerance, with some demonstrating superior adaptive responses. This hypothesis is supported by ecological and genetic evidence suggesting that domesticated chickpea has lost much of its cold resilience due to domestication reasons, while certain wild species have retained greater stress adaptability[2]. Given their origin in colder environments, we expected that these wild accessions would show measurable advantages in physiological, biochemical, and reproductive traits under cold stress[5,9,15]. Identifying such traits will not only enhance our understanding of cold tolerance mechanisms but also support future breeding strategies aimed at developing more resilient chickpea cultivars with broader environmental adaptability and improved food security potential.

Materials and methods

Field and growth chamber experiments

Raising of plants and cold stress treatment

Wild chickpea seeds, comprising thirty-six (36) accessions (Table 1), were sourced from the NBPGR (National Bureau of Plant Genetic Resources Regional Station), Phagli, Shimla, Himachal Pradesh, India. The selection of these genotypes was based on (i) representation of three major wild species—*Cicer judaicum*, *C. pinnatifidum*, and *C. reticulatum*—that are phylogenetically close to cultivated chickpea and are known to inhabit diverse and often harsh ecological niches; (ii) preliminary reports (unpublished) indicated possible resilience to abiotic stresses; and (iii) genetic diversity to ensure a broad range of responses to cold stress. This approach was intended to capture inter- and intra-specific variation in cold stress response and to identify potential donors of cold-tolerant traits for chickpea improvement programs. This has been included in the "Materials and methods" Section. Prior to sowing, the seeds were vernalized at 4 °C for one month. Subsequently, the seeds were implanted with *Mesorhizobium* sp. at a proportion of 1.95 g/kg of seeds. The plants were raised in earthen pots with a soil capacity of 4 kg, filled with a 3:1 sandy loam and sand mixture. Additionally, a portion of farmyard manure was mixed with three times the amount of the soil-sand mixture. Five seeds were sown per pot, and once they emerged, the plants were reduced to three per pot to ensure optimal growth. A completely randomized design was followed, with four pots per accession. These accessions were tested for cold tolerance under outdoor conditions as well as in a controlled growth chamber at the Department of Botany, Panjab University, Chandigarh, India. The plants were irrigated as required. Regular temperature measurements (both max and min) and relative humidity were recorded from the sowing date to the bud stage. To evaluate the effects of cold stress, plants were initially raised under outdoor conditions with a light intensity of 1,300–1,500 µmol m^{-2} s^{-1} and 60–70% relative humidity

Accession	Cicer species	Accession	Cicer species
1. ILWC 4	*Cicer judaicum*	19. ILWC 274	*Cicer judaicum*
2. ILWC 20	*Cicer judaicum*	20. ILWC 275	*Cicer judaicum*
3. ILWC 30	*Cicer judaicum*	21. ILWC 278	*Cicer judaicum*
4. ILWC 31	*Cicer judaicum*	22. ILWC 283	*Cicer judaicum*
5. ILWC 38	*Cicer judaicum*	23. ICC 13852	*Cicer judaicum*
6. ILWC 45	*Cicer judaicum*	24. ICC 17150	*Cicer judaicum*
7. ILWC 46	*Cicer judaicum*	25. ILWC 9	*Cicer pinnatifidum*
8. ILWC 48	*Cicer judaicum*	26. ILWC 22	*Cicer pinnatifidum*
9. ILWC 50	*Cicer judaicum*	27. ILWC 29	*Cicer pinnatifidum*
10. ICC 182	*Cicer judaicum*	28. ILWC 51	*Cicer pinnatifidum*
11. ICC 185	*Cicer judaicum*	29. ILWC 236	*Cicer pinnatifidum*
12. ILWC 207	*Cicer judaicum*	30. ILWC 263	*Cicer pinnatifidum*
13. ILWC 211	*Cicer judaicum*	31. ILWC 289	*Cicer pinnatifidum*
14. ILWC 223	*Cicer judaicum*	32. IG 135444	*Cicer pinnatifidum*
15. ILWC 229	*Cicer judaicum*	33. IG 136820	*Cicer pinnatifidum*
16. ILWC 256	*Cicer judaicum*	34. ILWC 36	*Cicer reticulatum*
17. ILWC 263	*Cicer judaicum*	35. ILWC 46	*Cicer reticulatum*
18. ILWC 273	*Cicer judaicum*	36. ILWC 229	*Cicer reticulatum*

Table 1. List of 36 wild chickpea accessions.

(Fig. 1) until the completion of the vegetative stage. At the commencement of the bud stage, according to the phenology and stage of the accessions, plants were transferred to a walk-in-growth chamber. The plants were gradually acclimatized to low temperatures of 15/7 °C (12 h. of daylight/12 hrs. of darkness; 600 µmol m^{-2} s^{-1} light intensity and 65–70% relative humidity) to full growth for exposure to cold stress. The diurnal temperature gradient was reduced by 1 °C per day until the target temperature (15/7 °C) was reached, maintained through podding (15 d), and subsequently increased by 2 °C per day to attain 30/23 °C (12 h light/12 h dark) until maturity, to simulate the field recovery conditions. A non-stressed control group was included in the study. For each of the 36 accessions, plants were grown under standard optimal temperature conditions (23/15 °C) for 15 days under the same light duration, relative humidity (RH), and time conditions. 'The 15-day time point was chosen based on our previous observations that indicated cold stress effects on reproductive and physiological traits typically manifest within 10–15 days of exposure. This duration aligns with key reproductive stages in chickpea, including pollen development, stigma receptivity, and pod formation, ensuring that measurements capture peak cold stress effects before recovery mechanisms become dominant'. This has been included in the "Materials and methods" Section. After harvest, the plants were noted for the filled pods, seed count, and total seed weight per plant. The following traits were assessed in the leaves of the plants after 15 days of cold stress.

Stress injury traits
The leaves (from the 2nd and 3rd topmost branches) were assessed for the following traits:

Electrolyte leakage % (as membrane damage): Membrane damage in the tissue was evaluated by quantifying electrolyte leakage (EL). At the outset, freshly picked leaves underwent a cleaning process with DI water (deionized) to get rid of any surface electrolytes. Following this, the cleaned leaf samples were transferred to sealed glass containers, each holding 10 ml of DI water. These containers were then set on a rotary shaker and kept for 12 h at 25 °C. Upon completion of the incubation period, the solution's conductivity (L1) was taken. The prepared samples underwent heating to 120 °C for 20 min in a heated water container and equilibrated at 25 °C. The subsequent conductivity value was remeasured, yielding the final value (L2). Damage was computed as EL (%) = (L1/L2) × 100[16].

Photosynthetic pigments: A SPAD chlorophyll meter (Apogee-SPAD meter) was used to measure the chlorophyll (SPAD value) of a tagged leaf between 10.00 and 11.00 a.m[17]. To determine carotenoid levels, 500 mg of leaf biomass was crushed in 15 mL of 80% acetone solution. The resulting mixture was then passed through the filter paper (Whatman). The filtrate's optical density was measured at wavelengths 440, 645, and 663 nm[18]. The calculation was used to get the carotenoid content (mg g^{-1} FW).

$$\text{Carotenoid content} = 4.69\,(\text{Abs440}) - 0.268\,(20.2\,(\text{Abs645}) + 8.02\,(\text{Abs663}))\,\text{V}/1000 \times \text{W}$$

Stomatal conductance: The leaf stomatal conductance was evaluated at 11.00 am utilizing a handheld SC1 leaf porometer (manufactured by Decagon Devices, Pullman, WA, USA). The measurements were recorded in units of $mmol^{-1}$ m^{-2} $s^{[-1}$[19].

Photosynthetic efficiency: The efficiency of photosynthesis, as implied by Photosystem II activity, was measured by using a chlorophyll fluorescence meter. This assessment was conducted at 11:00 a.m., utilizing the chlorophyll fluorescence technique to evaluate photosynthetic performance. The experiment utilized an OS1-FL-modulated chlorophyll fluorometer (Opti-Sciences, Tyngsboro, MA, USA) to perform a light deprivation test. The uppermost leaves were fitted with clamps to create a light-free environment, effectively stopping the

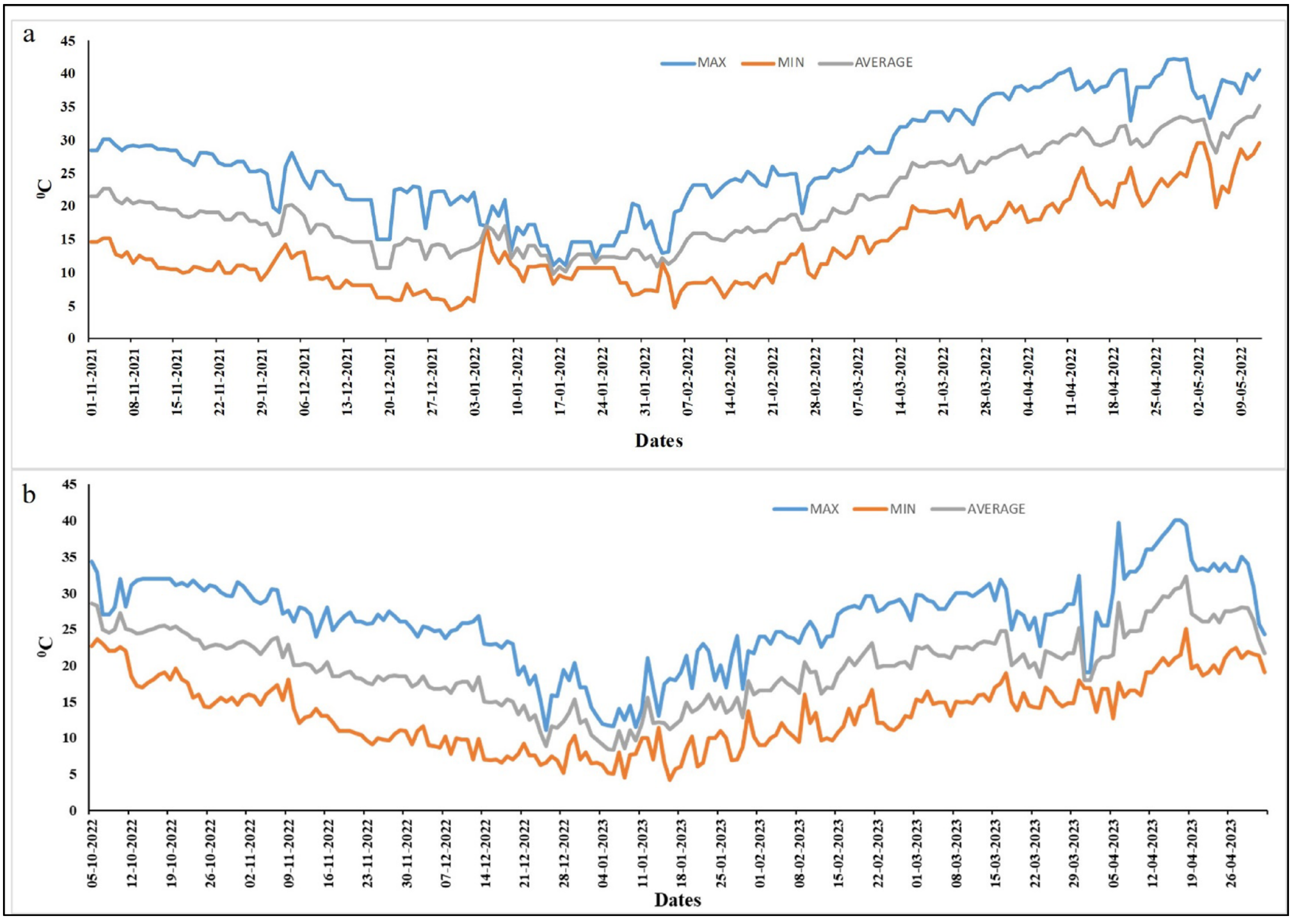

Fig. 1. :Temperature profile (Max., Min., Average) for plants grown in outdoor environment conditions for two consecutive years 2021–22 (**a**) and 2022–2023 (**b**) for cold stress screening.

photosynthetic light reactions for 45 min. Following this period, the instrument's optic fiber was connected to the clamps, and the valves were released. The instrument was activated, emitting modulated light at 695 nm through the optical fiber into the leaves. PE was measured as the Fv/Fm ratio, which indicates the optimum quantum efficiency of PSII photochemistry[19].

Relative leaf water content: To measure Relative Leaf Water Content (RLWC), leaves were harvested from the topmost (2nd and 3rd) branches. Their initial fresh weight was noted before submerging them in Petri dishes filled with distilled water for two hours. Following this immersion, the leaves were extracted, their surfaces dried using blotting paper, and their turgid weight was recorded. The leaves were subsequently transferred to a drying chamber set at 90 °C for 24 h to determine their dry mass. The RLWC was calculated using the methodology outlined by[20].

Reproductive function

Peterson stain treatment method was employed to assess pollen viability. The identification of viable pollen grains was based on the pollen absorption of the dye as well as its size and morphology (spherical or triangular). Pollen grains were gathered from newly dehisced flowers, pooled, and evaluated for their viability[21,22]. The taken-out pollen grains were germinated as per[23] protocol for in-culture pollen germination analysis. With a pH of 6.5, the germination media contained 1.3 mM calcium nitrate, 990 mM potassium nitrate, 812 mM magnesium sulfate, 1.64 mM boric acid, and 10% sucrose. When a pollen grain's tube length exceeded its diameter, it was said to have germinated. Nearly 100 pollen grains/replications were examined to calculate the germination percentage[24].

To measure pollen load, flowers bearing fully exposed anthers and pollen on the stigma were collected. The pollen grain count found on the stigma surface was scored on a range of 1 to 5, where 1 corresponds to a low pollen load and 5 corresponds to a high pollen load[25].

A TTC (2, 3, 5-triphenyl-2 H-tetrazolium chloride) reduction assay was employed to evaluate ovule viability. One day prior to the anthesis, fresh ovules were removed from the ovaries. The ovules were put in a petri dish lined with damp filter paper after being stained with a single droplet of TTC solution (0.5% TTC in 1% sucrose solution) was placed on a sterile glass slide and covered with a cover slip. After that, the dish was covered with black paper and placed in a growth chamber to incubate for 15 min at 25 °C in the dark. Their viability was assessed under a microscope by observing the level of the red color through the biochemical reduction of TTC to formazan, notably in the central region. The red color intensity, which reflected the oxidizing capacity of the cells and indicated ovule viability was scored on a range of 1 to 5, where 1 corresponds to the lowest intensity and 5 corresponds to the highest[24].

Cryoprotective molecules

For the evaluation of proline content in plant tissues, the samples underwent extraction with 3% sulfosalicylic acid, followed by centrifugation at 2150 × g for 20 min at 40 °C. The obtained supernatant was subsequently mixed into an acidic ninhydrin reagent, and the color that resulted was analyzed spectrophotometrically at 520 nm, with toluene as a reference. Proline concentrations were expressed in nmol g^{-1} DW[26].

The method described by[27] was used to measure the trehalose concentrations. Plant tissues were extracted in hot ethanol (80%) and centrifuged for 15 min at 3360× g. Subsequently, 0.1 mL of the supernatant was combined with 2 mL of TCA and analyzed following the protocol described by[28].

Total sugars were determined by homogenizing the harvested tissues in ethanol (80%), being before centrifuged for 10 min at 5000 rpm. The derived upper layer was collected and the process was carried out multiple times with the residue to assure complete sugar extraction. The entire supernatant was used to estimate the total sugars, while the residue was preserved in ethanol (20%) to determine starch content by conducting acid hydrolysis. Total sugars content was assessed by the methodology described by[29]. A 0.1 mL aliquot of ethanol extract was combined to 4 mL of chilled anthrone reagent. The solution was carefully mixed, then heated in boiling water for 10 min, followed by cooling in an ice bath. Using a spectrophotometer, the resulting blue-green liquid's absorbance (optical density) was quantified at 625 nm. The concentration of sugar (mg/g FW) was then determined using a glucose standard curve.

Oxidative stress and antioxidants

To assess malondialdehyde (MDA) content, fresh foliage samples were ground in trichloroacetic acid (0.1% TCA) and subjected to centrifugation for about 5 min at 3360× g. The resultant supernatant obtained was sampled by taking 0.1 mL and combining it with 4 mL of a mixture containing 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. This blend was then subjected to heat treatment at 95 °C for half an hour, after which it was cooled in ice and centrifuged for 10 min at 3360× g (4 °C). Afterward, the absorbance readings of the resulting supernatant were determined at 532 nm. The MDA concentration was then quantified using an extinction coefficient of 155 mM cm^{-1} and reported as nmol g^{-1} DW[30].

The concentration of hydrogen peroxide (H_2O_2) was determined with fresh leaf samples. Chilled 80% acetone (5 mL) was used to extract the samples, which were then collected utilizing filter paper (Whatman). The filtered solution obtained was mixed with 4 mL Ti (titanium) reagent and 25% ammonia solution (5 mL). This combination was subjected to centrifugation at 3360× g for 10 min. Subsequently, the deposits were mixed in 1 M H_2SO_4. The final solution's absorbance was determined at 410 nm. Using an extinction coefficient of 0.28 mmol cm^{-1}, the H_2O_2 concentration was calculated and expressed in nmol $g^{[-1}$[31].

Superoxide dismutase (SOD) activity was evaluated using newly collected tissue extracted in refrigerated PBS (phosphate buffer; 50mM, pH 7.0), afterward centrifuged for 5 min at 3360× g (4 °C). For the assay, a reagent mixture was prepared with a resulting volume of 3mL. This reaction medium consisted of 0.1 mL of extract, 0.05 M PBS (pH 7.8), 0.025 M nitro-blue tetrazolium chloride (NBT), 0.013 M methionine, and 0.1 mM EDTA (ethylene diamine tetra acetic acid). The solution was supplemented with 2 mM riboflavin and subsequently illuminated using fluorescent light (15 W) for a duration of 10 min. The absorbance readings were measured at 560 nm, and the activity level was determined following the method described by[32]with results expressed in units per mg of protein.

To measure Catalase (CAT) Activity, 0.1 mL of enzyme extract (prepared similarly to SOD) was combined with a mixture containing 0.05 M PBS (pH 7.0) and 0.2M H_2O_2. The absorbance readings were monitored at 410 nm for three minutes. The activity level was then calculated through an extinction coefficient of 40 mM cm^{-1} and expressed as mmol H_2O_2 broken down per mg of protein[33].

The measurement of ascorbate peroxidase (APX) activity involved the mixing of enzyme extract (0.1 mL) (prepared for SOD) to an experimental mixture. This mixture contained PBS (0.05 M; pH 7) and ascorbic acid (0.5 mM), with H_2O_2 as a substrate. The extinction coefficient of 2.8 mM cm^{-1} was used to quantify the activity[34] and expressed in terms of mmol oxidized donor decomposed min^{-1} mg^{-1} protein.

The glutathione reductase (GR) activity was evaluated by combining 0.1 mL of enzyme extract (prepared similarly to SOD) with an experimental mixture. This mixture consisted of 0.1 M PBS (1.5 mL; pH 7.6), NADP (0.35 mL), BSA (0.2 mL), and oxidized glutathione (0.1 mL). The activity level was determined by monitoring the drop in absorbance at 340 nm during a 3-minute period. Results were recorded as mmol oxidized donor decomposed min^{-1} mg^{-1} protein, following the method described by[35].

The concentration of ascorbic acid (AsA) was quantified using a method that involved extracting fresh tissue in TCA (6%) and centrifuging for 15 min at 3649.15× g. The filtered supernatant (4 mL) obtained was blended with 2 mL of 2% DNPH (dinitrophenylhydrazine) and one droplet of 10% thiourea. This experimental mixture was placed in a bath apparatus, heated for fifteen min, and cooled to normal temperature. Following this, 5 mL of ice-cooled H_2SO_4 was introduced, and the absorbance readings were measured at 530 nm. The AsA concentration was subsequently calculated using a standard curve and stated as mg g^{-1} DW[31].

To measure the reduced glutathione (GSH) concentration, fresh leaf samples underwent homogenization in metaphosphoric acid (2 mL) and then subjected to centrifugation for 15 min at 3650× g. The filtered supernatant (0.9 mL) was subsequently combined with 10% sodium citrate (0.6 mL). The reaction medium was created by combining extract (0.1 mL), distilled water (0.1 mL), 0.3 mM NADPH (0.7 mL), and 6 mM 5,5-dithio-bis-(2)-nitrobenzoic acid (DTNB; 0.1 mL). Thereafter, glutathione reductase enzyme (0.01 mL) (sourced from Sigma-Aldrich, located in Burlington, Missouri, USA) was introduced, and the optical density was recorded at 412 nm. The GSH quantity was subsequently calculated using a standard curve and expressed as nmol g^{-1} DW[36].

Yield traits

For each accession, measurements of the filled pods, total seed weight (g plant^{-1}), and number of seeds were taken from three samples per replicate. The collected data were combined, and average values were reported.

Experimental design and statistical analysis

The study applied a Completely Randomized Block Design (CRBD) with block arrangements for experimental setup to cultivate plants in both outdoor and growth chamber settings.Each accession was tested using a completely randomized block design (CRBD) with three independent replicates. For physiological and biochemical analyses, each replicate contained a single plant, totalling 3 plants per accession per treatment (cold-stressed and control). In contrast, for measuring yield traits, each replicate comprised three plants, totalling 9 plants per accession per treatment (cold-stressed and control). We used analysis of variance (ANOVA) with the stats package version 4.3.0 in R Studio (RCoreTeam,2023), gvlma package to analyze how accessions, treatment, replication, and genotype-by-treatment interaction affected traits at low temperatures.To investigate the relationships among various parameters, R software was employed to conduct principal component analysis (PCA) using factoextra and factominer packages, perform cluster analysis, and generate Peterson correlation and box plots.

Results

Figure 2 shows the plants grown in outdoor and growth chamber environments, effects of cold stress on leaves (chlorosis, necrosis, anthocyanin accumulation), and contrasting accessions, and Fig. 3 shows the effects of cold stress on flowers, pods, and reproductive organs of contrasting accessions.

Cluster analysis for identification of wild accessions contrasting in cold sensitivity based on yield traits

Cluster analysis was conducted to identify wild accessions with varying levels of cold sensitivity, based on yield traits. In the initial year, yield-based cluster analysis (Fig. 4a) classified 36 wild chickpea accessions into three primary clusters. Cluster I comprised of all cold-tolerant accessions, including *C. judaicum* ILWC 256 (FP-100), ICC 13,852 (FP-57), ILWC 263 (FP-86), ILWC 20 (FP-51), ILWC 30 (FP-54), and ILWC 223 (FP-40) which demonstrated high pod and seed production. Cluster II included accessions with moderate tolerance, while Cluster III consisted of cold-sensitive accessions such as *C. j.* ILWC 4 (FP-14), *C. j.* ILWC 38 (FP-16), and *C. j.* ILWC 45 (FP-16) was characterized by lower yields.

In the subsequent year, similar cluster analysis (Fig. 4b), based on yield attributes, identified three distinct clusters. Cluster I comprised of all cold-tolerant accessions, including *C. judaicum* ILWC 256 (FP-113), ICC 13,852 (FP-53), ILWC 263 (FP-52), ILWC 20 (FP-49), ILWC 30 (FP-54), and ILWC 223 (FP-58). Cluster II included moderately tolerant accessions, whereas Cluster III consisted of cold-sensitive accessions such as *C. judaicum* ILWC 4 (FP-13), ILWC 38 (FP-10), and ILWC 45 (FP-14).

To effectively illustrate the phenotypic diversity in reproductive traits under cold stress, a subset of accessions exhibiting the most extreme phenotypes (both high and low performers) were selected for detailed analysis. The findings are as follows:

Yield traits

Yield traits exhibited significant variation among wild chickpea accessions under both control and cold stress conditions, with a pronounced treatment × accession interaction ($P < 0.01$; Supplementary Table S1). Box plots demonstrated a shift toward lower median values and increased variability under cold stress for all three yield traits. The number of filled pods and seeds and the total seed weight were all negatively affected by cold stress, although the degree of reduction varied across accessions and screening years (Fig. 5).

Cicer accessions such as *C. j.* ILWC 256, *C. j.* ICC 13,852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30—exhibited limited reductions compared to the control conditions: 15.4% in the first year and 24.4% in the second year for filled pods, 20.9% and 21.7% for the number of seeds, and 37.7% and 44.4% for total seed weight.

In contrast, accessions, such as *C. j.* ILWC 4, *C. j.* ILWC 38, *C. j.* ILWC 45, and *C. j.* ICC 17,150 showed marked declines of 76.6% (first year) and 76.7% (second year) for filled pods, 82.5% and 72.2% for number of seeds, and 86.4% and 89.8% for total seed weight, respectively (Tables 2 and 3). These traits exhibited high heritability estimates for cold stress in both the first and second years (Table 4). The high heritability of these traits under stress (Table 4) suggests that yield performance under cold conditions is genetically regulated, and may be closely tied to reproductive and physiological resilience mechanisms. As explored below, reproductive traits such as pollen viability and ovule integrity—both sensitive to cold—may underlie these yield differences, particularly under stress.

Reproductive traits

We examined reproductive traits under the same conditions to understand the reproductive basis of yield variation. Significant differences were observed in the reproductive functions of wild chickpea accessions under control and cold stress conditions, along with a pronounced interaction between accessions and treatments across all traits ($P < 0.01$; Supplementary Table S1). Box plots illustrated a distinct downward shift in trait medians and increased dispersion under cold stress (Fig. 6). Under control conditions, tolerant accessions, such as *C. j.* ILWC 256, *C. j.* ICC 13,852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30—exhibited a lower percentage reduction in reproductive traits under cold stress (PV: 5.2% in the first year and 21.6% in the second year; PG: 4.6% in the first year and 13.6% in the second year; PL: 17.1% in the first year and 30% in the

Fig. 2. Wild chickpea crop in outdoor environemnt (**a**) and growth chamber conditions (**b**). Morphological effects of cold stress on wild chickpea accessions; Vegetative effects of cold stress; leaf chlorosis (**c**), leaf necrosis (**d**), accumulation of anthocyanin (**e**) and flower bud abortion at early stages (**f**), Wild chickpea crop yield under cold stress: high yield in tolerant accessions and low or poor yield in sensitive accessions (**g**, **h**).

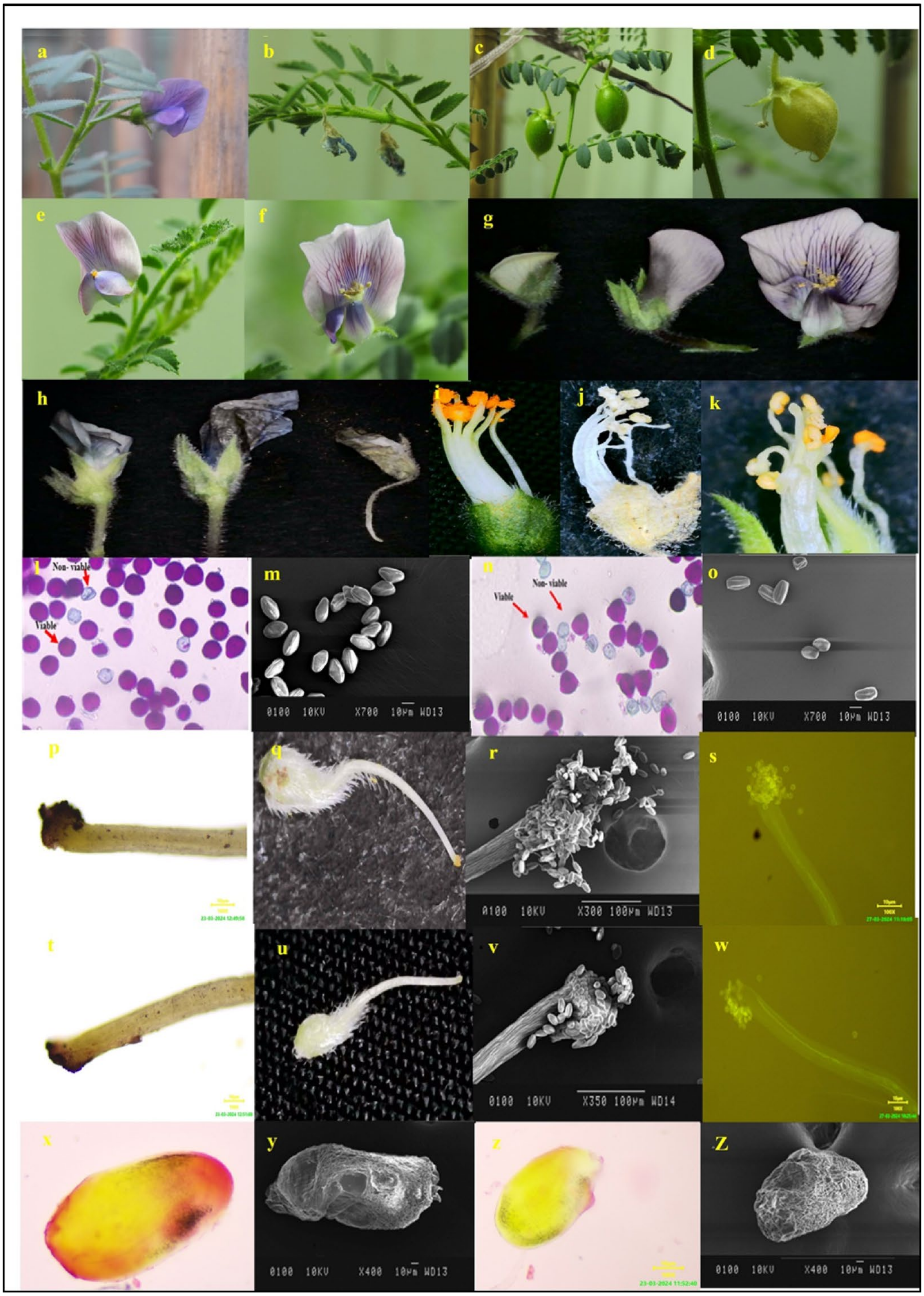

Fig. 3. Various effects of cold stress on the reproductive phase of wild chickpea plant, tolerant accessions under cold stress have healthy flower (**a**), healthy pod (**c**), normal flower development stages (**g**), more viable pollen grains (**l**, **m**),healthy anther dehiscence (**i**), healthy and highly receptive stigma (**p**, **q**), more pollen load (**r**, **s**) and viable and healthy ovule (**x**, **y**). Sensitive accessions under cold stress have aborted flower (**b**), aborted pods (**d**), exposed anther due to cold stress (**e**, **f**), stages of aborted flower development (**h**), distorted anthers under cold stress (**k**), non-viable pollen grains (**n**, **o**), poor anther dehiscence (**j**), low stigma receptivity (**t**, **u**), less pollen load (**v**, **w**), non-viable and distorted ovule (**z**, **Z**).

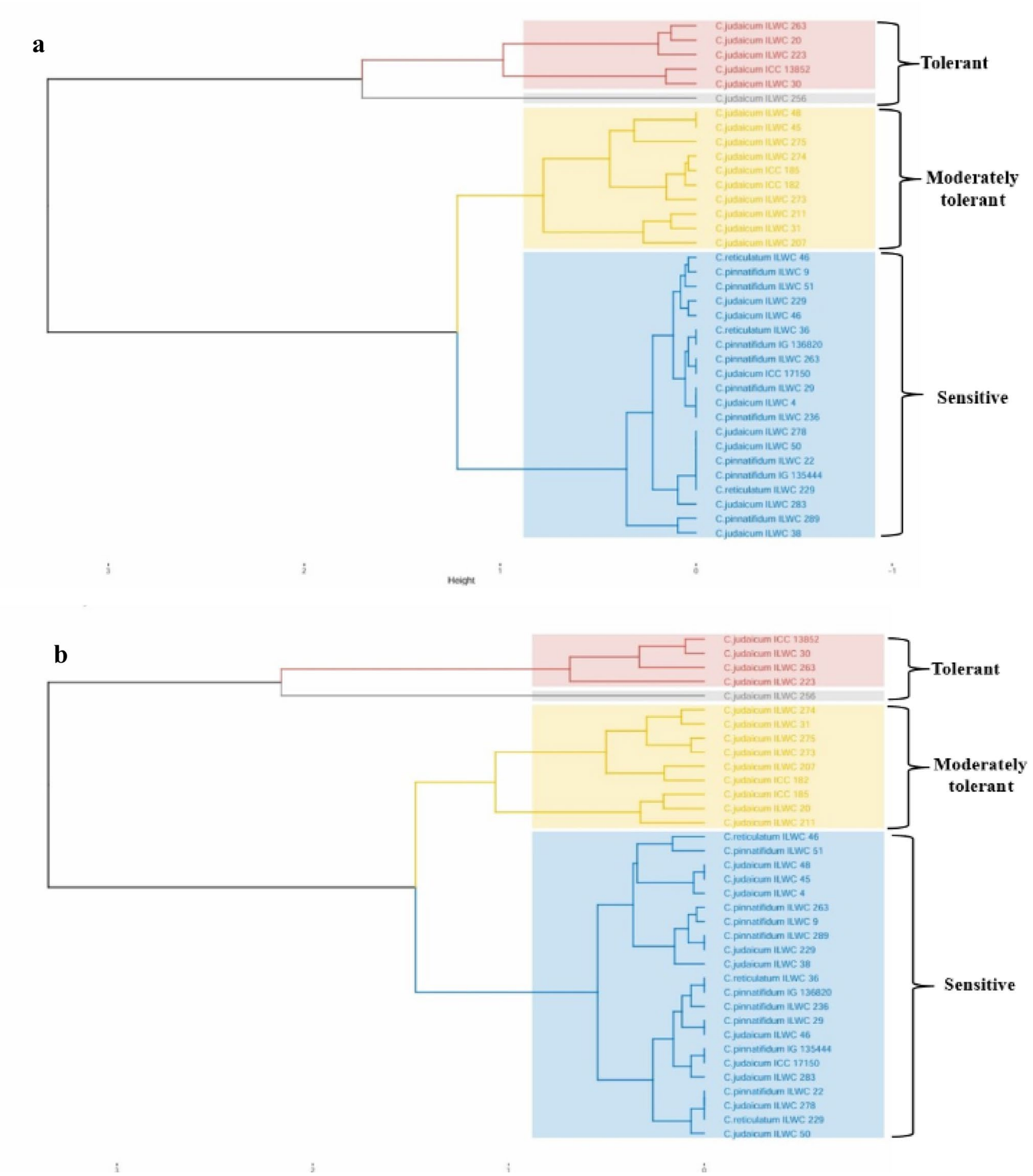

Fig. 4. Cluster analysis for the 2021–22 (**a**) and 2022–23 (**b**) screening, categorised accessions into different clusters as cluster I (tolerant), cluster II (moderately tolerant) and cluster III (sensitive) under cold stress.

second year; and OV: 11.8% in the first year and 31.2% in the second year). ILWC 4, *C. j.* ILWC 38, *C. j.* ILWC 45, and *C. j.* ICC 17,150, which showed higher reductions of 18.6% (first year) and 30.2% (second year) for PV; 19.3% and 19.7% for PG; 30.3% and 55% for PL; and 36.7% and 42.8% for OV, respectively (Tables 2 and 3). The observed pattern suggests that accessions with better maintained reproductive processes under cold stress also showed superior yield performance. This alignment supports the hypothesis that reproductive robustness under stress is a key determinant of resilience to yield. The high heritability of reproductive traits (Table 4) further strengthens their value as selection targets for breeding programs aimed at cold stress tolerance.

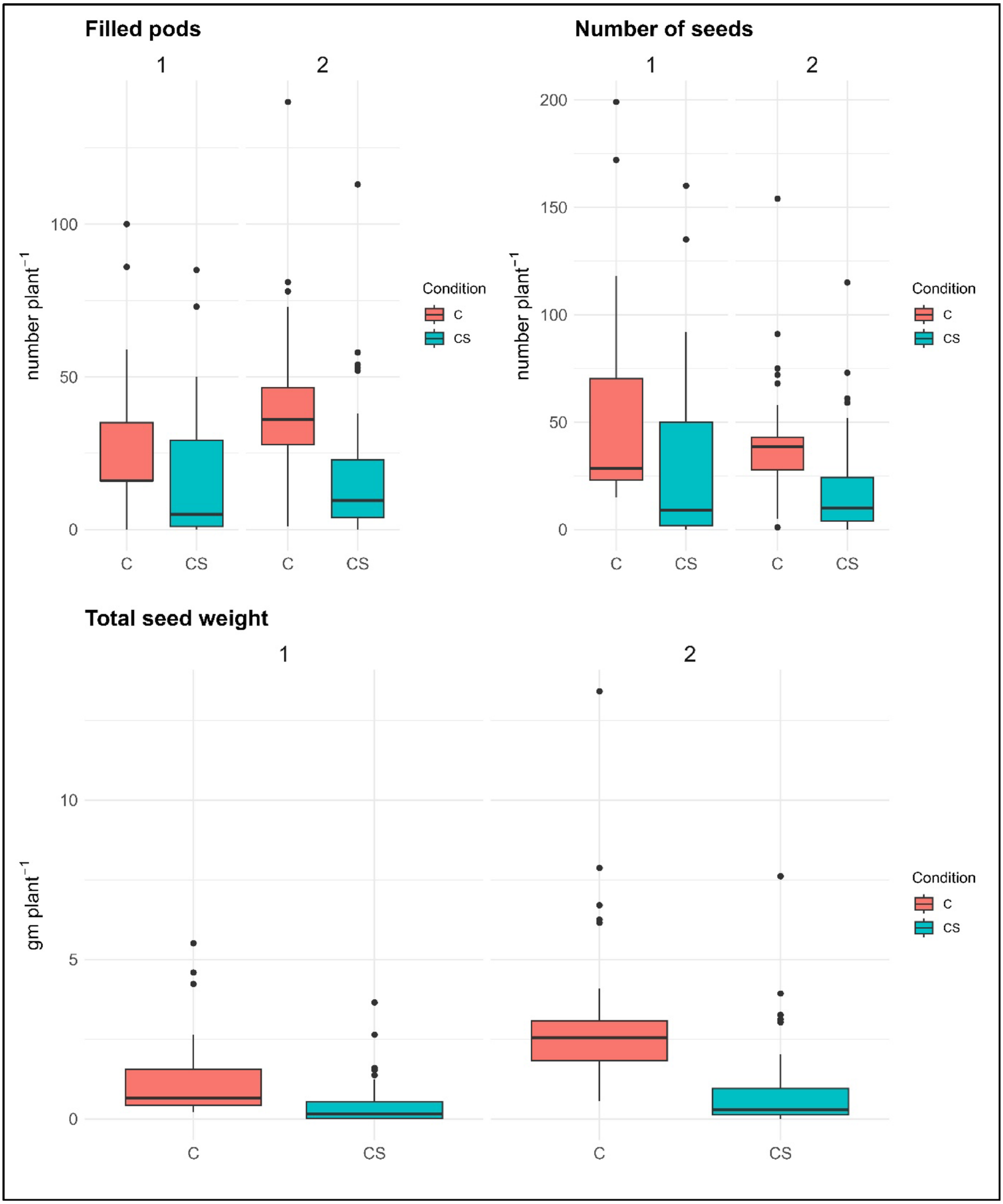

Fig. 5. Boxplots showing the effect of control (C) and cold stress (CS) conditions on the number of filled pods (FP), number of seeds(NS), and total seed weight (TSW) across two consecutive screening years (1 and 2). Boxes represent interquartile ranges; whiskers indicate variability outside the upper and lower quartiles; dots represent outliers.

Physiological traits

Building on the yield and reproductive responses described above, physiological traits provided further insight into the mechanisms underlying stress resilience in wild chickpea accessions. Wild chickpea accessions displayed substantial differences ($P<0.01$; Supplementary Table S1), as revealed by analysis of variance (ANOVA), indicating significant individual effects of accessions and treatments, as well as their combined interactions across all traits. Box plots (Fig. 7) highlighted clear divergence in trait behavior across accessions and years,

Traits	Cold tolerant accessions		% reduction	Cold sensitive accessions		% reduction
	Control	Cold stress		Control	Cold stress	
Filled pods (FP)	70	59	15.42	16	4	76.68
Number of seeds (NS)	142	112	20.99	22	4	82.51
Total seed weight (TSW)	3.310	2.060	37.764	0.370	0.050	86.486
Pollen viability (PV)	96.3	91.3	5.20	85.9	69.9	18.63
Pollen germination (PG)	93.4	89.1	4.57	84.8	68.4	19.32
Pollen load (PL)	4.7	3.9	17.14	2.8	1.9	30.30
Ovule viability (OV)	4.7	4.2	11.27	4.1	2.6	36.73
Electrolyte leakage (EL)	12.3	19.3	57.09	15.9	31.3	96.86
Relative leaf water content (RLWC)	85.0	76.1	10.48	81.2	68.1	16.06
Stomatal conductance (SC)	189.6	156.9	17.23	169.9	103.8	38.93
Chlorophyll content (SPAD)	38.6	32.7	15.16	30.9	23.4	24.20
Photosynthetic efficiency (PE)	0.75	0.70	6.98	0.72	0.60	16.32
Carotenoids (CART)	2.20	3.49	58.85	2.24	2.63	17.58
Proline (PRO)	17.4	25.4	46.53	15.2	20.0	31.33
Trehalose (TRE)	2.6	4.4	70.23	2.5	3.1	25.00
Total sugars (TS)	44.2	66.0	49.46	36.1	47.6	31.73
Malondialdehyde content (MDA)	9.3	15.0	60.97	8.6	23.2	170.69
Hydrogen peroxide (H_2O_2)	1.1	2.3	106.62	1.7	4.4	165.22
Superoxide dismutase (SOD)	15.2	23.8	56.86	14.2	18.0	27.28
Catalase (CAT)	3.4	5.2	52.14	2.4	3.0	26.50
Ascorbate peroxidase (APX)	3.6	5.8	59.90	2.3	3.0	27.85
Glutathione reductase (GR)	4.2	5.8	37.68	3.1	4.1	31.58
Ascorbic acid (AsA)	75.7	133.6	76.44	67.1	86.8	29.37
Glutathione reduced (GSH)	18.2	33.6	83.89	16.4	23.1	40.58

Table 2. Comparative percentage reduction for various yield, reproductive, physiological, oxidative and biochemical traits between cold-tolerant and cold-sensitive *Cicer* accessions under control and cold-stress conditions during first year screening (2021–22).

particularly for electrolyte leakage (EL) and relative leaf water content (RLWC) under cold stress. All traits exhibited varying percentage changes when comparing the tolerant accessions *C. j.* ILWC 256, *C. j.* ICC 13,852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30, with sensitive accessions *C. j.* ILWC 4, *C. j.* ILWC 38, *C. j.* ILWC 45, and *C. j.* ICC 17,150.

For instance, compared to the control conditions, EL decreased by 57.1% in the first year and 66.3% in the second year in tolerant accessions but by 96.8% and 83.1%, respectively, in sensitive accessions. RLWC decreased by 10.5% (first year) and 4.4% (second year) in tolerant accessions compared to 16.1% and 13.1%, respectively, in sensitive accessions. Similarly, stomatal conductance (SC) declined by 17.2% and 42.4% in tolerant accessions versus 38.9% and 55.1% in sensitive accessions over the two years. SPAD values decreased by 15.2% (first year) and 17.2% (second year) in tolerant accessions but by 24.2% and 39.4% in sensitive accessions. The photosynthetic efficiency (PE) was reduced by 6.9% and 13.3% in tolerant accessions compared to 16.3% and 25.7% in sensitive accessions, respectively.

In contrast, carotenoid content increased as a protective mechanism by 58.9% in the first year and 129.4% in the second year in tolerant accessions, compared to 17.5% and 73.8% in sensitive accessions (Tables 2 and 3). The pronounced genetic control over these traits was reflected in the high heritability estimates across both years under cold stress conditions (Table 4).

Notably, the observed physiological stability of tolerant accessions matched well with their superior reproductive function and yield retention under cold stress. Lower membrane injury (EL), improved water balance (RLWC), and sustained photosynthetic efficiency likely contribute to better pollen viability, ovule development, and seed set, thereby linking physiological robustness to reproductive integrity and final yield performance. This integrated resilience across functional levels highlights the adaptive value of these physiological traits under cold stress and their potential as selection criteria in breeding programs.

Osmolytes and cryoprotectants

Complementing the physiological responses, the accumulation of compatible solutes under cold stress provided further biochemical evidence of stress mitigation strategies in tolerant wild chickpea accessions. The boxplots (Fig. 8) showed a pronounced increase in proline (PRO), trehalose (TRE), and total sugars (TS) under cold stress compared to the control, with a consistent trend of higher metabolite accumulation across both years. ANOVA results across wild chickpea accessions, treatments, and significant treatment × accession interactions revealed strong effects on proline, trehalose, and total sugar content ($P < 0.01$; Supplementary Table S1).

Traits	Cold tolerant accessions			Cold sensitive accessions		
	Control	Cold stress	% reduction	Control	Cold stress	% reduction
Filled pods (FP)	90	68	24.44	43.0	10	76.74
Number of seeds (NS)	92	72	21.74	36.0	10	72.22
Total seed weight (TSW)	8.10	4.50	44.44	2.55	0.26	89.80
Pollen viability (PV)	97	76	21.65	86.0	60	30.23
Pollen germination (PG)	93	80.3	13.66	86.0	69	19.77
Pollen load (PL)	5	3.5	30.00	4.0	1.8	55.00
Ovule viability (OV)	4.8	3.3	31.25	3.5	2.0	42.86
Electrolyte leakage (EL)	10.4	17.3	66.35	14.8	27.1	83.11
Relative leaf water content (RLWC)	84.4	80.7	4.38	80.8	70.2	13.12
Stomatal conductance (SC)	204.4	117.7	42.42	195.2	87.6	55.12
Chlorophyll content (SPAD)	43.7	36.2	17.16	36.8	22.3	39.40
Photosynthetic efficiency (PE)	0.8	0.69	13.75	0.74	0.55	25.68
Carotenoids (CART)	1.7	3.9	129.41	1.7	2.99	73.84
Proline (PRO)	16.2	29	79.01	14.9	19.6	31.54
Trehalose (TRE)	2.7	4.4	62.96	1.8	2.3	27.78
Total sugars (TS)	51.6	75.2	45.74	35.5	44.1	24.23
Malondialdehyde content (MDA)	8.3	12.7	53.01	8.4	22.7	170.24
Hydrogen peroxide (H_20_2)	1.2	2.4	100.00	1.3	4	207.69
Superoxide dismutase (SOD)	10.7	24.4	128.04	9.8	14.6	48.98
Catalase (CAT)	2.9	5.5	89.66	2.4	3.5	45.83
Ascorbate peroxidase (APX)	3.2	6.6	106.25	2.8	4.1	46.43
Glutathione reductase (GR)	3.5	5.8	65.71	2.1	3.1	47.62
Ascorbate (ASC)	62.5	120.1	92.16	62.0	86.3	39.19
Glutathione reduced (GSH)	14.9	34.9	134.23	13.3	17.5	31.58

Table 3. Comparative percentage reduction for various yield, reproductive, physiological, oxidative and biochemical traits between cold-tolerant and cold-sensitive *Cicer* accessions under control and cold-stress conditions during second year screening (2022–23).

In tolerant accessions such as *C. j.* ILWC 256, *C. j.* ICC 13,852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30, proline levels increased by 46.5% in the first year and 79.0% in the second year. Similarly, trehalose levels increased by 70.2% in the first year and 62.9% in the second year compared to the control, whereas total sugar levels increased by 49.5% in the first year and 45.7% in the second year.

In contrast, sensitive accessions such as *C. j.* ILWC 4, *C. j.* ILWC 38, *C. j.* ILWC 45, and *C. j.* ICC 17,150 exhibited lower osmolyte accumulation: 31.3% (first year) and 31.5% (second year) for proline; 25.0% and 27.7% for trehalose; and 31.8% and 27.7% for total sugars, respectively, indicating a less effective osmoprotective response (Tables 2 and 3).

These trends support the above physiological observations, where tolerant accessions maintained better membrane integrity, water status, and photosynthetic efficiency under cold stress. The enhanced accumulation of osmolytes and cryoprotectants in tolerant accessions likely acted synergistically with physiological adjustments to minimize oxidative and structural damage during chilling episodes. Furthermore, the high heritability of these biochemical traits under stress conditions (Table 4) supports their potential utility as stable selection markers for cold tolerance.

Oxidative stress and antioxidant defense

Based on the observed physiological and osmoprotective responses, the extent of oxidative damage and efficiency of antioxidant defense systems were assessed to understand their role in cold stress tolerance among wild chickpea accessions. Cold stress triggered a substantial oxidative burst in wild chickpea accessions, reflected by elevated levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), with highly significant treatment × accession interactions for both traits ($P < 0.01$; Supplementary Table S1). Boxplots (Fig. 9) showed divergence in oxidative responses across all accessions under control and cold stress conditions.

Tolerant accessions such as *C. j.* ILWC 256, *C. j.* ICC 13,852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30 exhibited moderate increases in MDA (60.9% in the first year and 53.0% in the second year) and H_2O_2 (106.6% in the first year and 100.0% in the second year) compared to the control. In contrast, sensitive lines such as *C. j.* ILWC 4, *C. j.* ILWC 38, *C. j.* ILWC 45, and *C. j.* ICC 17,150 recorded sharp elevations of 170.6% (first year) and 170.2% (second year) for MDA, and 165.2% (first year) and 207.7% (second year) for H_2O_2 under cold stress (Tables 2 and 3). These oxidative traits exhibited high heritability under cold stress in both years, indicating a strong genetic component (Table 4).

	Mean		Range		Heritability	
Variable	**1st year**	**2nd year**	**1st year**	**2nd year**	**1st year**	**2nd year**
Filled pods	18	18.6	85–0	113–0	99.4	91.4
Number of seeds	31.2	20	160–0	115–0	99.6	91.5
Total seed weight	0.51	0.95	3.65–0	7.61–0	97.8	93.9
Pollen viability	67.1	65.9	98–36	78–0	95.8	92.3
Pollen germination	62.5	60.8	92–35	81–0	46.1	94.7
Pollen load	2.65	2.69	5–1	4-0	62	81.9
Ovule viability	3.04	2.65	5–1	5–0	74	74.8
Electrolyte leakage	24.3	23.7	32.4–14.2	28.5–17.4	92.2	93.1
Relative Leaf water content	71.2	73.4	77.8–60.7	81.9–63.9	80.02	84.3
Stomatal conductance	115.5	92.7	190.7–80.7	135.8–80.7	75.9	64.2
Chlorophyll content	25.2	24.3	36.2–21.1	38.6–18.9	69.8	67.4
Photosynthetic efficiency	0.58	0.63	0.73-0.45	0.73-0.54	86.1	75.8
Carotenoids	2.57	3.06	3.69–1.75	4.30–2.37	67.5	69.9
Proline	21	22.4	30.5–14.9	32.2–18.9	80.7	87.3
Trehalose	3.24	3	4.9–2.3	4.57–1.83	83.7	88.8
Total sugars	52.3	52.1	73.7–41.6	76.1–35.6	88	93.7
Malondialdehyde content	21.3	19.9	25.8–12.8	25.2–12.5	85.4	94.9
Hydrogen peroxide	3.22	3.36	4.46–2.18	4.01–2.31	56.6	48.8
Superoxide dismutase	19.01	17.2	24.8–14.1	25.1–13.5	79.1	92.2
Catalase	3.32	4	5.9–2.4	5.8–2.8	86	79.4
Ascorbate peroxidase	3.56	4.33	6.35–2.27	7.53–2.21	90.6	93.3
Glutathione reductase	4.23	3.64	6.05–2.50	6.05–2.41	80.2	89
Ascorbic acid	102.2	97.3	146.7–73.4	124.3–76.2	90.1	87.7
Glutathione reduced	24.7	22.1	36.1–15.6	36.9–15.6	84.8	90.4

Table 4. General statistics for various traits for wild Chickpea accessions under cold stress in both the years.

Enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), along with non-enzymatic antioxidants such as ascorbate (AsA) and reduced glutathione (GSH), displayed highly significant effects ($P<0.01$) in the analysis of variance (ANOVA), with significant individual effects of accessions and treatments, as well as their interactions (Supplementary Table S1). Boxplots (Fig. 10) revealed clear differences in antioxidant responses between the control and cold stress conditions across accessions.

To mitigate oxidative damage, the activities of key enzymatic antioxidants were significantly upregulated in tolerant lines under cold stress, showing increases of 56.9% (first year) and 128.1% (second year) for SOD, 52.1% and 89.6% for CAT, 59.9% and 106.2% for APX, and 37.6% and 65.7% for GR in *C. j.* ILWC 256, *C. j.* ICC 13,852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30, respectively. In contrast, sensitive accessions such as *C. j.* ILWC 4, *C. j.* ILWC 38, *C. j.* ILWC 45, and *C. j.* ICC 17,150 exhibited lower antioxidant accumulation, with SOD levels of 27.3% (first year) and 48.9% (second year), CAT of 26.5% and 45.8%, APX of 27.9% and 46.4%, and GR of 31.5% and 47.6%, respectively (Tables 2 and 3).

Similarly, non-enzymatic antioxidants also showed significant differences: AsA increased by 76.4% in (first year) and 92.1% in (second year), and GSH increased by 83.9% and 134.2% in the tolerant accessions, respectively. In contrast, the sensitive lines recorded lower levels of AsA, 29.3% (first year) and 39.2% (second year); GSH, 40.5% (first year), and 31.6% (second year) (Tables 2 and 3).

Importantly, these antioxidant traits also demonstrated high heritability under cold stress, indicating strong genetic regulation (Table 4), similar to osmolyte-related traits described earlier. The enhanced osmoprotective capacity of the tolerant accessions was complemented by a more effective antioxidant defense system, which is crucial for limiting cold-induced oxidative damage. The capacity of tolerant genotypes to effectively detoxify reactive oxygen species (ROS) through integrated enzymatic and non-enzymatic antioxidant responses is likely a central mechanism conferring cold resilience.

Principal component analysis

To further understand the interrelationships among the physiological, biochemical, and oxidative stress-related traits under cold stress, Principal Component Analysis (PCA) was conducted.

For the first year, the PCA (Fig. 11) depicts the relationships between the 24 variables and their contributions to the variance in the data under cold stress. The first principal component (PC1) explained 65.8% of the total variance, whereas the second principal component (PC2) accounted for 8.2%, capturing a combined variance of 74%. Each variable is depicted as an arrow, with the direction indicating its influence on the principal components, and the length reflecting its contribution strength. The variables closer to the edge of the correlation circle had stronger contributions, whereas those near the origin contributed less. Variables such as catalase

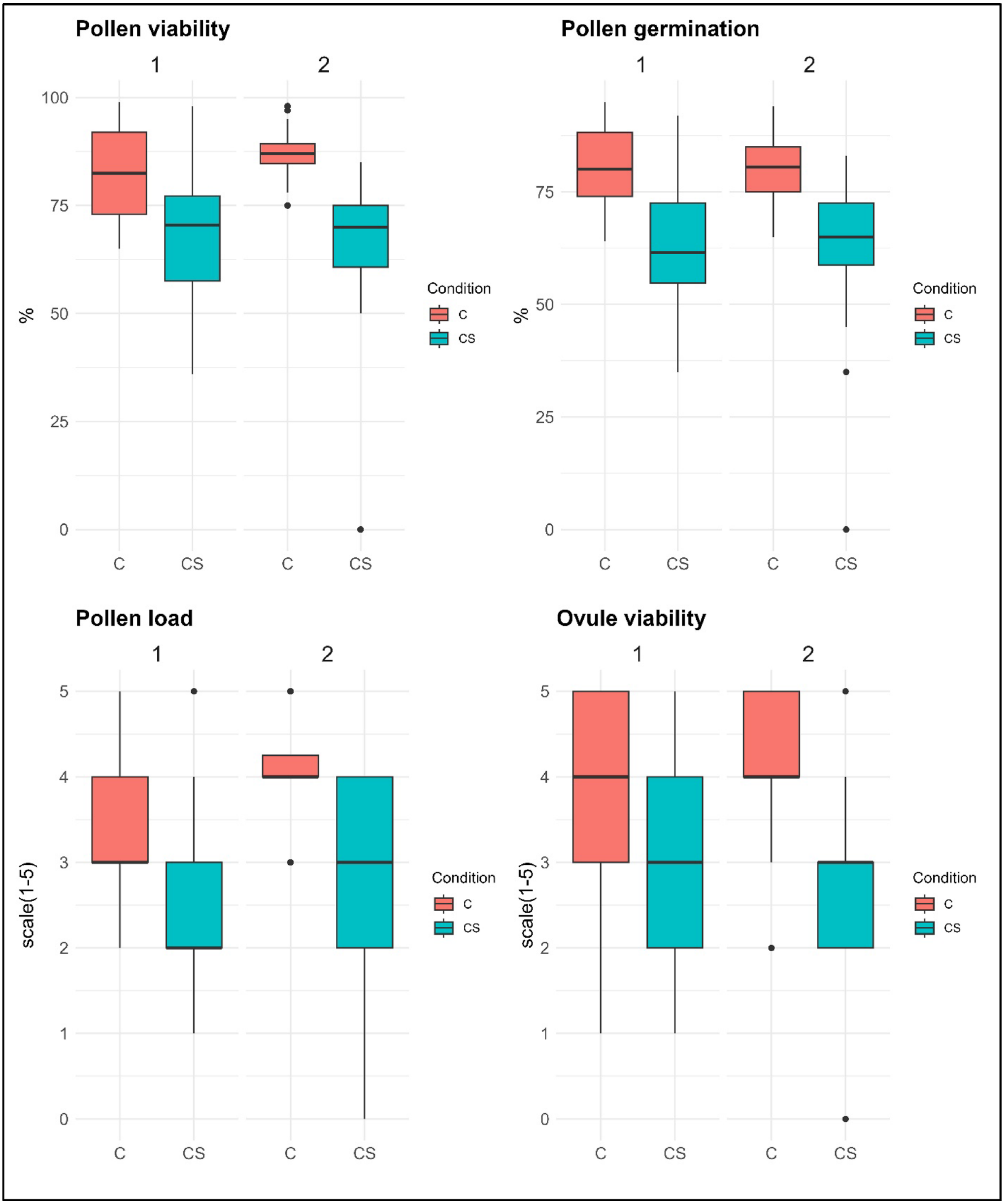

Fig. 6. Boxplots showing the effect of control (C) and cold stress (CS) conditions on the pollen viability (PV), pollen germination (PG), pollen load (PL) and ovule viability (OV) across two consecutive screening years (1 and 2). Boxes represent interquartile ranges; whiskers indicate variability outside the upper and lower quartiles; dots represent outliers.

(CAT: 0.233), reduced glutathione (GSH: 0.228), and malondialdehyde content (MDA: -0.234) showed higher contributions, as indicated by their factor loadings for PC1. The chlorophyll content (SPAD: 0.224), total sugars (TS: 0.219), and ascorbate peroxidase (APX: 0.217) clustered on the right side and showed strong associations with PC1, suggesting a positive contribution. Conversely, MDA and EL (-0.181), which were located on the left side, exhibited a negative relationship with PC1. Variables such as pollen germination (PG: 0.182), ovule viability

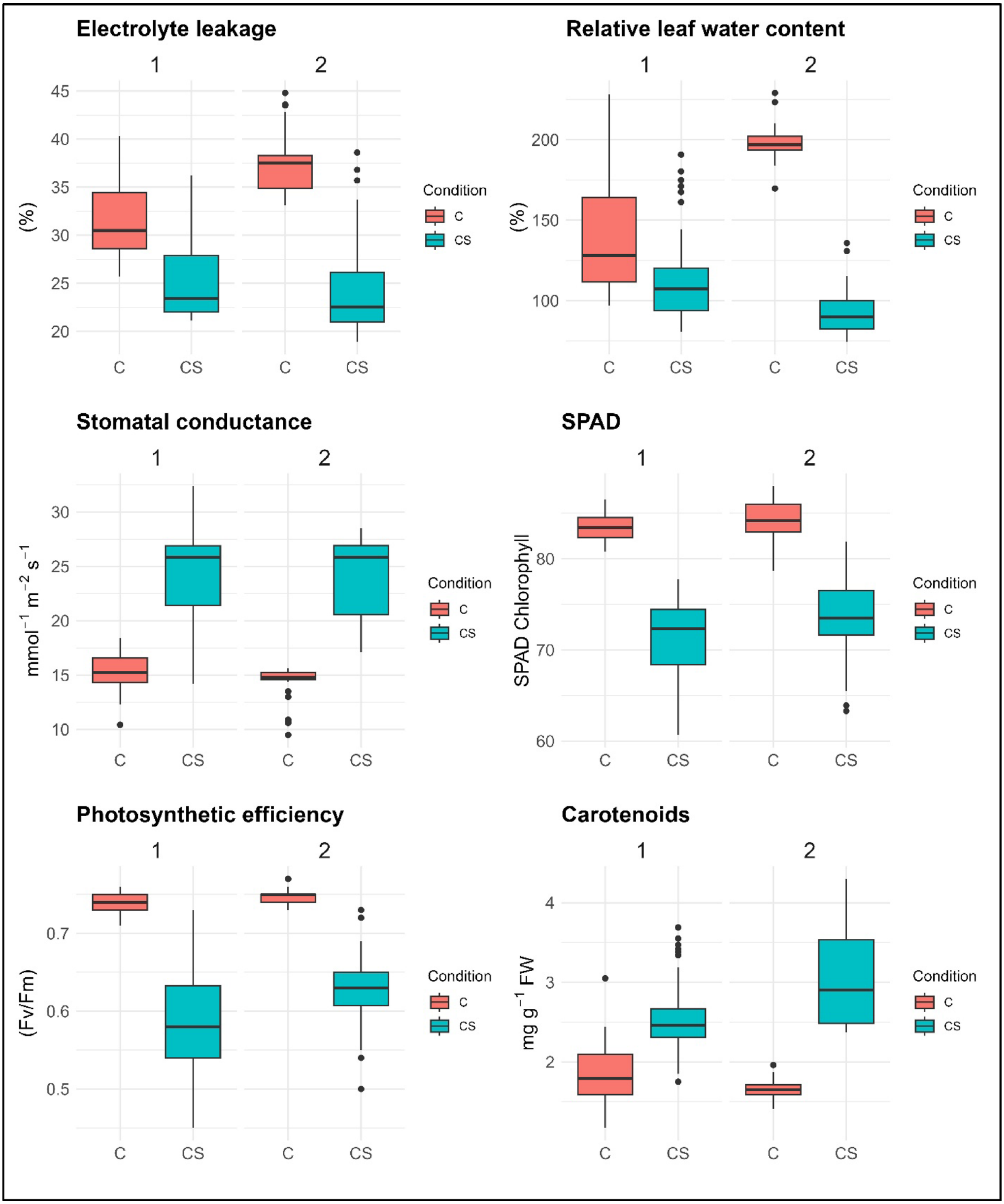

Fig. 7. Boxplots showing the effect of control (C) and cold stress (CS) conditions on the electrolyte leakage (EL), relative leaf water content (RLWC), stomatal conductance (SC), chlorophyll content (SPAD), photosynthetic efficiency (PE), and carotenoids (CART) across two consecutive screening years (1 and 2). Boxes represent interquartile ranges; whiskers indicate variability outside the upper and lower quartiles; dots represent outliers.

(OV: 0.181), pollen load (PL: 0.203), filled pods (FP: 0.21), and the number of seeds (NS: 0.215) also contributed positively to PC1. Proline (PRO: 0.201) and trehalose (TRE: 0.224) were positively correlated and significantly contributed to PC1. Variables such as reduced glutathione (GSH) and ascorbic acid (AsA) contribute uniquely and are situated closer to the periphery, indicating their strong influence on PC1 and their role in antioxidant

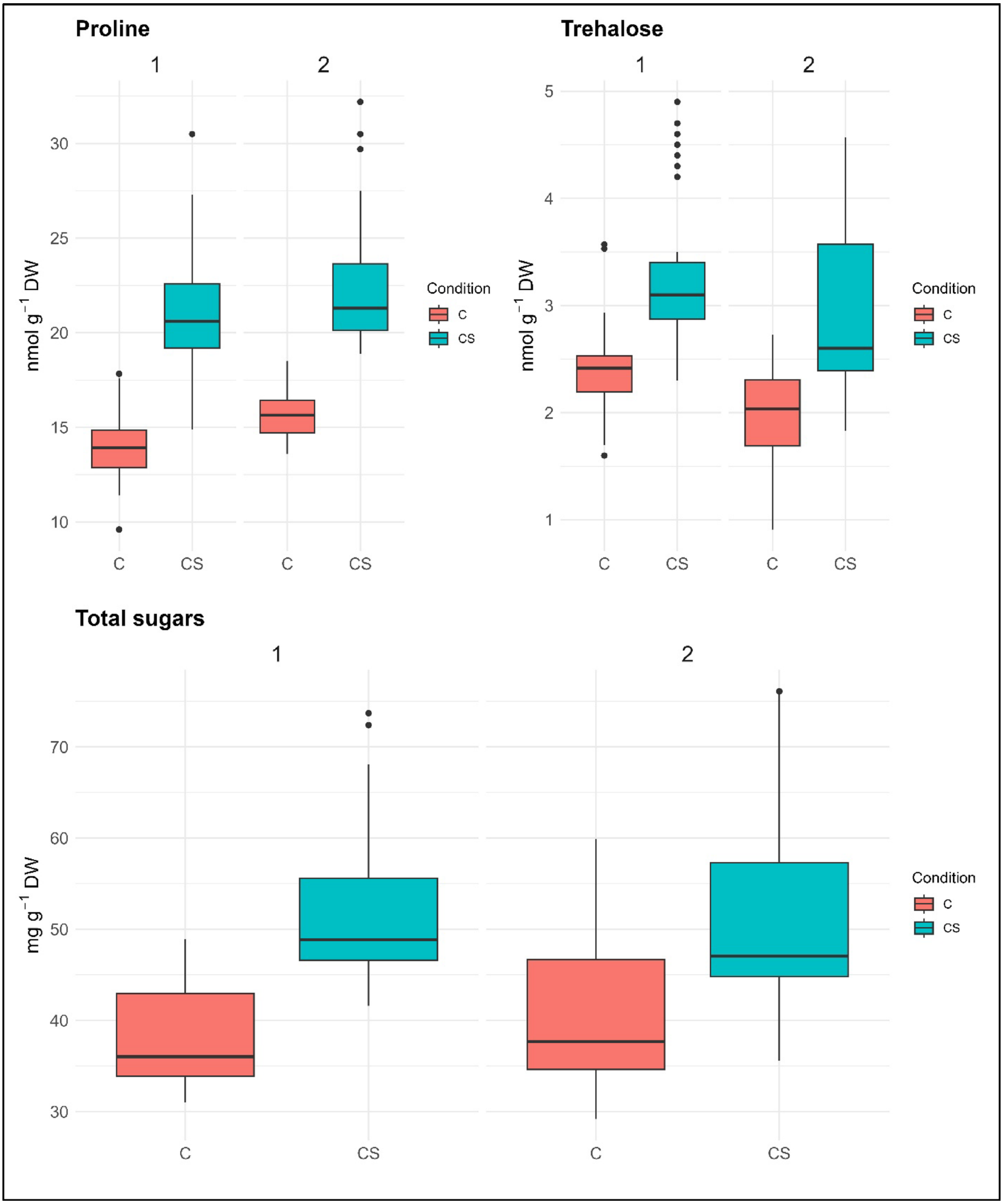

Fig. 8. Boxplots showing the effect of control (C) and cold stress (CS) conditions on the cryoprotectants including proline (PRO), trehalose (TRE), and total sugar (TS) across two consecutive screening years (1 and 2). Boxes represent interquartile ranges; whiskers indicate variability outside the upper and lower quartiles; dots represent outliers.

defence mechanisms. H_2O_2 (-0.291) contributed strongly and negatively to PC2, as indicated by its vertical orientation and factor loading score. This suggests its unique role compared with other variables, emphasizing its association with stress-induced effects.

Similarly, for the second-year crop (Fig. 12), the dataset explained 64.2% of the variance in PC1 and the second principal component (PC2) accounted for 9.3%. MDA (-0.232) and EL (-0.239) for PC1 and H_2O_2 (-0.140) for

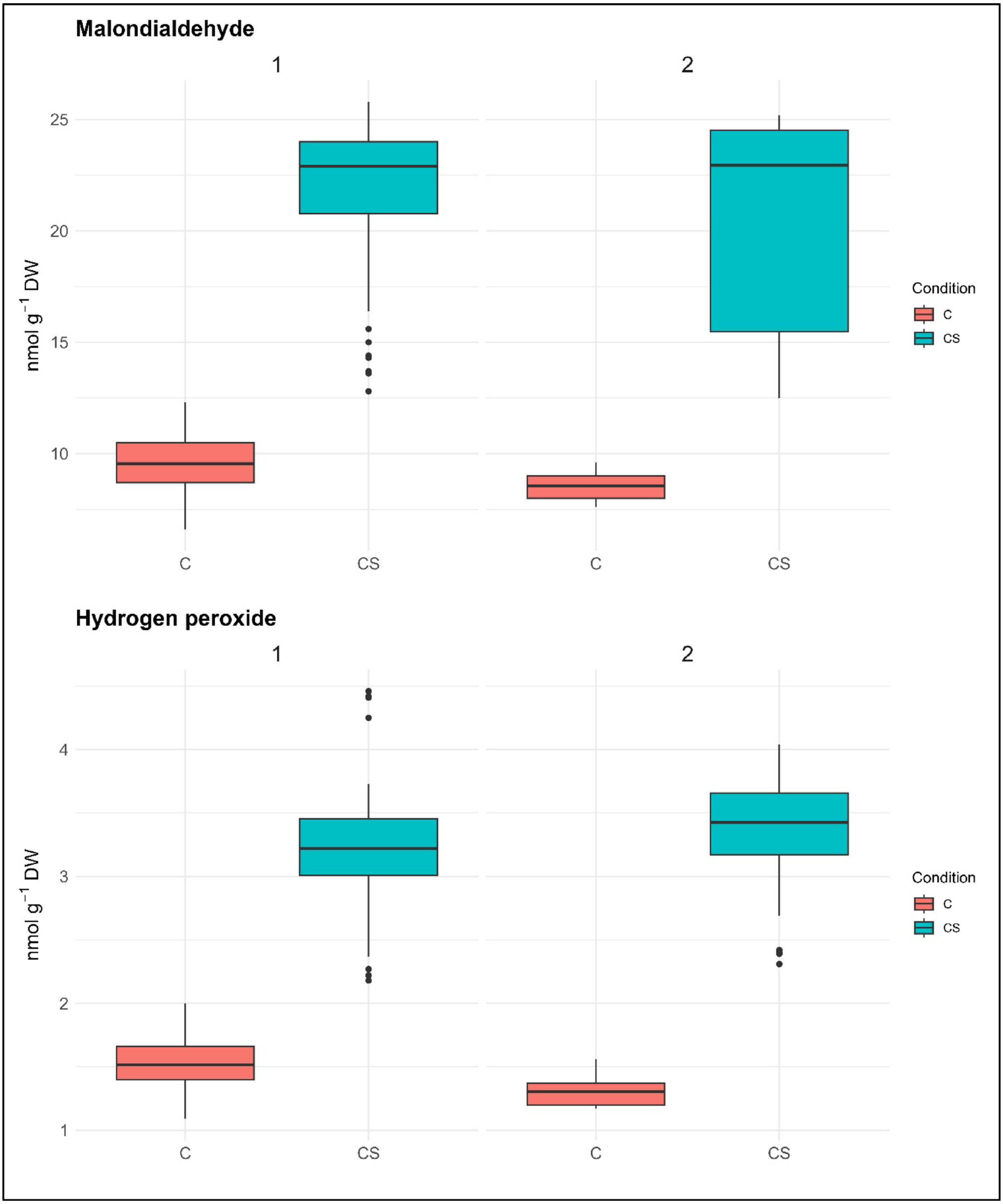

Fig. 9. Boxplots showing the effect of control (C) and cold stress (CS) conditions for the oxidative stress markers malondialdehyde content (MDA) and hydrogen peroxide (H_2O_2) across two consecutive screening years (1 and 2). Boxes represent interquartile ranges; whiskers indicate variability outside the upper and lower quartiles; dots represent outliers.

PC2 were positioned on the left side of the plot and contributed negatively, as indicated by their factor loading. TS (0.229), PRO (0.231), and TRE (0.236) were the key metabolic variables that significantly contributed to PC1 (red orange). Variables such as SPAD (0.181), NS (0.22), SC (0.193), and RLWC (0.124) also contributed positively to PC1. Other variables, such as PG (0.146), PL (0.119), OV (0.134), and TSW (0.199), exhibited

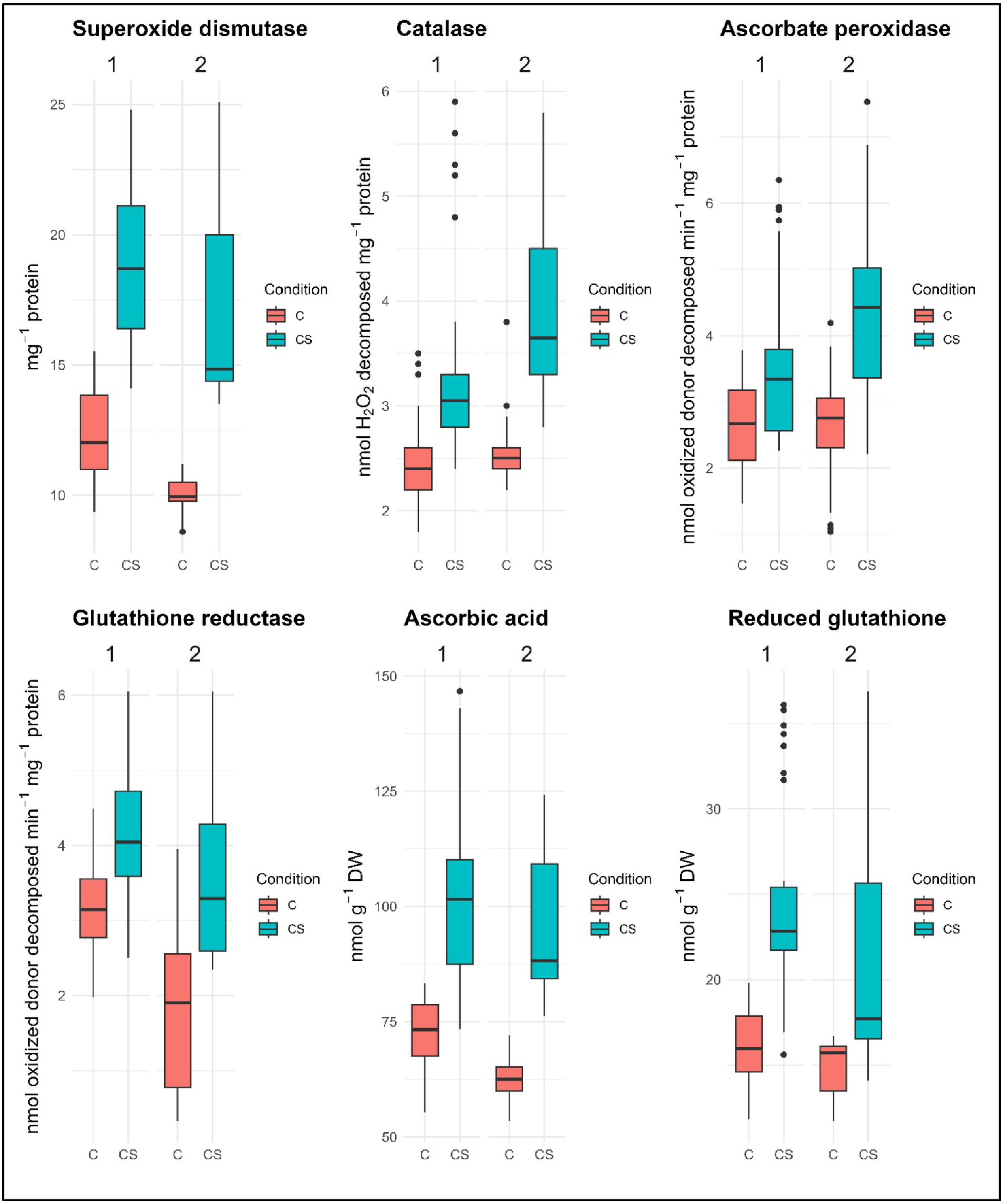

Fig. 10. Boxplots showing the effect of control (C) and cold stress (CS) conditions for antioxidants response including enzymatic and non-enzymatic antioxidants, super-oxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), and reduced glutathione (GSH) across two consecutive screening years (1 and 2). Boxes represent interquartile ranges; whiskers indicate variability outside the upper and lower quartiles; dots represent outliers.

significant contributions and were positioned toward the right side of the plot, suggesting their importance in the reproduction and yield of wild chickpea crops.

This multivariate approach enabled the identification of key traits contributing to variability among wild chickpea accessions and helped to distinguish tolerant and sensitive genotypes based on trait clustering.

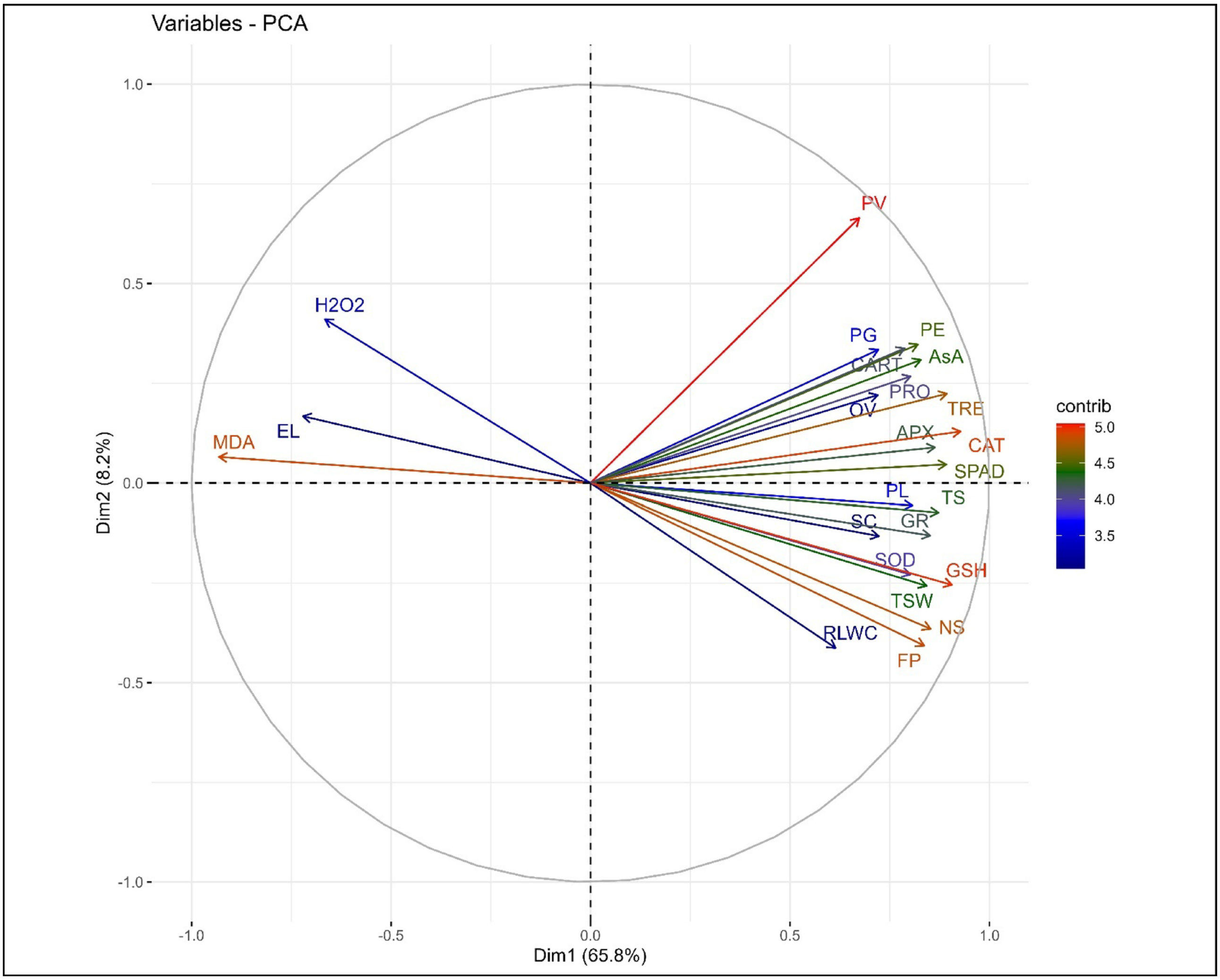

Fig. 11. Principal component analysis (PCA) plot for 24 traits in wild chickpea accessions during first year screening (2021–22). SPAD: Chlorophyll content, SC: Stomatal conductance, FP: Filled pods, TSW: Total seed weight, NS: Number of seeds,, PV: Pollen viability, PG: Pollen germination, PL: Pollen load, OV: Ovule Viability, PE: Photosynthetic efficiency, PRO: Proline, TRE: Trehalose, TS: Total sugars, CART: Carotenoids, RLWC: Relative leaf water content, EL: Electrolyte leakage, MDA: Malondialdehyde content, H_2O_2: Hydrogen peroxide, SOD: Superoxide dismutase, CAT: Catalase, APX: Ascorbate peroxidase, GR: Glutathione reductase, AsA: Ascorbic acid, GSH: Reduced glutathione.

Correlation analysis

To complement the PCA and further dissect the relationships among physiological, osmoprotective, and antioxidant traits under cold stress, Pearson correlation analysis was performed.

The correlation plot (Figs. 13 and 14) illustrates Pearson's correlation coefficients among various yield, reproductive, physiological, oxidative, and biochemical traits under cold stress conditions. Overall, the correlations were positive and negative, indicating highly significant behavior. Traits such as filled pods (FP) and total seed weight (TSW) showed strong positive correlations with physiological traits such as chlorophyll content (FP: 0.73***; TSW: 0.75***), photosynthetic efficiency (PE) (FP: 0.58***; TSW: 0.62***), relative leaf water content (RLWC) (FP: 0.58***; TSW: 0.51**), and stomatal conductance (SC) (FP: 0.65***; TSW: 0.62***). Similar results were observed for second-year correlation analysis with SPAD (FP: 0.77***; TSW: 0.80***), PE (FP: 0.51**;: 0.47***), RLWC (FP: 0.50**; TSW: 0.57***) and SC (FP: 0.73***; TSW: 0.67***). A strong and positive association between SPAD and total sugars (TS) (1st year: 0.83***; 2nd year: 0.71***), proline (PRO), and PE (1st year: 0.74***; 2nd year: 0.57***) was observed.

Significant negative associations between FP and TSW and electrolyte leakage (FP: 1st year: -0.52**, 2nd year: -0.71***; TSW: 1st year: -0.49**, 2nd year: -0.59***), hydrogen peroxide (FP: 1st year: -0.67***, 2nd year: -0.57***; TSW: 1st year: -0.61***, 2nd year: -0.64***), and malondialdehyde content (FP: 1st year: -0.81***, 2nd year: -0.68***; TSW: 1st year: -0.79***, 2nd year: -0.54***) were observed for first- and second-year crops, respectively. Importantly, catalase (CAT) activity was negatively correlated with H_2O_2 under cold stress (1st year: -0.63***; 2nd year: -0.36*), indicating a stronger detoxification role of antioxidants. Osmolyte accumulation,

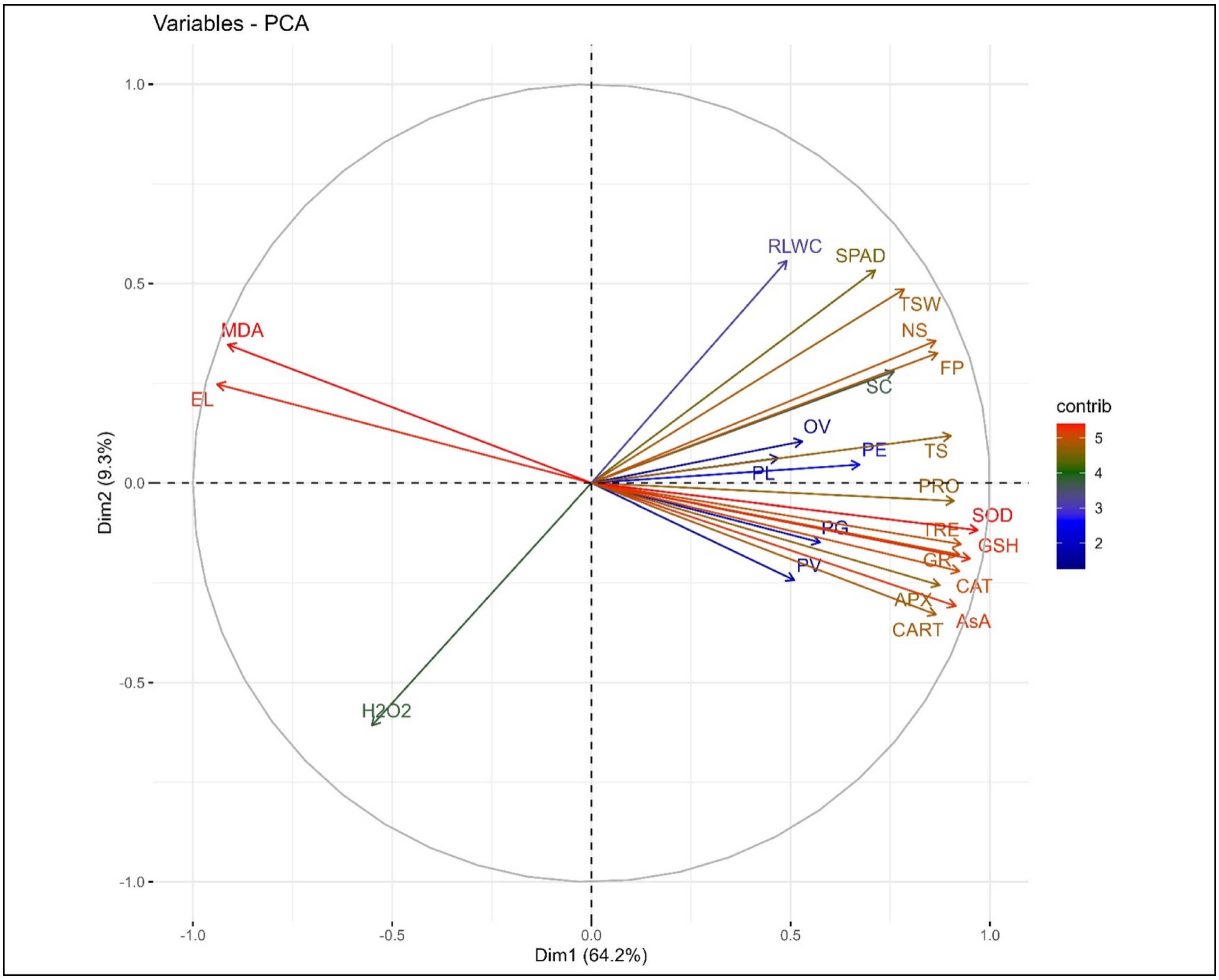

Fig. 12. Principal component analysis (PCA) plot for 24 traits in wild chickpea accessions during second year screening (2022–23). SPAD: Chlorophyll content, SC: Stomatal conductance, FP: Filled pods, TSW: Total seed weight, NS: Number of seeds,, PV: Pollen viability, PG: Pollen germination, PL: Pollen load, OV: Ovule Viability, PE: Photosynthetic efficiency, PRO: Proline, TRE: Trehalose, TS: Total sugars, CART: Carotenoids, RLWC: Relative leaf water content, EL: Electrolyte leakage, MDA: Malondialdehyde content, H_2O_2: Hydrogen peroxide, SOD: Superoxide dismutase, CAT: Catalase, APX: Ascorbate peroxidase, GR: Glutathione reductase, AsA: Ascorbic acid, GSH: Reduced glutathione.

such as proline, also became more tightly linked with photosynthesis-related traits, such as SPAD (1st year: 0.78***; 2nd year: 0.63***) and PE (1st year:0.74***; 2nd year: 0.57***), during stress, highlighting its protective role.

Under cold stress, all physiological traits (chlorophyll content, photosynthetic efficiency, stomatal conductance, and relative leaf water content), yield traits (filled pods and total seed weight), and biochemical traits were significantly and negatively correlated with electrolyte leakage (EL), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) content, which are indicators of oxidative stress, suggesting that these traits are essential for screening cold-tolerant wild chickpea accessions.

These correlations provide additional evidence supporting the physiological and biochemical mechanisms that differentiate between tolerant and sensitive genotypes.

Discussion

Climate change necessitates the identification of new sources of tolerance to abiotic stresses; however, the narrow genetic base of cultivated chickpea (*C. arietinum*) and a limited number of cold-tolerant accessions in chickpea[2] mandate the search for additional sources of cold tolerance in wild relatives of chickpea. The present study fills this gap by identifying six new sources of cold tolerance (*C. j.* ILWC 256, *C. j.* ICC 13852, *C. j.* ILWC 263, *C.j.* ILWC 223, *C. j.* ILWC 20, and *C. j.* ILWC 30) in the wild chickpea species (*C. judaicum*) that can be exploited by breeders to develop cold-tolerant varieties.

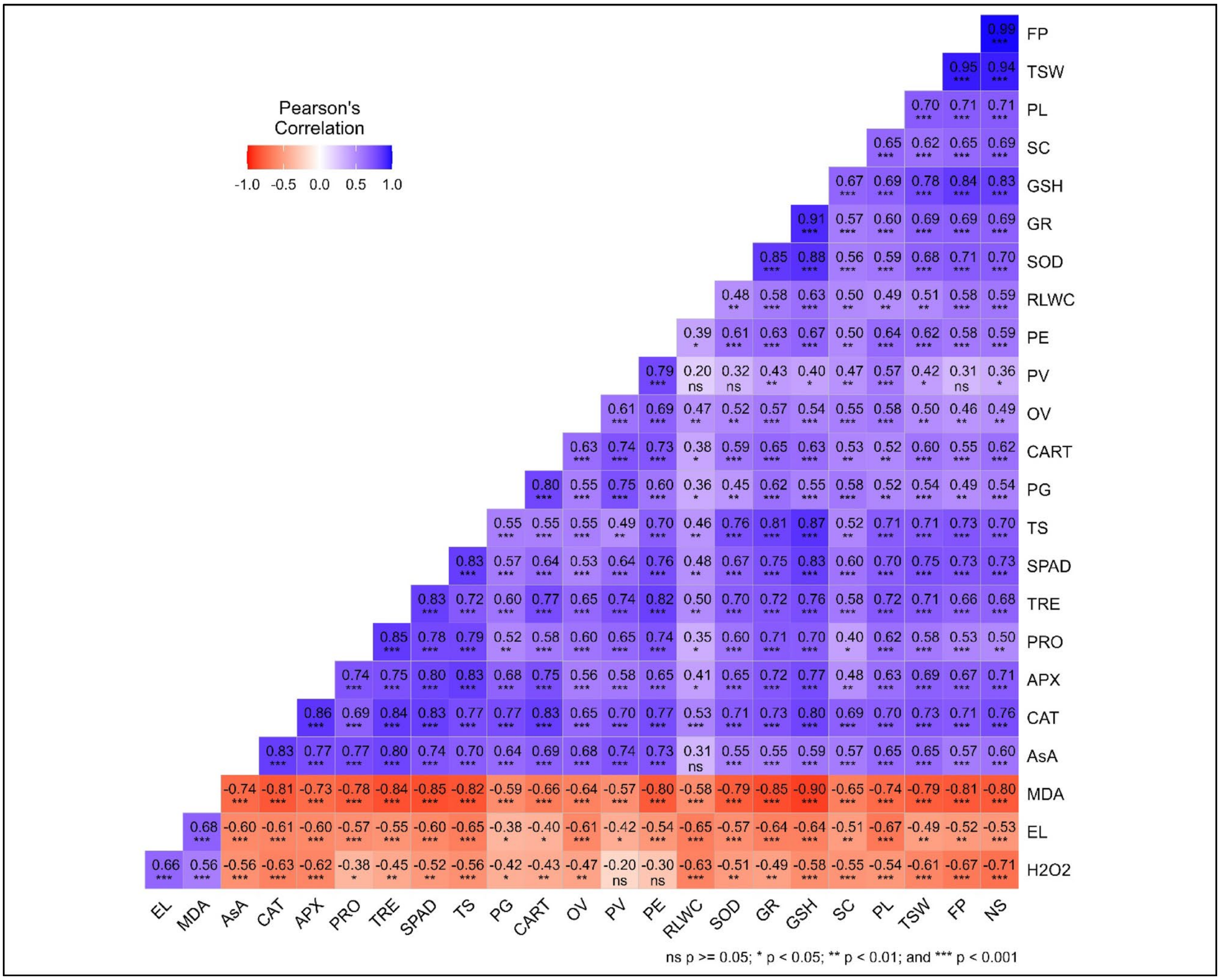

Fig. 13. Pearson corelation matrix for 24 traits in wild chickpea accessions during first year screening (2021–22). SPAD: Chlorophyll content, SC: Stomatal conductance, FP: Filled pods, TSW: Total seed weight, NS: Number of seeds,, PV: Pollen viability, PG: Pollen germination, PL: Pollen load, OV: Ovule Viability, PE: Photosynthetic efficiency, PRO: Proline, TRE: Trehalose, TS: Total sugars, CART: Carotenoids, RLWC: Relative leaf water content, EL: Electrolyte leakage, MDA: Malondialdehyde content, H_2O_2: Hydrogen peroxide, SOD: Superoxide dismutase, CAT: Catalase, APX: Ascorbate peroxidase, GR: Glutathione reductase, AsA: Ascorbic acid, GSH: Reduced glutathione.

Owing to their adaptation to harsh climates, wild chickpeas are widely regarded as valuable sources of abiotic stress tolerance[2,37]. In the present study, several wild chickpea accessions exhibited strong cold tolerance traits, supporting their potential for use in cold stress breeding. While earlier studies have indicated lower cold resilience in cultivated chickpea than the wild types[2,9]direct comparisons were not made in this study. In contrast to *C. judaicum*, none of the *C. pinnatifidum* and *C. reticulatum* accessions evaluated in the present study were tolerant to cold, suggesting screening of all available wild germplasm to identify additional sources of tolerance to cold.

This study also indicates a possible combination of traits that can be used as indicators of cold tolerance, especially in wild chickpeas. We used several morphological, physiological, and biochemical parameters in our study, and cluster analysis (Fig. 3) involving yield-related traits, such as pod number, seed weight, and seed count, which were directly affected by cold stress, served as strong indicators of the accession's resilience to cold tolerance. The wide variability in the reaction of accessions to cold stress also provides a basis for further investigations into the specific mechanisms contributing to cold tolerance in high performers and cold susceptibility in low performers.

Physiological resilience to cold stress

Physiological traits, including chlorophyll content, photosynthetic efficiency, stomatal conductance, electrolyte leakage, and leaf water content, are considered crucial for the selection of cold-tolerance[15]. In our investigation, wild chickpea accessions that demonstrated superior reproductive and yield traits under cold stress conditions

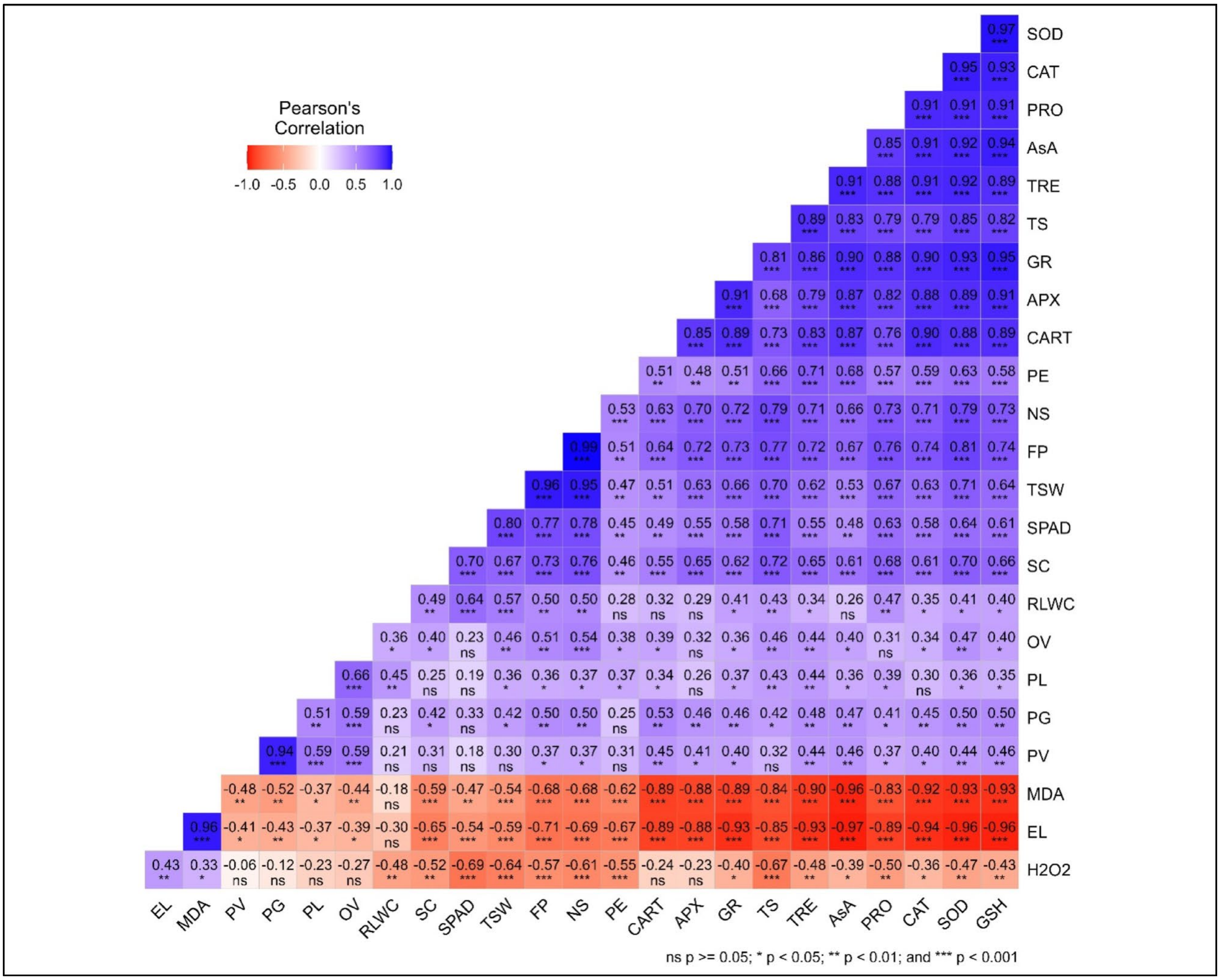

Fig. 14. Pearson corelation matrix for 24 traits in wild chickpea accessions during second year screening (2022–23). SPAD: Chlorophyll content, SC: Stomatal conductance, FP: Filled pods, TSW: Total seed weight, NS: Number of seeds,, PV: Pollen viability, PG: Pollen germination, PL: Pollen load, OV: Ovule Viability, PE: Photosynthetic efficiency, PRO: Proline, TRE: Trehalose, TS: Total sugars, CART: Carotenoids, RLWC: Relative leaf water content EL: Electrolyte leakage, MDA: Malondialdehyde content, H_2O_2: Hydrogen peroxide, SOD: Superoxide dismutase, CAT: Catalase, APX: Ascorbate peroxidase, GR: Glutathione reductase, AsA: Ascorbic acid, GSH: Reduced glutathione.

exhibited superiority in these traits under cold stress. These characteristics identified those accessions as more cold-tolerant, consistent with previous findings in cultivated chickpea[3,6,15]. Cold stress damaged chlorophyll, resulting in greater loss in cold-sensitive accessions, thereby causing a greater reduction in photosynthetic efficiency in sensitive genotypes[15]. Previous studies have indicated that cool-season grasses that maintain high chlorophyll levels during cold stress exhibit enhanced growth performance[38]whereas enhanced chlorophyll retention in mung bean is associated with reduced oxidative damage and improved cold tolerance[39]. Similarly, higher chlorophyll levels were observed in cold tolerant chickpea cultivars exposed to cold stress[40]. Our findings are consistent with those of previous studies demonstrating that cold-tolerant maize genotypes exhibit higher photosynthetic efficiency during cold stress, and consequently, improved grain yield[41]. Similarly, maintenance of elevated photosynthetic efficiency has been associated with enhanced cold tolerance in sweet potato[42] and rice[43]. In the present study, cold-tolerant chickpea accessions also exhibited higher carotenoid content than sensitive accessions, suggesting that these pigments may support better chlorophyll retention and membrane stability under cold stress, suggesting a possible auxiliary role in tolerance mechanisms[44]. Furthermore, the antioxidant capacity of carotenoids may enhance stress tolerance in chickpea plants[45].

The physiological traits utilized in the present study also indicated the involvement of additional mechanisms associated with cold tolerance in chickpeas. For instance, the relative leaf water content (RLWC) is directly influenced by root hydraulic conductivity, which can be adversely affected by cold stress[46]. In the present study, high-performing wild accessions maintained a higher RLWC, thereby reducing water loss and sustaining turgor pressure, which is essential for photosynthesis and growth[47]ultimately enhancing reproductive success and increasing pod and seed quantities, as well as seed mass. Similarly, wild chickpea accessions with higher stomatal conductance exhibited superior performance under cold stress, probably because of their increased efficiency in regulating water loss and maintaining hydration. Furthermore, enhanced stomatal conductance may have improved water-use efficiency, enabling plants to maintain leaf turgor and metabolic functions under cold stress.

Cryoprotectants as components of cold stress tolerance

Another category of biomolecules associated with cold tolerance in wild chickpeas is cryoprotectants such as proline, trehalose, and total sugars (present study). Wild accessions with improved reproductive success under cold stress accumulated significantly higher amounts of proline, sugars, and trehalose than low-yielding/cold-sensitive accessions, suggesting that these molecules are part of the key adaptive mechanisms in wild chickpea to mitigate cold stress. Proline, a crucial osmo- and cryoprotectant, potentially enhances cold tolerance in wild chickpea accessions by maintaining cell turgor and osmotic balance, scavenging ROS to reduce oxidative stress, and stabilizing proteins and cellular membranes under cold stress conditions, which are essential for cellular integrity[3,4]. Studies on other crops such as sorghum[48] and olive[49] have demonstrated that genotypes with higher proline content exhibit greater resilience to cold stress, supporting the hypothesis that proline accumulation enhances stress tolerance and yield potential.

Sugar accumulation in stressed plants, on the other hand, functions as a buffer to provide energy, sustain growth, and stabilize cellular components and membranes[50].Increased sugar accumulation has already been demonstrated to contribute to cold tolerance and reproductive success in rice[51] and chickpea[4,6]. While the present study focused on sugar accumulation at the physiological level, previous studies have identified sugar metabolism genes such as *UGPase*, *CWINs*, *GBSS2*, and *BAM3*as important components of the cold tolerance machinery in chickpea leaves and anthers[6,52] thereby supporting the physiological patterns observed here. Trehalose, an osmo- and cryoprotectant, facilitates the stabilization of proteins and cellular membranes during cold stress by inhibiting protein denaturation and maintaining membrane fluidity, which are essential for cellular functions under stress conditions[53]. Our study, and others on Brassica seedlings[53]rapeseed[54]and rice[55]indicate a key role of trehalose in mitigating cold stress.

Antioxidants in cold stress mitigation

Stress-induced induction of ROS in plants and their scavenging by a variety of antioxidants in tolerant genotypes are well-documented in the literature[4,15]. Malondialdehyde (MDA) and hydrogen peroxide are the most important indicators of cold stress in crop plants, reflecting degree of oxidative damage to cellular membranes and other cellular components[56,57]. The present study demonstrated lower MDA and H_2O_2 levels in accessions with higher reproductive success, whereas the opposite was true for accessions with low reproductive success, supporting the significance of antioxidants in maintaining cellular homeostasis, and promoting growth and yield stability. Increased MDA and H_2O_2 accumulation were consistent with previous findings in cold-stressed wild and cultivated chickpea[4,15]and wild clover[58] plants. Elevated MDA and H_2O_2 levels are associated with increased stress sensitivity and greater damage from ROS[57]. Studies on other crops, including rice[59]wheat[60]and chickpea[61]have corroborated the importance of managing oxidative stress to improve growth under cold conditions.

Cold tolerance is associated with the elevated expression of various antioxidants in diverse plant species, including rice[62] and maize[63]. High-performing wild chickpea accessions in our study exhibited increased SOD, CAT, APX, and GR activities, whereas low-performing accessions exhibited reduced activities, suggesting that elevated expression of these enzymes is an efficient ROS-scavenging system essential for mitigating oxidative stress at the cellular level. SOD serves as a primary defense mechanism against oxidative stress by catalyzing the conversion of superoxide radicals to hydrogen peroxide in cold-stressed plants[64,65]. Similar to chickpea, increased CAT activity under cold stress has also been observed in other plant species, such as rice[66]. APX reduces H_2O_2 using ascorbate as an electron donor, which is essential for maintaining the cellular redox balance under stress conditions[67]. Elevated APX activity enhances stress resilience, mitigates oxidative stress, preserves cellular functions[67]and enhances cold tolerance (present study[68]), . GR regenerates reduced glutathione, a key antioxidant that detoxifies ROS, thereby enhancing the antioxidant capacity of plants to cope with cold-induced oxidative stress[69]. Overexpression of GR genes in Arabidopsis and transgenic tomato plants improves cold tolerance by enhancing ROS scavenging[70]. A previous study demonstrated that cold-tolerant rice genotypes exhibit higher GR activity, which contributes to oxidative stress mitigation and cellular homeostasis[71]. Maize and rice also exhibited increased GSH content and GR activity under cold stress, supporting these findings[69]. Previous studies have also reported increased expression of these antioxidants under cold stress in various other crops, including chickpea[6]wild *Cicer microphyllum*[72]rice[71]and wheat[38]where cold-tolerant genotypes exhibited greater expression of antioxidants[73,74]consistent with our findings.

Reproductive traits and yield

The reproductive stage is particularly susceptible to the adverse effects of cold stress in all plants, including chickpea[6,73,75]. Maintaining reproductive functions under cold stress is essential for successful fertilization and seed set, which directly influence yield[75]. This study demonstrated greater reductions in susceptible accessions, whereas tolerant accessions retained efficient reproductive functions, resulting in improved yield-related traits under cold stress conditions. Effective germination of pollen under cold conditions is crucial for reproductive success[73]. Cold-tolerant chickpea[15] and rice[76] lines produced more pollen, a higher percentage of fertile pollen, a lower percentage of sterile pollen, and a greater quantity of seeds than the cold-sensitive lines. This robust performance in reproductive traits translates to increased seed production in high-performing wild genotypes. Similar trends have been documented for rice, demonstrating that genotypes with higher pollen viability and load produce more grains per spike when exposed to low temperatures[76]. Additionally, higher stigma receptivity and ovule viability in tolerant accessions indicate their superior capacity to maintain reproductive function despite adverse conditions, contributing to improved yield potential[75]. In wheat, higher stigma receptivity is correlated with improved fruit set and seed development, particularly under stressful conditions[77]. This study revealed a strong correlation between reproductive traits and yield-related parameters such as filled pods per plant, total seed weight per plant, and number of seeds per plant. High heritability estimates for reproductive traits indicated a strong genetic basis for these traits under cold stress, suggesting that selection for these traits could enhance cold tolerance in breeding programmes[2].

Correlations and principal component analysis (PCA)

Correlation studies revealed significant relationships among the measured traits. Notably, electrolyte leakage (EL) exhibited a highly negative correlation with most physiological traits such as chlorophyll content, photosynthetic efficiency, stomatal conductance, and yield-related traits. This finding suggests that accessions with lower EL values are better performers under cold stress, indicating that membrane integrity is crucial for maintaining physiological functions and overall plant health[45]. Furthermore, the positive correlations observed between physiological traits and yield-related traits support the importance of maintaining optimal physiological performance to enhance the yield potential. For instance, higher chlorophyll content and photosynthetic efficiency are associated with increased pod number and seed yield, supporting the idea that physiological traits are critical determinants of reproductive success and overall productivity in cold-stressed environments[73].

PCA provided a comprehensive overview of the relationships among various traits, revealing strong positive correlations among physiological, reproductive, biochemical, and yield-related traits. The PCA results indicated that the first few principal components (PC1 and PC2) accounted for a significant proportion of the total variability in the data, highlighting the importance of these traits in explaining the observed differences among accessions. The PCA graphs demonstrated a clear distinction between cold-tolerant and cold-sensitive accessions based on their trait profiles. Tolerant accessions clustered together and exhibited higher values for physiological traits, whereas sensitive accessions showed higher EL and MDA levels. The ability of some accessions to maintain lower EL and MDA levels while exhibiting robust physiological and reproductive traits is indicative of their potential cold tolerance.

Cluster analysis

The identification of cold-tolerant accessions in cluster 1 suggests that these accessions can serve as valuable genetic resources for breeding programs. Accessions exhibiting high pod set and seed yield under cold stress conditions are particularly promising candidates for selection. The high heritability estimates observed for yield-related traits, such as filled pods per plant and total seed weight, further support the potential for successful selection and introgression of favorable traits into cultivated chickpea varieties. The results of cluster analysis also highlight the importance of integrating physiological and biochemical assessments in the selection process. Accessions that cluster together based on their physiological performance under cold stress can be prioritized for further evaluation and breeding efforts. This targeted approach can enhance the efficiency of breeding programs by focusing on accessions that exhibit robust performance under adverse conditions.Although earlier studies have reported cold tolerance in *C. pinnatifidum* and *C. reticulatum*, the accessions evaluated in this study did not show significant tolerance, unlike several *C. judaicum* accessions, likely due to weakly expressed defence mechanisms. These findings indicate that cold tolerance in wild *Cicer* is accession-specific rather than species-wide, with *C. judaicum* accessions in this study potentially possessing more effective mechanisms for cold stress adaptation.

Conclusions

In the present study, we screened 36 wild chickpea accessions and identified promising cold-tolerant accessions based on their yield traits. These accessions were then subjected to an in-depth analysis to evaluate their responses to cold stress, involving a comprehensive assessment of physiological, biochemical, and reproductive traits. These findings highlight the importance of various physiological traits for conferring cold tolerance. Cold-tolerant accessions exhibited less cellular damage, coupled with a strong antioxidant defense. Additionally, the study highlighted the role of carotenoids in mitigating oxidative damage and the significance of cryoprotectants such as proline, trehalose, and carbohydrates in maintaining cellular stability. These traits collectively contributed to the ability of accessions to sustain reproductive success under cold stress, as evidenced by their superior reproductive traits and seed set. The insights obtained from this research provide a valuable foundation for breeding programs aimed at enhancing cold tolerance in cultivated chickpea varieties by utilizing the inherent resilience of the wild germplasm to improve crop performance under cold stress.

Data availability

The data is provided within the manuscript.

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Author contributions

S.K., D.P., U.J., K.D. performed the experiments, conduced analysis and prepared the manuscript. P.V., S.K.P., M.S., K.H.M. and H.N. reviewed the manuscript and finalized the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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