



Cold stress modulates osmolytes and antioxidant system in *Calendula officinalis*

Nelofer Jan¹ · Umer Majeed¹ · Khurshid Iqbal Andrabi¹ · Riffat John¹

Received: 20 October 2017 / Revised: 13 March 2018 / Accepted: 16 March 2018 / Published online: 22 March 2018
© Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków 2018

Abstract

Understanding the mechanism of adaptation to low-temperature stress is very crucial for developing cold-tolerant crop plants. The present study was used to investigate the response of *Calendula officinalis* (*C. officinalis*) to cold stress (CS). Seeds of *C. officinalis* were grown at normal temperature of 25 °C for 14 days and then shifted to a growth chamber set at 4 °C. The response of *C. officinalis* seedlings to cold stress was evaluated by estimating the relative growth changes, chlorophyll, electrolyte leakage (EL) and the content of malondialdehyde (MDA), proline, glycine betaine (GB), total soluble sugars, trehalose, total protein content and fatty acid profile. Enzymatic activity of antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) was also assessed. Moreover, transcript expression of cold-responsive genes [APX, CAT, dehydration-responsive element-binding protein (*DREB1*), GR and SOD] was also evaluated on homology basis. We measured and compared these indices of seedlings leaves under low temperature (4 °C) and normal temperature (25 °C) and observed that on exposure to CS, *C. officinalis* shows higher accumulation of osmoprotectants (proline, soluble sugars, glycine betaine and trehalose), phenolics and proteins, and increased antioxidant enzyme activity (APX, GR and SOD) except CAT activity, which declined in cold-stressed plants. Transcript expression of cold-responsive genes (SOD, CAT, APX, GR and *DREB1*) was found to be upregulated under cold stress. Overall, our study suggests that cold exposure/CS, besides eliciting various biochemical/physiological responses, triggers various pathways causing differential gene expression, consequently leading to differential protein expression. Further, this is the first report of *C. officinalis* under cold stress that may help us in exploring the mechanism of cold tolerance in future.

Keywords Cold stress · Proline · Glycine betaine · *DREB1*

Introduction

Low temperature is a major abiotic stress affecting plant growth and development, and limiting their spatial distribution across the globe (John et al. 2016; Sanghera et al. 2011). Generally, overwintering plants from temperate regions on brief exposure to low temperature during their life cycle acquire tolerance to subsequent chilling/cold stress, a phenomenon called ‘cold acclimation’. Cold acclimation is a complex and energy-intensive process that involves multifaceted biochemical changes and intricate signal transduction and modulation of gene expression (Zuther et al. 2015).

Cold stress (CS) elicits diverse responses in plants that range from modulated gene expression to differential growth pattern and morphology. Based on the physiological and molecular changes during low temperature/CS, many tolerance mechanisms have been proposed (Arbona et al. 2017). Plants have the unique ability to modify metabolic processes in response to different environmental perturbations, and it has been observed that during CS the metabolism is modified to adjust to low temperature by producing osmolytes such as glycine betaine (GB) and proline (Pro) that stabilize cellular structures and proteins and/or maintain the cellular turgidity by redox metabolism and osmotic adjustment (Janska et al. 2010). The total soluble sugars in plants are highly sensitive to various environmental cues. They play dual role as signaling molecules and as substrates in cellular respiration and/or also act as osmolytes for maintaining cellular homeostasis (Janmohammadi 2012). Levitt (1980) proposed that the cell membrane is the main target of cold

Communicated by J. Gao.

✉ Riffat John
riffatminhaj@kashmiruniversity.ac.in

¹ University of Kashmir, Srinagar, India

stress. The reduction in temperature also alters the saturated to unsaturated fatty acids ratio and ensures the transformation of membrane lipids from liquid to crystalline state into the solid state in plants (Steponkus 1984; Murata and Los 1997). The conversion of fatty acid in the plant cells may provide chilling resistance to plants at low temperatures (Karabudak et al. 2014).

Under normal conditions, plant cells have steady-state level of ROS and the redox state; however, various stress conditions including CS leads to the overproduction of ROS (Suzuki and Mittler 2006). Although ROS have dual role as being cytotoxic compound as well as stress-signaling molecule in plants (Raja et al. 2017), plants under cold stress quench the excessive ROS by enhancing the activities of various antioxidant enzymes, such as SOD, CAT, APX and GR (Tahmasebi and Pakniyat 2015). Synthesis of these antioxidants may be a part of a complex mechanism of low-temperature stress involving both protection against ROS production and avoidance of ROS production (Canev et al. 2012). SOD belonging to a group of metalloenzymes converts the superoxide (O_2^-) to H_2O_2 and O_2 . H_2O_2 is neutralized through the activity of the Asada-Halliwell scavenging cycle operating in the chloroplast and cytosol. The Asada-Halliwell cycle, activating various enzymes such as APX, GR, MDHAR and DHAR, leads to the reduction and oxidation of glutathione and ascorbate (Apel and Hirt 2004). APX causes the reaction between ascorbic acid and H_2O_2 , and GR leads to ascorbic acid regeneration. CAT also reduces H_2O_2 to water, but has lower affinity to H_2O_2 compared with APX (Choudhary et al. 2016).

In response to CS, gene expression gets altered and epigenetic regulation has a critical role in regulating the gene expression (Khraiweh et al. 2012; John et al. 2016). Dehydration-responsive element-binding proteins (*DREBs*), being important transcription factors, regulate the expression of various downstream stress-inducible genes in association with *DRE/CRT cis*-element of many abiotic stress-responsive genes (Xiong and Fei 2006). So far, the best characterized key regulatory pathway for cold stress signalling is *CBF/DREB1*-dependent cold-signaling pathway (Miura and Furumoto, 2013). The *CBF/DREB1* genes being transiently and quickly elicited by CS activate the target genes involved in low temperature/freezing tolerance and cold acclimation (Shinozaki et al. 2003).

It is estimated that we can increase crop yield up to 70% under optimum environmental conditions, and one of the main factors affecting plant productivity and yield is cold/freezing stress (Tuteja and Gill 2016).

Calendula officinalis (Asteraceae) is an annual plant grown worldwide as an ornamental (Martin and Deo 2000) and medicinal plant containing different active phytochemicals of therapeutic importance (Maysa et al. 2015; Asif 2015). *C. officinalis* flourishes well in temperate

climate, growing throughout the year at high altitudes of the Kashmir Himalayan region (Dar et al. 2014; Jan and John 2017). Interestingly, the plant flowers even in the harsh winter season of Kashmir and has not been explored much for its response to low temperature/cold stress. In the present study, *C. officinalis* was subjected to two temperatures: cold (4 °C) and 25 °C as control/reference to understand the mechanism of cold acclimation/CS tolerance by evaluating changes in compatible osmolytes, fatty acid profile, level of antioxidant enzymes and change in gene expression pattern of selected cold stress-related genes.

Materials and methods

Plant material and growth condition

Seeds of *C. officinalis*, obtained from Kashmir University Botanical Garden (KUBG), Srinagar, were thoroughly washed and sown in plastic pots containing autoclaved farm soil and sand mix (3:1). The seeds germinated and grew in a growth chamber (Blue Star, ICo No: NKL-750) under a controlled condition with temperature of 25 ± 2 °C, 70% relative humidity and 16 h light/8 h dark photoperiod. After 14 days of germination, the pots containing seedlings were divided into two sets. One set (control) was allowed to grow continuously at 25 °C, while the other set was exposed to 4 °C (Fig. 1). Shoots were harvested after an interval of 24 h, i.e. 24, 48, 72, 96 and 120 h from both the control and cold-stressed plants and flash frozen at -80 °C until further analysis. However, the fresh tissue was used for measurement of root growth and shoot growth and estimation of various physio-biochemical parameters including chlorophyll and carotenoid estimation, relative electrolytic leakage and fatty acid profile. All the experiments were done in triplicate and also repeated thrice.

Estimation of shoot and root growth rate

The main shoot length and primary root length were measured from the stem-root transition zone to the tip of the shoot and root, respectively (Fig. 2). For both, shoot and root, the growth was expressed in terms of relative growth rate, which was measured by $(\text{the length under cold stress} / \text{the length under control}) \times 100$ (Wang et al. 2015).

Estimation of pigments

Chlorophyll and carotenoid contents were determined using the protocol described by Lichtenthaler and Wellburn (1983) and absorbance was read against blank (80% acetone) at 663 and 645 nm, and 470 nm for chlorophyll and carotenoid, respectively, on (UV-VIS)

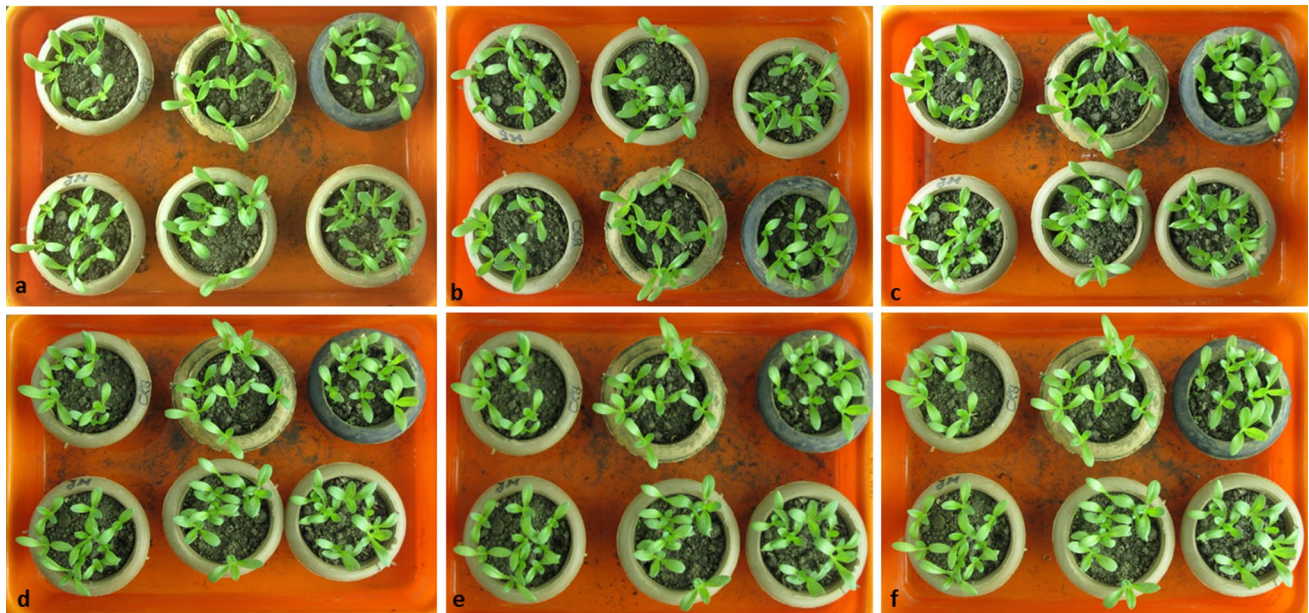


Fig. 1 *Calendula officinalis* seedlings under different temperature regimes. **a** Control (25 °C) and **b** 24 h, **c** 48 h, **d** 72 h, **e** 96 h and **f** 120 h of 4 °C cold stress



Fig. 2 Growth of 14-day-old *Calendula officinalis* seedlings under different time periods of normal temperature (**a** 24, **b** 48, **c** 72, **d** 96 and **e** 120 h) and different treatment periods of 4 °C cold stress (**f** 24, **g** 48, **h** 72, **i** 96, **j** 120 h) with a common control (control)

spectrophotometer (Shimadzu, Japan). The total pigment content was calculated using the following formulas (Rahimi et al. 2016):

$$\text{Total chlorophyll} = (19.3 \times A_{663} - 0.86 \times A_{645}) V/100 W + (19.3 \times A_{645} - 3.6 \times A_{663}) V/100 W,$$

$$\text{Carotenoids} = 100 (A_{470} - 3.27 (\text{mg chl. } a) - 104 (\text{mg chl. } b)/227,$$

where A is the absorbance of light at wavelengths of 663, 645 and 470 nm, V the volume of the supernatant and W the fresh weight of the sample (grams).

Estimation of lipid peroxidation and relative electrolytic leakage (REL)

The end product of lipid peroxidation, i.e. malondialdehyde (MDA), was measured following the method described in de Azevedo Neto et al. (2006) and absorbance of the supernatant was read at 600 and 532 nm by a UV–VIS spectrophotometer (Shimadzu, Japan). The MDA content was calculated with an extinction coefficient (155/mm²/cm).

The REL assay was determined according to Yan et al. (2006) with a slight modification. The leaves were first rinsed with deionized water. The rinsed leaves (0.5 g) were placed in deionized water for 3 h at normal temperature. The conductivity was read using a conductivity meter. The conductivity was again measured after incubating the samples at 90 °C for 2 h. The total electrical conductivity (REL) was then expressed as the ratio of initial and final conductivity.

Proline assay

Proline quantification was done according to Bates et al. (1973) and optical density was read spectrophotometrically at 520 nm in UV–VIS spectrophotometer (Shimadzu, Japan) using toluene as a blank.

Estimation of glycine betaine

Glycine betaine was determined according to Greive and Grattan (1983) and absorbance was measured at 365 nm by UV–VIS spectrophotometer (Shimadzu, Japan).

Estimation of protein

Protein was extracted according to Subba et al. (2013) and later quantified according to Bradford (1976). The protein concentration was estimated by quantification of Coomassie Brilliant Blue G-250 binding to unknown protein compared to the known standard (bovine serum albumin).

Estimation of trehalose

Trehalose content was determined by using megazyme kit (K-TREH 02/16). For estimation of trehalose content, 0.2 mL of sample solution was added to a reaction mixture containing solution 1 (buffer pH 7), solution 2 (NADP⁺/ATP), suspension 3 (HK/G-6-PDH) and distilled water and the absorbance (A_1) was read at 340 nm. To this reaction mixture, suspension 4 (trehalase) was added and absorbance (A_2) was again read at 340 nm. The trehalose content

is measured by increase in the absorbance of NADPH at 340 nm by UV–VIS spectrophotometer (Shimadzu, Japan).

Estimation of total sugar

Total sugar was estimated according to Dey (1990) and the absorbance was measured at 485 nm using UV–VIS spectrophotometer (Shimadzu, Japan). Amount of the sugar produced was calculated from the standard curve that was prepared by using sucrose solution.

Estimation of phenolic content

Phenolic content was estimated by the modified method of Apak et al. (2008) with a slight modification. The plant material (50 mg) was homogenized in 80% acetone and centrifuged at 10,000g for 10 min. To 100 μ L of supernatant, 2 mL distilled water and 1 mL folin reagent was added and shaken vigorously. To this mixture, 20% sodium carbonate was added and the volume was made up to 10 mL with distilled water. The absorbance was taken in UV–VIS spectrophotometer (Shimadzu, Japan) at 750 nm.

Estimation of free fatty acids

Fatty acids were extracted from fresh leaf tissue and analysed by gas chromatography (GC) according to the method described in Cyril et al. (2002).

Antioxidant enzyme activity assay

For enzymatic antioxidants, the extraction was done according to Hossain et al. (2013). 0.5 g of plant material was homogenized in 5 mL of 50 mM ice-cold potassium phosphate buffer (pH 7.0) which contained 100 mM potassium chloride, 5 mM β -mercaptoethanol, 1 mM ascorbate and 10% (w/v) glycerol. The homogenate was centrifuged at 11000g for 15 min at 4 °C and the supernatant was used in enzymatic assay after total protein estimation. For antioxidant enzyme activity, equal quantity of the total protein was taken for each assay performed.

Superoxide dismutase (EC 1.15.1.1)

The SOD activity was examined according to Zhang et al. (2005) with further slight modification. For analysis of SOD activity, the reaction containing a mixture of 1.5 mL phosphate buffer (pH 7.8), 0.3 mL 750 μ mol/L nitroblue tetrazolium chloride (NBT), 0.3 mL 130 mmol/L methionine, 0.3 mL 100 μ mol/L EDTA-Na₂, 0.3 mL 20 μ mol/L riboflavin and 0.05 mL of enzyme extract was placed under two fluorescent lamps (15 W) for 15 s for reaction to occur. The reaction was then quenched by placing the tubes containing

the reaction mixture under dark for a period of 5 min and absorbance was measured at 560 nm in a UV–VIS spectrophotometer (Shimadzu, Japan). A unit of SOD activity is described as the quantity of enzyme that is required to cause 50% inhibition of reduction of NBT and the specific activity of enzyme was expressed in terms of units per mg protein/min.

Catalase activity (EC 1.11.1.6)

The CAT activity was determined according to Zhang et al. (2005) with slight modification. For CAT activity, the reaction containing a mixture of 2.25 mL of 0.05 M sodium phosphate buffer (pH 7.8), 0.45 mL 0.1 M H₂O₂, 1.5 mL deionized water and 0.05 mL of the enzyme extract was used. The enzyme activity was estimated by monitoring the decreasing absorbance at 240 nm due to H₂O₂ consumption and its activity was expressed in terms of units per mg protein/min.

Ascorbate peroxidase (EC 1.11.1.11)

APX activity was determined as described in Harb et al. (2015). 40 µL of the plant extract was used in the reaction containing a mixture of 25 mM potassium phosphate buffer (pH 7.0), 0.25 mM ascorbate, 0.1 mM EDTA and 1 mM H₂O₂. The enzyme activity was determined by measuring the decline in absorbance at 290 nm for 1 min and activity was expressed in terms of units per mg protein/min.

Glutathione reductase (EC 1.8.1.7)

The GR activity was determined according to the method of Halliwell and Foyer (1978) with some modification. For analysing GR activity, 0.06 mL enzyme extract was added to the reaction containing a mixture of 0.5 mM glutathione, 950 µL of 0.15 mM NADPH and 3 mM MgCl₂ in 50 mM Tris (pH7.5) and the activity was measured by monitoring NADPH oxidation by glutathione at 340 nm in UV–VIS spectrophotometer (Shimadzu, Japan).

Expression analysis of antioxidant genes

RNA extraction and cDNA synthesis

Trizol reagent (Invitrogen, Carlsbad, CA) was for total RNA extraction. The extracted RNA was analysed for quality by agarose gel electrophoresis and quantified by spectrophotometric analysis. The first strand of complementary DNA (cDNA) was prepared with 2 µg of RNA using SuperScript cDNA Synthesis Kit (Invitrogen).

Semi-quantitative PCR

Primers for the cold-induced genes were ordered from Integrated DNA Technologies (IDT), USA (Table 1). For each gene, a PCR mixture of 20 µL was used containing 1× PCR buffer, gene-specific primer (forward and reverse, 2.5 mM), 1× Taq DNA polymerase, nuclease-free water and 1 µL cDNA. The PCR programme with 35 cycles of initial denaturation (95 °C for 5 min), denaturation (95 °C for 30 s), annealing (specific temperature for specific gene for 30 s), extension (72 °C for 40 s) and final extension (72 °C for 10 min) was used for amplification of genes that were amplified (Fig. 5).

Quantitative real-time RT-PCR

Quantitative PCR (q-PCR) was carried out using the Light Cycler (Roche) with the Light Cycler Fast Start DNA Master SYBR Green kit (Roche) for the amplification of SOD, APX, CAT, GR and *DREB1* gene using the manufacturer's protocol. Each reaction contains 12.5 µL SYBR Premix, 1.5 µL cDNA, 0.3 µL of primer (forward and reverse) and 5.4 µL dH₂O with a final total volume of 20 µL. The appropriate primers were used (Table 1) and all the genes were tested in triplicate along with tubulin, used as a reference gene. The data of gene expression was calculated relative to α-tubulin and C_t values of the used target genes were normalized using the C_t values of tubulin. The levels of mRNA were also normalized with tubulin and its value was expressed relative to that of control (0 h), which was given an arbitrary value 1 (Liu et al. 2012). The relative differential gene expression

Table 1 Sequences of primers and their corresponding gene accession number used in quantitative real-time PCR

Gene	Genebank accession no.	Forward (5'–3')	Reverse (5'–3')
CuZn-SOD	EF495351.1	GCACCATCCACTTCACCCAA	CCCTGAAGTCCAATGATCCCA
CAT	L28740.1	TCAAGTGCCATAATGCCCC	CAGCACATGTGAGGGCAGTA
APX	EF495352.1	AGAGTTCCCCATCCTCTCGT	GCCTTCTCATCCGCAGCATA
GR	AB158514.1	ATTTGGGCCGTTGGAGATGT	ACTCTATCGGGAAGGCCTGA
<i>DREB1</i>	NM001247760.1	GGGGTAAATGGGTTGCTGAGA	AAGAGCACCATCAACCGGAC
Tubulin	XM018616849.1	GATAACTGTACTGGACTGCAAGG	GGATGGCTTCGTTATCCAAGAG

was measured according to the equation $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001). Data were derived from three experimental replicates.

Statistical analysis of data

The significant difference of data was analysed by two-way analysis of variance, and Pearson's correlation coefficient was used for the correlation analyses of data using SPSS 16.0 software. The data were analysed for three replicates and standard error (SE) was calculated. The temperature stress and stress duration were taken as different plots and data were depicted as mean \pm SE.

Results

Shoot and root growth rate

No significant difference in the shoot growth rate was found between CS and control plants. However, in contrast root growth rate showed a significant increase with increased stress duration as observed at 48 and 120 h of cold stress, and the maximum increase in the root growth rate (1.839%) was observed at 120 h duration (Table 2).

Change in chlorophyll and carotenoid content

The chlorophyll content showed a slight increase with increase in the duration of stress. However, significant increase was seen only after 48 h (13.91%) and 72 h (21.64%), and further exposure to CS did not yield any significant change (Fig. 3a). Carotenoid content increased significantly at 48 h (16.27%), 72 h (34.88%) and 96 h (48.83%), remaining constant at 120 h. With increased stress duration from 24 h, the content showed significant increase up to 96 h and remained unchanged with further exposure to CS (Fig. 3b).

Table 2 Effect of cold stress on relative shoot growth and root growth of *C. officinalis*

Time period	Relative shoot growth	Relative root growth
Control	1 \pm 0.00 ^a	1 \pm 0.00 ^a
24 h	1.222 \pm 0.039 ^b	1.113 \pm 0.044 ^a
48 h	1.292 \pm 0.021 ^b	1.558 \pm 0.031 ^b
72 h	1.267 \pm 0.066 ^b	1.532 \pm 0.019 ^b
96 h	1.200 \pm 0.015 ^b	1.595 \pm 0.055 ^b
120 h	1.185 \pm 0.007 ^b	1.839 \pm 0.035 ^c

^a, ^b and ^c represents the significant difference

Change of malondialdehyde (MDA) content and relative electrolytic leakage

Calendula seedlings exhibited a slight increase in MDA content at 24 h of low-temperature stress exposure (16.79%) with a slight decrease at 48 h (12.21%). The continued cold stress led to the decline in MDA content and then remained constant at the value as that of control plants (1.314 μ g/g fwt) (Fig. 3c). The REL showed the same trend as MDA content. We observed significant increase in REL after 24 h (11.788%) and decrease at 48 h (11.641%), though the decline was non-significant. Later continued exposure did not affect the REL much and level was found same as that of control plants grown at 25 °C (11.438%) (Fig. 3d).

Change in free proline content

The proline content showed an increased accumulation with increase in the duration of CS compared to control plant grown at 25 °C (73.318 μ g/g fwt). We observed the proline accumulated significantly right from 24 h (31.96%), 48 h (55.68%), 72 h (103.11%) and 96 h (363.96%) and reached the maximum (396.95%) at 120 h stress (Fig. 3e).

Change in glycine betaine

The amount of GB, an important osmolyte, initially increased and then declined on exposure to 4 °C. The content increased significantly after 24 h (40.81%), 48 h (75.88%) and 72 h (115.15%), but showed a significant decline after 96 h (71.63%) and 120 h (29.64%) reaching the same level as that of the control plants growing at 25 °C (Fig. 4a).

Change in protein content

The protein content increased significantly after 24 h (5.82%), 48 h (16.01%) and 72 h (18.2% fwt) of CS, remaining constant after 96 and 120 h of low-temperature stress (18.68%) (Fig. 4b). Statistical analysis also showed that protein content was higher at all the levels compared with the control plants grown at 25 °C (4.121 μ g/g fwt).

Change in trehalose content

Trehalose content remained initially constant on exposure to CS and content was same as that of control plants grown at 25 °C. However, the trehalose elevated significantly on continued exposure to CS in *C. officinalis* as we observed increase in trehalose content up to 48.73% (48 h), 90.01%

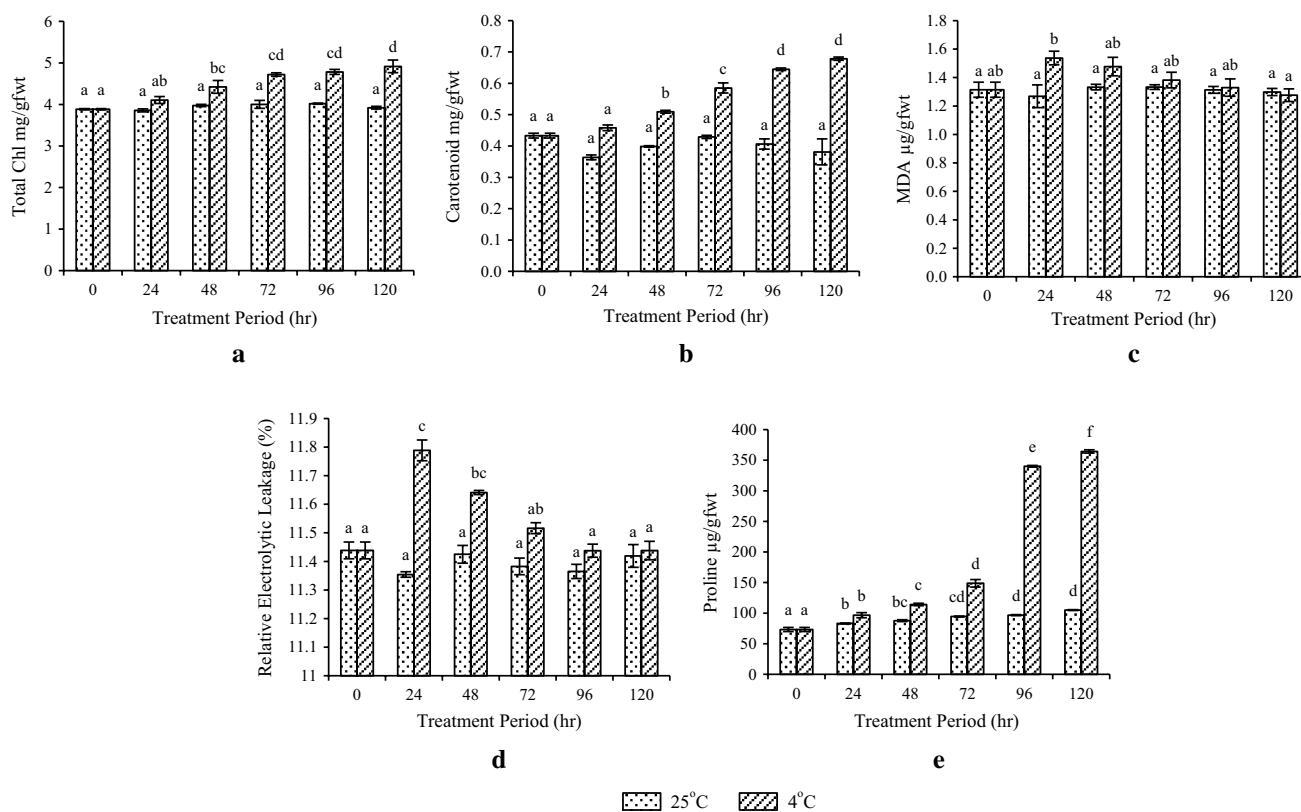


Fig. 3 Effect of cold stress on total chlorophyll, carotenoid, MDA, relative electrolytic leakage and proline content of *C. officinalis* (a–e). Values are the mean of three replicates with standard error. Error bars

represent standard error and the different letters indicate the significant difference between the control and 4 °C, and between different time points at $p < 0.05$

(720 h), 162.53% (96 h) and the highest level of 387.56% at 120 h of CS (Fig. 4c).

Change in sugar content

The sugar content significantly increased in seedlings of *C. officinalis* when grown at 4 °C and kept on increasing with the duration of cold stress and then later remained constant. We observed significant increase after 24 h (35.91%) of cold stress, but remained almost stable/unchanged at 48 h. Again, sugars increased significantly after 72 h (89.5%) and then remained constant at 96 and 120 h stress (Fig. 4d).

Change in phenolic content

The phenolic content remained almost constant at 24 h of low-temperature stress, but increased significantly after 48 h (58.27%), 72 h (76.38%), 96 h (200.02%) and 120 h (241.74%) when compared with control. The phenolic content showed a slight increase between control and 24 h, 24 and 48 h, and between 48 and 72 h. However, there was a linear and significant increase at 96 and 120 h stress (Fig. 4e). So, the level of total phenolic content is higher

for low-temperature treatment compared to control. The level of total phenolic content, higher in 4 °C treatment, suggests that cold stress leads to an increased accumulation of phenolics. This increase in phenolic content under low-temperature stress increases the antioxidant activity (free radical-scavenging activity).

Change in fatty acid composition

The analysis of total fatty acid revealed the presence of cerotic, palmitic and behenic acid. In control, the most abundant fatty acid is behenic acid followed by palmitic acid. These saturated fatty acids showed a decreased level with increased stress duration. The cerotic acid showed a 2.26–1.60% decline from 24 to 120 h duration of stress, but do not appear in control. The palmitic acid decreased from 0.89 to 0.21% in the control and 48 h and does not appear with later increase in stress duration. Behenic acid also showed a uniform decrease (2.38–0.68%) with increased stress duration, but exhibited a slight increase at 120 h (0.90%) (Fig. 5).

However, unsaturated fatty acids appear with increase in the duration of stress. 11,14,17 eicosatrienoic acids

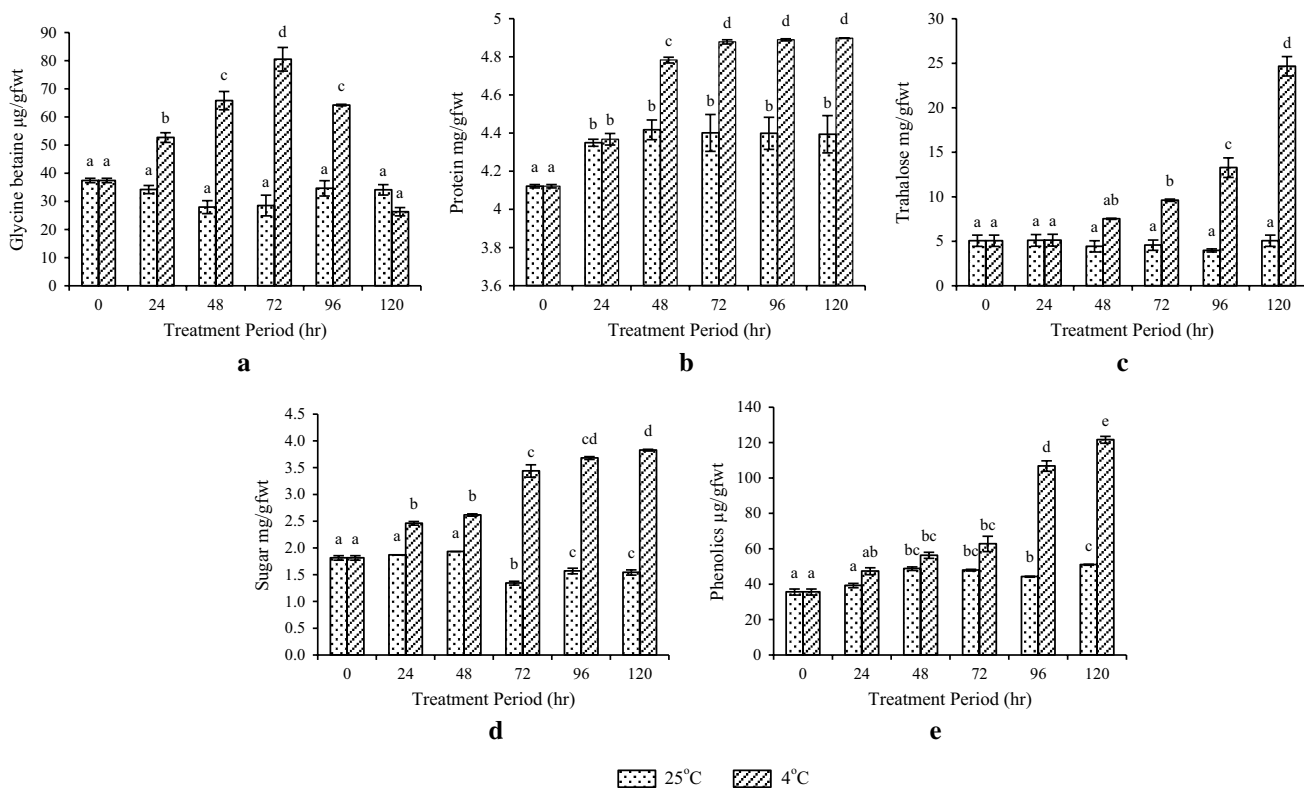


Fig. 4 Effect of cold stress on glycine betaine, protein, trehalose, total soluble sugar and phenolic content of *C. officinalis* (a–e). Values are the mean of three replicates with standard error. Error bars represent

standard error and the different letters indicate the significant difference between the control and 4 °C, and between different time points at $p < 0.05$

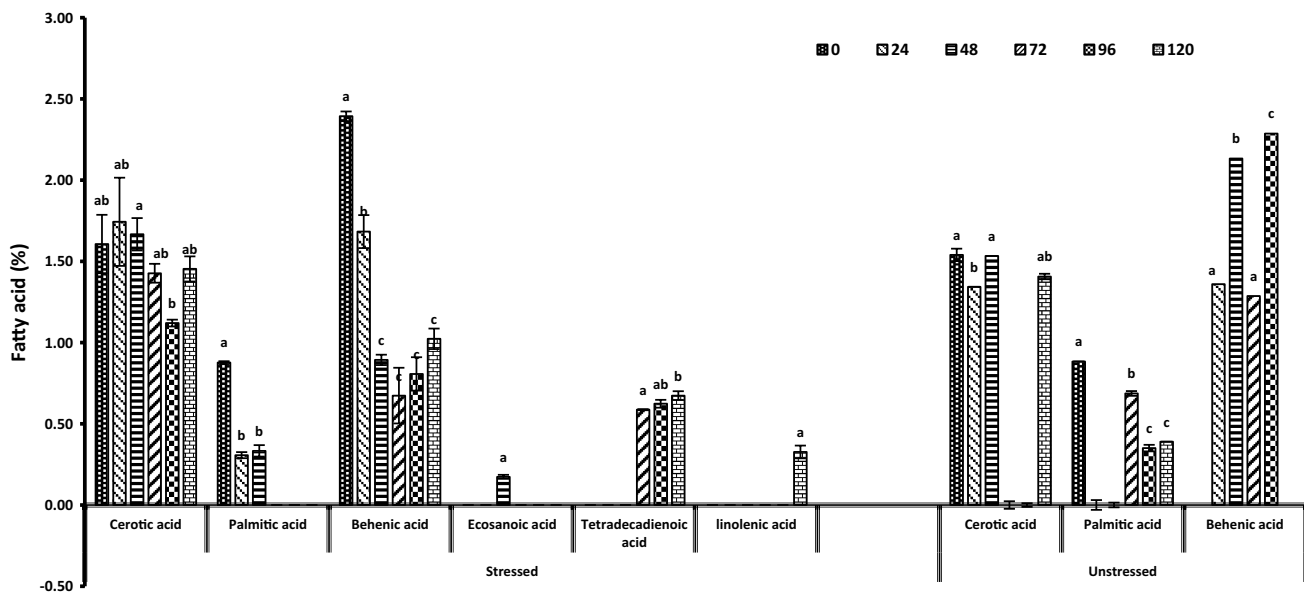


Fig. 5 Effect of cold stress on the fatty acid composition of *C. officinalis*. Values are the mean of three replicates with standard error. Error bars represent standard error and the different letters indicate

the significant difference between the control and 4 °C, and between different time points at $p < 0.05$

accumulated (0.15%) at 48 h duration of stress, α -linolenic acid appeared at 120 h duration of stress (0.25%) and 8,12-tetradecadienoic acid appeared at 72 h (0.58%) duration of stress and later showed a uniform increase in accumulation with increased stress duration (0.59 and 0.62% at 96 and 120 h stress duration) (Fig. 5).

Change in antioxidant enzyme activities

We evaluated the response of *C. officinalis* to cold stress in terms of activity of various antioxidant enzymes. SOD activity showed significant increase after 24 h (25.03%) of low temperature, but remained almost unchanged at 48 h. The SOD activity again increased sharply and linearly after 72 h (31.04%), 96 h (40.44%) and 120 h (46.25%). Thus, SOD activity showed significant increase with increased duration of cold stress (Fig. 6a). CAT activity decreased gradually with increased duration of stress. The CAT activity started to decrease significantly at 24, 48, 72 and 96 h by 7.7, 16.18, 22.06 and 25.33%, respectively, and remained almost

constant at 120 h (Fig. 6b). Statistical analysis revealed that CAT activity decreased significantly at all levels compared to the control plants grown at 25 °C.

The activity of APX enhanced significantly at 24, 48, 72 and 96 h by 71.25, 78.96, 80.25 and 82.72%, respectively, and reached the highest level at 120 h (3.05 U/mg protein/min) compared to the control (Fig. 6c). The GR activity exhibited an increase with increased stress duration. The GR activity initially increased significantly at 24 h (42.96%), remained constant at 48 h, then increased significantly and then increased significantly and linearly at 72 (134.77%), 96 (151.14%) and 120 h stress (161.25%) (Fig. 6d).

Change in gene expression

The gene expression profile of antioxidants exhibited a rapid increase in the transcription of SOD, APX, CAT and GR at 24 h. SOD increased 3.99-fold at 24 h of low-temperature exposure, but remained almost constant at 48 h and showed a slight decrease (2.41-fold) in expression at 72 h and then

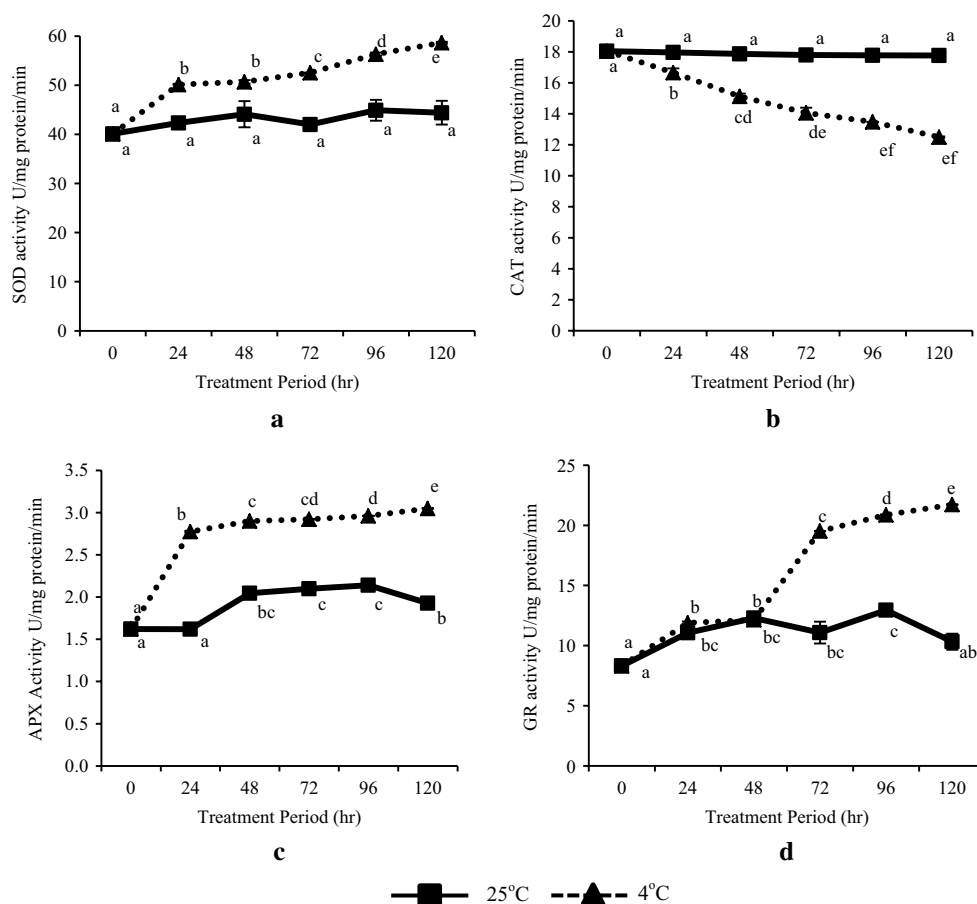


Fig. 6 Effect of cold stress on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities of *C. officinalis* (a–d). Values are the means of three repli-

cates with standard error. Error bars represent the standard error and the different letters indicate the significant difference between the control and 4 °C, and between different time points at $p < 0.05$

remained constant at 96 and 120 h of low-temperature stress. CAT and APX expression increased (14.89- and 10.97-fold, respectively) at 24 h and decreased significantly at 48 h (8.86- and 5.00-fold, respectively) remaining constant with

later increase in stress duration. However, GR showed a gradual increase in its expression from 24 h (6.95-fold) up to 96 h (10.05-fold) and remained constant at 120 h of cold stress. *DREB1* transcripts accumulated gradually with the

Fig. 7 Semi-quantitative PCR of cold-responsive genes in *C. officinalis* under different time periods of cold stress (4 °C). The bands represent a change in gene expression with reference to internal control (tubulin)

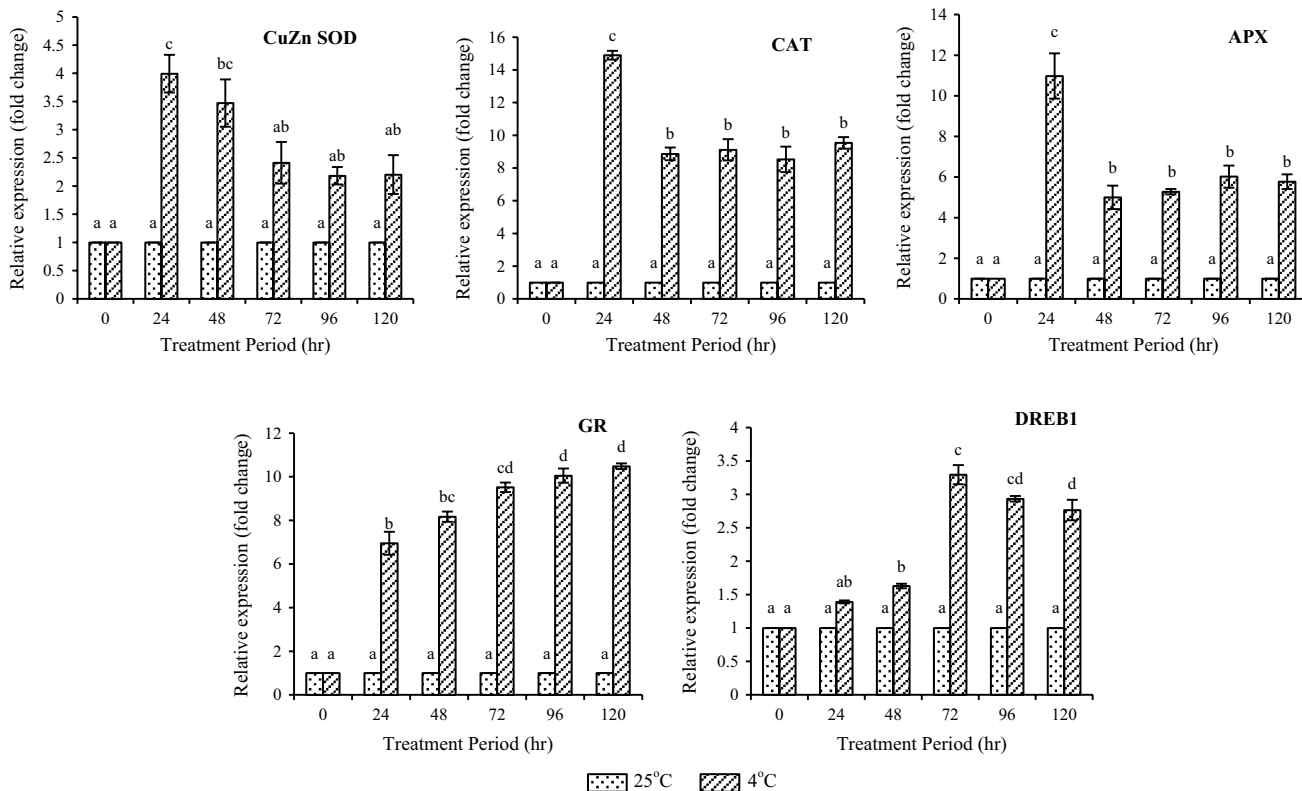
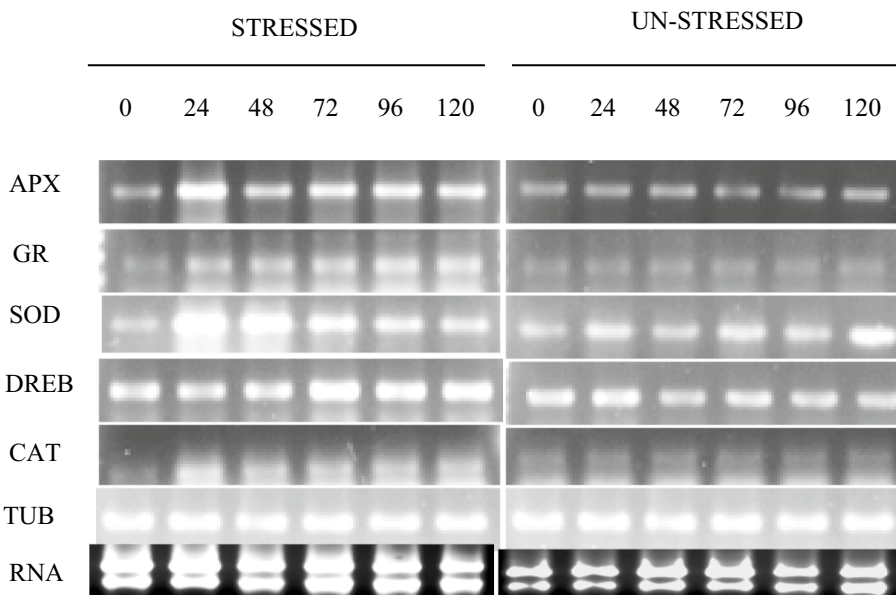


Fig. 8 Quantitative real-time PCR of different cold-responsive genes in *C. officinalis* under different time periods of cold stress (4 °C). Values are the mean of three replicates with standard error. Error bars

represent standard error and the different letters indicate the significant difference ($p < 0.05$)

highest expression at 72 h (3.29-fold) of cold stress and then declined slightly and gradually at 120 h (Figs. 7, 8).

Discussion

Low temperature/CS elicits a wide variety of responses in *C. officinalis* as it responds to CS by altering its morphology, biochemical profile, antioxidant enzymes, fatty acid profile and gene expression. We observed significant changes in the root and shoot growth rate in 14-day-old *C. officinalis* seedling at 4 °C as compared to those grown at 25 °C. Root developmental morphology of plants is associated with its CS tolerance (Presterl et al. 2007; Hund et al. 2004) and it has also been suggested to be used as criterion/marker for CS tolerance (Chassot and Richner 2002). Generally, plants adapted to cold environments by natural selection tend to have high root/shoot ratio (Janska et al. 2010), as we observed that root growth is favoured relative to shoot growth in *C. officinalis* and can be due to the fact that longer roots explore the soil more thoroughly for water (Hsiao and Xu 2000). In maize, the varieties having an increased root/shoot growth ratio under chilling stress were found to be more cold tolerant (Di Fenza et al. 2017) and longest root lengths have been also reported in various corn hybrids under low-temperature stress (Wijewardana et al. 2016). Moreover, overexpression of NAC transcription factor (GmNAC20) in *Arabidopsis* enhanced freezing tolerance and promoted lateral root formation (Hao et al. 2011). The root system offers an important system for studying the effect of environmental cues on the growth and development of plants (Malamy 2005; Ostonen et al. 2007), and a well-developed root system has been found to be very crucial for cold tolerance of plants (Hund et al. 2007; Lee et al. 2002).

We found significant increase in chlorophyll after 48 and 72 h on exposure to CS and carotenoids too got elevated significantly after 48, 72 and 92 h of 4 °C exposure to CS as compared to control plants grown at 25 °C in *C. officinalis*. The reduced photochemical inactivation of the photosystem II complex occurs in response to CS which simultaneously leads to the elevation of GB (Hayashi et al. 1997) and, since GB is synthesized in chloroplast, its accumulation accelerates the recovery of photosystem II from the photo-inhibited state (Hayashi et al. 1997). Cold tolerance involves the increased accumulation of chlorophyll and reduced photosynthetic sensitivity (Sanghera et al. 2011). Increased chlorophyll accumulation has been observed in cold-tolerant lines of rice (*japonica*) than cold-sensitive lines (*indica*) (Sanghera et al. 2011). Previously, it was reported by Haldimann (1999) that the chilling-tolerant maize genotypes exhibited higher accumulation of chlorophyll and carotenoid than the sensitive ones. Bano et al. (2015) had also reported three cold-tolerant maize accessions (EV-134, B-304 and

F-151) showing higher chlorophyll contents with outstanding photosynthetic activity. All these studies are consistent with our results.

Malondialdehyde (MDA), a degradation product of lipid peroxidation, and REL increased after 24 h, but then showed a declining trend after the 48 h time point as compared to plants grown at 25 °C. The key sites of injury during chilling/CS are thylakoids and plasma membrane (Leshem 1992), the observed initial increase in MDA and REL and then decline points to the reversible damages in biophysical characteristics of membrane due to CS (Campos et al. 2003). The constant level of MDA and REL at later stages of CS may be due to an effective antioxidant mechanism preventing the lipid peroxidation, i.e. production of MDA (Wu et al. 2014) and electrolyte leakage, as our statistical analysis showed that MDA level and REL have a negative correlation with almost all the antioxidants, suggesting the increase in the level of antioxidants with increased duration of CS inhibits the membrane damage in *C. officinalis*.

Plant are capable of accumulating non-toxic compatible solutes such as proline, sucrose, GB, etc. to withstand osmotic stress, which is a very common feature of various abiotic stresses such as drought, salinity, low- and high-temperature stress and metal stress (Gill et al. 2014). We observed accumulation of proline on exposing 14-day-old *C. officinalis* to 4 °C through all the time points. Increased free proline maintains osmotic equilibrium between the symplast and apoplast and helps in resisting low-temperature injury by maintaining the functional integrity of cellular membranes besides directly acting as ROS scavenger and regulator or cellular redox (Szabados and Savoure 2010; Ershadi et al. 2016). We observed that *C. officinalis* also accumulated GB after 24, 48 and 72 h and then its concentration decreased after 92 and 120 h. GB, a quaternary ammonium compound and a crucial osmolyte, plays a critical role in osmotic adjustment in chloroplast (Genard et al. 1991) and protects the structure of protein and macromolecules and membrane integrity against abiotic stress (Niu et al. 2014). However, the subsequent decrease in GB in *C. officinalis* could be due to less availability of substrate (choline) or owing to the reduced transportation of choline into the chloroplast (Huang et al. 2000; Mc Neil et al. 2000), as GB is synthesized in the chloroplast in the two-step biosynthetic pathway from choline by dehydrogenation reaction (Shan et al. 2016). The exposure of 14-day-old seedling of *C. officinalis* to the CS of 4 °C led to the significant increases in the sugar content after 24 and 72 h and then the values remained constant. Total sugars increased by 35.91% after 24 h CS and by 89.5% after 72 h of exposure to 4 °C. Trehalose has been implicated in protection against cold stress by acting as a stress protectant (Elbein et al. 2003), a signalling molecule and an antioxidant, stimulating the genes for detoxification and protecting the protein synthesis machinery (Abdallah et al. 2016; John

et al. 2017). The higher accumulation of total sugar improves cold tolerance, being an osmoprotectant, an energy source and substrates for various physiological processes (Yue et al. 2015). It has been reported that under low-temperature stress, polysaccharides get hydrolysed to soluble sugars, thus increasing the cytoplasm osmotic potential and lowering the freezing temperature (Zhang et al. 2012). Cold-acclimated plants showed high expression of glycolysis enzymes when compared with non-acclimated ones (Janmohammadi 2012).

We analysed the CS response in *C. officinalis* in terms of protein content and observed significant increase in the protein levels when exposed to 4 °C. The elevated protein levels in the plant in response to CS points to synthesis of some specific proteins and their accumulation (Koc et al. 2010), such as antifreeze proteins that protect the plant against cold stress by preventing chlorophyll loss, stabilizing cell membrane, maintaining stomatal conductance and retaining water to maintain survival of many biochemical activities (Aras and Eşitken 2013). Similarly, Azyimi et al. (2012) observed higher accumulation of sugar, proline and protein under chilling stress. *Capsicum annuum* L. and rice genotypes also showed higher accumulation of soluble protein under low-temperature stress (Koc et al. 2010; Aghae et al. 2011).

The phenolics, particularly flavonoids, function as anti-oxidants and enhance the enzymatic antioxidant systems during stress, apart from acting as osmoregulators (Oh et al. 2009; Lalrinzuali et al. 2016). The increased phenolic content indicates its role in plant acclimation to CS in *C. officinalis*. High phenolic content has been associated with better radical-scavenging activity and stronger reducing power in grapevine under CS (Król et al. 2015). However, in soybean seedlings, the phenolic compounds decreased suddenly on exposure to cold stress (Posmyk et al. 2005) that is in contrast with our results.

One of the major changes in plants during cold acclimation is the membrane stabilization against the freezing-induced injury and membrane damage and the best recognized change is in lipid composition to change the membrane fluidity (Jan et al. 2009). We observed that the immediate result of change in membrane fluidity is the alteration in the fatty acid composition that includes the appearance of unsaturated fatty acid and the disappearance of saturated fatty acid with increase in the duration of stress. The palmitic acid disappeared while the 8, 12-tetradecadienoic acid (polyunsaturated fatty acid) accumulated after 72 h duration of cold stress and showed a uniform increase along with time. Similarly, α -linolenic acid showed abrupt increase after continued cold stress for 120 h. This shows that *C. officinalis* maintains membrane fluidity through the unsaturation of fatty acids as an adaptive response to cold stress, as higher levels of membrane lipid unsaturation is one of the

principle factors responsible for optimum membrane function at low temperature (Jan et al. 2009).

SOD is the first defence line against oxidative stress causing the conversion of O_2^- into O_2 and H_2O_2 (de Carvalho 2008). Further, this H_2O_2 is decomposed by CAT and APX in peroxisomes and cytosol, respectively (Zhang et al. 2015). However, we observed that in *C. officinalis*, CS induced SOD and APX activity and declined CAT activity significantly, as observed in bermudagrass under cold stress (Fan et al. 2014) and rice roots under salt stress (Pan et al. 2006), pointing that APX has a higher capacity of H_2O_2 decomposition generated by SOD. CAT having lower affinity to H_2O_2 scavenges most of H_2O_2 , while APX having more affinity to H_2O_2 is responsible for the absolute regulation of ROS (de Carvalho 2008). The unpredicted decline in CAT activity can be due to its translation inhibition and photo-inactivation under stress (Dat et al. 2000). The low CAT activity may be due to the effect of stress on the structure and function of CAT protein and subsequently the inhibition of enzyme activity (Cavalcanti et al. 2004). Chakraborty and Bhattacharjee (2015) have also observed a decreased CAT activity in rice under cold stress and CAT activity declined in cowpea under salinity stress (Cavalcanti et al. 2004). For instance, in many plant species, catalase activity inhibition under oxidative stress has been correlated with salicylic acid accumulation (Cavalcanti et al. 2004). Moreover, light-dependent decrease in CAT protein and its activity has been reported under salt stress (Cavalcanti et al. 2004).

A significant and positive correlation has been found between SOD, APX and GR, while CAT shows a negative correlation with all these antioxidants. GR, NADPH-dependent oxidoreductase, maintains the reduced form of GSH by converting oxidized glutathione (GSSG) to reduced glutathione (GSH) and sustains increased GSH/GSSG ratio under various abiotic stresses (Trivedi et al. 2013). GR maintains the reducing environment of the cell, important for protein function, and plays a very important role in regulating the stress tolerance in various plants under different abiotic stresses (Gill and Tuteja 2010). An increased GR activity has also been reported in chilling-tolerant rice cultivar and leaves of *E. adenophorum* (Guo et al. 2006; Lu et al. 2008) that are consistent with our results. However, a further decreased GR activity with increased sensitivity to oxidative stress was observed in tobacco plants (Aono et al. 1995; Ding et al. 2009) that is in contrast with our results.

There is a unique means of gene expression and activity of enzymatic antioxidants in *C. officinalis* under CS. Our analysis of data obtained showed that the gene expression profile of important enzymatic antioxidants such as SOD, APX, CAT and GR show no direct correlation with their corresponding enzymatic activities. Harb et al. (2015) reported similar studies in barley under drought stress.

The antioxidant activity of APX, SOD and GR increased linearly with the duration of CS, except CAT which showed a decline in its activity when *Calendula* was subjected to 4 °C. However, gene expression analysis revealed that SOD, CAT and APX gene expressions are highly upregulated after 24 h with subsequent downregulation at 72 h (SOD) and 48 h (APX and CAT) and then remain constant up to 120 h. Gene expression of GR is linearly upregulated up to 96 h. This difference in the enzyme activity and their gene expression can be due to the presence of different antioxidant enzyme isoforms (Gill and Tuteja 2010). The antioxidant activity assayed is of more than isoforms, whereas real-time gene expression is the analysed expression of only one isoform (Furlan et al. 2013). Moreover, due to the complex mechanism of gene expression regulation, antioxidant gene expression cannot be correlated directly with its activity (Harb et al. 2015).

CBFDREB1 family of transcription factors get triggered by freezing stress/Cs and acts as a master switch inducing many downstream cold-responsive genes, thus imparting CS tolerance and/or cold acclimation (Kidokoro et al. 2017). We observed an increased accumulation of *DREB1* transcripts in *C. officinalis* when exposed to CS as compared to plants growing at 25 °C. The maximum expression was obtained at 72 h (3.29-fold) of CS and the transcription declined gradually at 120 h. The later decline in the transcript levels of *DREB1* could be due to the de novo production of mRNA-degrading enzymes and/or labile transcriptional repressors (Fujimoto et al. 2000). The Arabidopsis plants that overexpressed *DREB1A* had increased protection against freezing stress (Kasuga et al. 1999; Mizoi et al. 2012).

Conclusions

Calendula owes its cold resistance to the change in the levels of osmoprotectants, antioxidant activity and expression of various stress-responsive genes. Our studies suggest that the enhanced level of these osmolytes, antioxidant activity and expression of cold-responsive genes help the plants to withstand the CS. These results along with many of the others further our understanding of the basic mechanism the plants have evolved for their survival under low temperatures. In addition, the findings have potential practical applications, as CS is the single major factor that limits the plant geographical distribution.

Author contribution statement NJ and UM conducted the experiments and drafted the manuscript. KIA and RJ edited the paper and did the statistical analysis of the data.

Acknowledgements Authors acknowledge the financial support by Department of Biotechnology (DBT) and Science and Engineering Research Board (SERB). NJ also acknowledges the grant by Department of Science and Technology (DST) under Women Scientist Scheme (WOS).

References

- Abdallah MMS, Abdelgawad ZA, El-Bassiouny HMS (2016) Alleviation of the adverse effects of salinity stress using trehalose in two rice varieties. *S Afr J Bot*. <https://doi.org/10.1016/j.sajb.2015.09.019>
- Aghaee A, Moradi F, Zare-Maivan H, Zarinkamar F, Irandoost HP, Sharifi P (2011) Physiological responses of two rice (*Oryza sativa* L.) genotypes to chilling stress at seedling stage. *Afr J Biotechnol* 10:7617–7621
- Aono M, Saji H, Fujiyama K, Sugita M, Kondo N, Tanaka K (1995) Decrease in activity of glutathione reductase enhances paraquat sensitivity in transgenic *Nicotiana tabacum*. *Plant Physiol* 107:645–648
- Apak R, Güçlü K, Özyürek M, Çelik SE (2008) Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay. *Microchim Acta* 160:413–419
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399
- Aras S, Eşitken A (2013) Effects of antifreeze proteins and glycine betaine on strawberry plants for resistance to cold temperature. *ICABT* 60:21 (IACSIT Press, Singapore)
- Arbona V, Manzi M, Zandalinas SI, Vives-Peris V, Pérez-Clemente RM, Gómez-Cadenas A (2017) Physiological, metabolic and molecular responses of plants to abiotic stress. In stress signaling in plants. *Genom Proteom Perspect* 2:1–35
- Asif M (2015) Pharmacological activities and phytochemistry of various plant containing coumarin derivatives. *Curr Sci Perspect* 1:77–90
- Azimi S, Sofalian O, Jahanbakhsh GS, Khomari S (2012) Effect of chilling stress on soluble protein, sugar and proline accumulation in cotton (*Gossypium hirsutum* L.) genotypes. *Int J Agric Crop Sci* 4:825–830
- Bano S, Aslam M, Saleem M, Basra SMA, Aziz K (2015) Evaluation of maize accessions under low temperature stress at early growth stages. *J Anim Plant Sci* 25:392–400
- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–208
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Campos PS, Quartin V, Ramalho JC, Nunes MA (2003) Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. *J Plant Physiol* 160:283–292
- Cansev A, Gulen H, Celik G, Eris A (2012) Alterations in total phenolic content and antioxidant capacity in response to low temperatures in olive (*Olea europaea* L. “Gemlik”). *Plant Arch* 12:489–494
- Cavalcanti FR, Oliveira JTA, M-Miranda AS, Viegas RA, Silveira JAG (2004) Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. *New Phytol* 163:563–571
- Chakraborty A, Bhattacharjee S (2015) Differential competence of redox-regulatory mechanism under extremes of temperature determines growth performances and cross tolerance in two indica rice cultivars. *J Plant Physiol* 176:65–77
- Chassot A, Richner W (2002) Root characteristics and phosphorus uptake of maize seedlings in a bilayered soil. *Agron J* 94:118–127

- Choudhary DK, Kasotia A, Jain S, Vaishnav A, Kumari S, Sharma KP, Varma A (2016) Bacterial-mediated tolerance and resistance to plants under abiotic and biotic stresses. *J Plant Growth Regul* 35:276–300
- Cyril J, Powell GL, Duncan RR, Baird WV (2002) Changes in membrane polar lipid fatty acids of *Seashore Paspalum* in response to low temperature exposure. *Crop Sci* 42:2031–2037
- Dar GH, Malik AH, Khuroo AA (2014) A Contribution to the flora of Rajouri and Poonch districts in the Pir Panjal Himalaya (Jammu & Kashmir), India. *Check List* 10:317–328
- Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inzé D, Van Breusegen D (2000) Dual action of the active oxygen species during plant stress responses. *Cell Mol Life Sci* 57:779–795
- de Azevedo Neto AD, Prisco JT, Eneas-Filho J, de Abreu CEB, Gomes-Filho E (2006) Effect of salt stress on anti-oxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ Exp Bot* 56:87–94
- de Carvalho MC (2008) Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signal Behav* 3:156–165
- Dey PM (1990) Oligosaccharides. In: Dey PM, Harborne JB (eds) *Methods in plant biochemistry, carbohydrates*, vol 2. Academic Press, London, pp 189–218
- Di Fenza M, Hogg B, Grant J, Barth S (2017) Transcriptomic response of maize primary roots to low temperatures at seedling emergence. *Peer J* 5:e2839
- Ding S, Lu Q, Zhang Y, Yang Z, Wen X, Zhang L, Lu C (2009) Enhanced sensitivity to oxidative stress in transgenic tobacco plants with decreased glutathione reductase activity leads to a decrease in ascorbate pool and ascorbate redox state. *Plant Mol Biol* 69:577–592
- Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multi-functional molecule. *Glycobiology* 13:17–27
- Ershadi A, Karimi R, Mahdei KN (2016) Freezing tolerance and its relationship with soluble carbohydrates, proline and water content in 12 grapevine cultivars. *Acta Physiol Plant* 38:2
- Fan J, Ren J, Zhu W, Amombo E, Fu J, Chen L (2014) Antioxidant responses and gene expression in Bermuda grass under cold stress. *J Am Soc Hortic Sci* 139:1–7
- Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 12:393–404
- Furlan A, Bianucci E, Tordable M, Castro S, Dietz KJ (2013) Antioxidant enzyme activities and gene expression patterns in peanut nodules during a drought and rehydration cycle. *Funct Plant Biol* 41:704–713
- Genard H, Le Saos J, Hillard J, Tremolieres A, Boucaud J (1991) Effect of salinity on lipid composition, glycine betaine content and photosynthetic activity in chloroplasts of *Suaeda maritime*. *Plant Physiol Biochem* 29:421–427
- Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48:909–930
- Greive CM, Grattan SR (1983) Rapid assay for determination of water-soluble quaternary amino compounds. *Plant Soil* 70:303–307
- Guo Z, Ou W, Lu S, Zhong Q (2006) Differential responses of antioxidant system to chilling and drought in four rice cultivars differing in sensitivity. *Plant Physiol Biochem* 44:828–836
- Haldimann P (1999) How do changes in temperature during growth affect leaf pigment composition and photosynthesis in *Zea mays* genotypes differing in sensitivity to low temperature? *J Exp Bot* 50:543–550
- Halliwell B, Foyer CH (1978) Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. *Planta* 139:9–17
- Hao YJ, Wei W, Song QX, Chen HW, Zhang YQ, Wang F, Zou HF, Lei G, Tian AG, Zhang WK, Ma B, Zhang JS, Chen SY (2011) Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. *Plant J* 68:302–313
- Harb A, Awad D, Samarah N (2015) Gene expression and activity of antioxidant enzymes in barley (*Hordeum vulgare* L.) under controlled severe drought. *J Plant Interact* 10:109–116
- Hayashi H, Mustardy L, Deshnum P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the codA gene for choline oxidase; accumulation of glycine betaine and enhanced tolerance to salt and cold stress. *Plant J* 12:133–142
- Hossain M, Mostofa M, Fujita M (2013) Cross protection by cold-shock to salinity and drought stress-induced oxidative stress in mustard (*Brassica campestris* L.) seedlings. *Mol Plant Breed* 4:50–70
- Hsiao TC, Xu LK (2000) Sensitivity of growth of roots versus leaves to water stress: biophysical analysis and relation to water transport. *J Exp Bot* 51:1595–1616
- Huang J, Hirji R, Adam L, Rozwadowski KL, Hammerlindl JK, Keller WA, Selvaraj G (2000) Genetic engineering of glycine betaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol* 122:747–756
- Hund A, Fracheboud Y, Soldati A, Frascaroli E, Salvi S, Stamp P (2004) QTL controlling root and shoot traits of maize seedlings under cold stress. *Theor Appl Genet* 109:618–629
- Hund A, Richner W, Soldati A, Fracheboud Y, Stamp P (2007) Root morphology and photosynthetic performance of maize inbred lines at low temperature. *Eur J Agron* 27:52–61
- Jan N, John R (2017) *Calendula officinalis*—an important medicinal plant with potential biological properties. *Proc Indian Nat Sci Acad* 83(4):769–787
- Jan N, Hussain M, Andrabi KI (2009) Cold resistance in plants: a mystery unresolved. *Electron J Biotechnol* 12:15
- Janmohammadi M (2012) Metabolomic analysis of low temperature responses in plants. *Curr Opin Agric* 1:1
- Janska A, Marsik P, Zelenkova S, Ovesna J (2010) Cold stress and acclimation: what is important for metabolic adjustment? *Plant Biol* 12:395–405
- John R, Anjum NA, Sopory SK, Akram NA, Ashraf M (2016) Some key physiological and molecular processes of cold acclimation. *Biol Plant* 60:603–618
- John R, Raja V, Ahmad M, Jan N, Majeed U, Ahmad S, Yaqoob U, Kaul T (2017) Trehalose: metabolism and role in stress signaling in plants. In: Sarwat M et al (eds) *Stress signaling in plants: genomics and proteomics perspective*, vol 2. <https://doi.org/10.1007/978-3-319-42183-4-11>
- Karabudak T, Bor M, Özdemir F, Türkan İ (2014) Glycine betaine protects tomato (*Solanum lycopersicum*) plants at low temperature by inducing fatty acid desaturase 7 and lipoxygenase gene expression. *Mol Biol Rep* 41:1401–1410
- Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol* 17:287–291
- Khraiwesh B, Zhu JK, Zhu J (2012) Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants—gene regulatory mechanisms. *Biochim Biophys Acta* 1819:137–148
- Kidokoro S, Yoneda K, Takasaki H, Takahashi F, Shinozaki K, Yamaguchi-Shinozaki K (2017) Different cold-signaling pathways function in the responses to rapid and gradual decreases in temperature. *Plant Cell* 29 (4):760–774
- Koc E, Islek C, Ustun AS (2010) Effect of cold on protein, proline, phenolic compounds and chlorophyll content of two pepper (*Capsicum annum* L.) varieties. *GU J Sci* 23:1–6

- Król A, Amarowicz R, Weidner S (2015) The effects of cold stress on the phenolic compounds and antioxidant capacity of grapevine (*Vitis vinifera* L.) leaves. *J Plant Physiol* 189:97–104
- Lalrinzuali K, Vabeiryureilai M, Jagetia GC (2016) The analysis of antioxidant activity and phenolic contents of selected medicinal plants of Mizoram. *Genom Appl Biol* 28:6
- Lee E, Staebler M, Tollenaar M (2002) Genetic variation in physiological discriminators for cold tolerance—early autotrophic phase of maize development. *Crop Sci* 42:1919–1929
- Leshem YY (1992) Plant membranes: a biophysical approach to structure, development and senescence. Kluwer Academic Publishers, Dordrecht
- Levitt J (1980) Responses of plants to environmental stress—chilling, freezing and high temperature stresses, vol 1. Academic Press, New York
- Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochem Soc Transact* 603:591–592
- Liu SQ, Chen CM, Chen GJ, Cao BH, Chen QH, Lei JJ (2012) RNA sequencing tag profiling of the placenta and pericarp of pungent pepper provides robust candidates contributing to capsaicinoid biosynthesis. *Plant Cell Tissue Organ Cult* 110:111–121
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25:402–408
- Lu P, Sang WG, Ma KP (2008) Differential responses of the activities of antioxidant enzymes to thermal stresses between two invasive Eupatorium species in China. *J Integr Plant Biol*. <https://doi.org/10.1111/j.1744-7909.2007.00583.x>
- Malamy J (2005) Intrinsic and environmental response pathways that regulate root system architecture. *Plant Cell Environ* 28:67–77
- Martin RJ, Deo B (2000) Effect of plant population on calendula (*Calendula officinalis* L.) flower production. *N Z J Crop Hortic Sci* 28:37–44
- Maysa M, Mallah E, Mohamed A (2015) Hepatoprotective effect of *Calendula officinalis* Linn (Asteraceae) flowers against CCl₄-induced hepatotoxicity in rats. *World Appl Sci J* 33:949–1959
- Mc Neil SD, Rhodes D, Russell BL, Nuccio ML, Shachar-Hill Y, Andrew D, Hanson AD (2000) Metabolic model identifies key constraints on an engineered glycine betaine synthetic pathway in tobacco. *Plant Physiol* 12:153–162
- Miura K, Furumoto T (2013) Cold signaling and cold response in plants. *Int J Mol Sci* 14:5312–5337
- Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim Biophys Acta* 1819:86–96
- Murata N, Los DA (1997) Membrane fluidity and temperature perception. *Plant Physiol* 115:875
- Niu X, Xiong F, Liu J, Sui Y, Zeng Z, Lu BR, Liu Y (2014) Co-expression of ApGSMT and ApDMT promotes biosynthesis of glycine betaine in rice (*Oryza sativa* L.) and enhances salt and cold tolerance. *Environ Exp Bot* 104:16–25
- Oh SJ, Kim YS, Kwon CW, Park HK, Jeong JS, Kim JK (2009) Over-expression of the transcription factor AP37 in rice improves grain yield under drought conditions. *Plant Physiol* 150:1368–1379
- Ostonen I, Püttsepp Ü, Biel C, Alberton O, Bakker MR, Löhmus K, Majdi H, Metcalfe D, Olsthoorn AFM, Pronk A et al (2007) Specific root length as an indicator of environmental change. *Plant Biosyst* 141:426–442
- Pan Y, Wu LJ, Yu ZL (2006) Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (*Glycyrrhiza uralensis* Fisch). *J Plant Growth Regul* 49:157–165
- Posmyk MM, Bailly C, Szafran'ska K, Jasan KM, Corbineau F (2005) Antioxidant enzymes and isoflavonoids in chilled soybean (*Glycine max* L. Merr.) seedlings. *J Plant Physiol* 162:403–412
- Presterl T, Ouzunova M, Schmidt W, Möller EM, Röber FK, Knaak C, Ernst K, Westhoff P, Geiger HH (2007) Quantitative trait loci for early plant vigour of maize grown in chilly environments. *Theor Appl Genet* 114:1059–1070
- Rahimi A, Jahanbin Sh, Salehi A, Farajee H (2016) Changes in content of chlorophyll, carotenoids, phosphorus and relative water content of medicinal plant of Borage (*Borago officinalis* L.) under the influence of mycorrhizal fungi and water stress. *J Biol Sci* 17:28–34
- Raja V, Majeed U, Kang H, Andrabi KI, John R (2017) Abiotic stress: interplay between ROS, hormones and MAPKs. *Environ Exp Bot* 137:142–157
- Sanghera GS, Wani SH, Hussain W, Singh NB (2011) Engineering cold stress tolerance in crop plants. *Curr Genom* 12:30–43
- Shan T, Jin P, Zhang Y, Huang Y, Wang X, Zheng Y (2016) Exogenous glycine betaine treatment enhances chilling tolerance of peach fruit during cold storage. *Postharvest Biol Technol* 114:104–110
- Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6:410–417
- Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Annu Rev Plant Physiol* 35:543–584
- Subba P, Barua P, Kumar R, Datta A, Soni KK, Chakraborty S, Chakraborty N (2013) Phosphoproteomic dynamics of chickpea (*Cicer arietinum* L.) reveals shared and distinct components of dehydration response. *J Proteome Res* 12:5025–5047
- Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiol Plant* 126:45–51
- Szabados L, Savaouré A (2010) Proline: a multifunctional amino acid. *Trends Plant Sci* 15:89–97
- Tahmasebi A, Pakniyat H (2015) Comparative analysis of some biochemical responses of winter and spring wheat cultivars under low temperature. *Int J Agric Agric* 7:14–22
- Trivedi D, Gill SS, Yadav S, Tuteja N (2013) Genome-wide analysis of glutathione reductase (GR) genes from rice and Arabidopsis. *Plant Signal Behav* 8:1
- Tuteja N, Gill SS (2016) Abiotic stress response in plants. Wiley, New York
- Wang L, Fan XW, Pan JL, Huang ZB, Li YZ (2015) Physiological characterization of maize tolerance to low dose of aluminum, highlighted by promoted leaf growth. *Planta* 242:1391–1403
- Wijewardana C, Henry WB, Hock MW, Reddy KR (2016) Growth and physiological trait variation among corn hybrids for cold tolerance. *Can J Plant Sci* 96:639–656
- Wu FZ, Wang BC, Yang CP (2014) Proteomic analysis of the cold stress response in the leaves of birch (*Betula platyphylla* Suk). *Plant Omics J* 7:195–204
- Xiong Y, Fei SZ (2006) Functional and phylogenetic analysis of a DREB/CBF-like gene in perennial ryegrass (*Lolium perenne* L.). *Planta* 224:878–888
- Gill SS, Gill R, Anjum NA (2014) Target osmoprotectants for abiotic stress tolerance in crop plants—glycine betaine and proline. In: *Plant adaptation to environmental change: significance of amino acids and their derivatives*, vol 10, pp 97–108
- Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006) Comparative proteomic analysis provides new insights into chilling stress responses in rice. *Mol Cell Proteom* 3:484–496
- Yue C, Cao H, Wang L et al (2015) Effects of cold acclimation on sugar metabolism and sugar-related gene expression in tea plant during the winter season. *Plant Mol Biol* 88:591–608
- Zhang HY, Jiang YN, He ZY, Ma M (2005) Cadmium accumulation and oxidative burst in garlic (*Allium sativum*). *J Plant Physiol* 162:977–984
- Zhang T, Zhao X, Wang W, Pan Y, Huang L, Liu X, Zong Y, Zhu L, Yang D, Fu B (2012) Comparative transcriptome profiling of

- chilling stress responsiveness in two contrasting rice genotypes. PLoS ONE 7:e43274
- Zhang YP, E ZG, Jiang H, Wang L et al (2015) A comparative study of stress-related gene expression under single stress and intercross stress in rice. Genet Mol Res 14:3702–3717
- Zuther E, Juszczak I, Lee YP, Baier M, Hincha DK (2015) Time-dependent deacclimation after cold acclimation in *Arabidopsis thaliana* accessions. Sci Rep. <https://doi.org/10.1038/srep12199>