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# Acquisition of Freezing Tolerance in Non-Acclimated *Alcea rosea* L. 'nigra' by Exposure to Different Duration of Cold Preconditioning

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## ABSTRACT

**Lower winter temperatures may negatively affect winter survival by preventing maximum cold acclimation before freezing temperatures. The process of cold acclimation requires adaptation to both light and low temperatures, depending on the duration of exposure. Accordingly, research is needed to identify strategies to promote cold acclimation and increase freezing tolerance. Therefore, this experiment was conducted to investigate whether a shorter or more extended preconditioning cold treatment can improve the cold hardiness of hollyhock and its threshold of winter hardiness. The results showed that both 14 ($CP_1$) and 28 days of Cold Preconditioning ($CP_2$) decreased electrolyte loss, increased the activity of SOD, CAT, and APX enzymes, inhibited accumulation of Hydrogen peroxide ($H_2O_2$), and delayed the increase of Malondialdehyde (MDA) content, while non-acclimated plants experienced a decrease in MDA. No difference was observed in antioxidant activity and photosynthetic parameters between $CP_1$ and $CP_2$. Although proline and water-soluble sugar contents were higher in plants subjected to 28 days of cold preconditioning than in those treated for 14 days, no significant difference was found in survival percentage. Low temperatures decreased photosynthetic parameters, while increasing leaf contents of Abscisic Acid (ABA) and phenolic. The results suggested that 14-day cold preconditioning could be used to increase cold tolerance for non-acclimated hollyhocks to grow in the field at -4°C.**

**Keywords**: Antioxidant activity, Hollyhock, Cold tolerance, Freezing resistance, Hormonal changes.

## INTRODUCTION

*Alcea rosea* belongs to the Malvaceae family and is a medicinal plant that produces large flowers in many colors (Ghahreman *et al*., 2000). *Alcea rosea* is a precious plant due to its pigments and colored substances. The flowers of this plant species contain detoxifying, diuretic and emollient substances (Fahamya *et al*., 2016), which are effective in curing constipation, dysmenorrhea, bleeding, and breast pain, and a decoction is used to improve blood circulation (Kim *et al*. 2017).

Many abiotic and biotic stresses affect winter survival, but cold tolerance is one of the most critical factors for winter plant survival (Chang *et al*. 2020). Low temperatures are crucial to plant survival, as they regulate plant growth and yield (Oraee *et al*., 2018). Maximum cold tolerance of plants is associated with subsequent periods of cold acclimation, which is achieved by Short Day length (SD) and low temperatures (Welling *et al*., 2002). These factors affect cold tolerance and survival in susceptible plants at temperatures below freezing (Yadav 2010). Cold acclimation is sequential and successional in woody species (Baniulis *et al*. 2020) and various herbaceous plants (Chen and Li 1980) in response to temperature reductions.

Cold stress reduces plants' photosynthesis,

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protein synthesis, and general metabolic functions. Various physiological, morphological, biochemical, and molecular changes occur when a given plant is exposed to stress conditions (Supplementary Figure S1). Chilling damage is classified into two categories based on direct and indirect effects, which are called primary and secondary events. The primary symptom causes a phase transition of the membrane from a liquid to a solid gel phase, decreasing membrane permeability, i.e., fluidity, and promoting the expression of the secondary symptom (Liang *et al.* 2020). Plant countermeasures counter cold stress through stress detection, signal perception, signal transduction, and induction of cold-tolerant genes (Guo *et al*., 2018). Numerous genes have been found in plants that respond to cold. In addition to those that respond directly to cold stress, some genes regulate other molecular activities (Lissarre *et al*., 2010). Plants' physiological and biochemical processes, such as photosynthesis and respiration, water balance, and other metabolic processes are more susceptible to stress at low temperatures (Hassan *et al*. 2021). Cold stress disrupts the normal electron transport chain, resulting in electron loss that generates ROS, causing oxidative stress and degrading the membrane (Dreyer and Dietz, 2018). The duration of cold stress leads to an increase or decrease in respiration rate, as a short period increases the respiration rate because the normal electron transport chain switches to the cyanide-resistant pathway. In contrast, a long or very low temperature leads to cell damage and death, and decreases the rate (Hassan *et al*. 2021). When a plant undergoes cold stress, several morphological alterations occur. Cold stress causes changes in plants, such as reduced plant growth and loss of vigor, which can decrease survival percentages (Hussain *et al*. 2018).

The speed and timing of acclimation are critical for survival, especially in winter. In contrast, premature transfer of unacclimated plants can increase the risk of frost damage unless the plants can tolerate cold stress. Changes in the plant environment due to cultural practices such as containers can affect plant growth control, acclimation, and the maximum degree of plant hardiness. Plant growth control and cold hardiness thresholds can reduce plant losses due to injury by understanding a better understanding of plant growth control and cold hardiness thresholds will aid in developing methods to reduce plant losses due to injury. (Wisniewski *et al*. 2018).

Plants growing in greenhouses can experience frost stress when transferred to urban green space. Many researchers have reported the effects of pre-cold conditioning on increasing resistance to cold stress. However, the present study tried to assess the shorter- (14 days) and longer-term (28 days) effects of cold to substitute for the process of cold acclimation. In the present study, the frost hardiness of *Alcea rosea* 'nigra' was supposed to be affected by cold conditioning. The hypothesis was that prolonged exposure to cold could increase hollyhocks' frost tolerance without cold acclimation by increasing antioxidant enzyme activity and osmoregulation. This experiment aimed to evaluate the effects of cold duration on the increase in hollyhock's resistance to cold stress.

## MATERIALS AND METHODS

In June 2021, hollyhock (*Alcea rosea* 'nigra') seeds were soaked in water for 12 h and then kept at room temperature (21±1°C). The seeds were planted in a soilless medium and grown at a temperature of 22±1°C under intermittent misting (10 s misting h-1) until transplanting. Plants were irrigated with 0.45% Hoagland's solution, pH= 6. The macro nutrients for Hoagland nutrient solution were; $Ca(NO_3)_2{\cdot}4H_2O$: 212 g $L^{-1}$; and $KNO_3$: 90.9 g $L^{-1}$ and $KH_2PO_4$: 61.2 g $L^{-1}$ and micronutrients were $H_3BO_3$: 1.3 g $L^{-1}$; $MnCl_2$. $4H_2O$: 0.81 g $L^{-1}$; $ZnSO_4{\cdot}7H_2O$: 0.099 g $L^{-1}$; $CuSO_4{\cdot}5H_2O$: 0.036 g $L^{-1}$; $Na_2MoO_4{\cdot}2H_2O$: 0.054 g $L^{-1}$, FeEDTA: 0.54 g $L^{-1}$. After every fourth

application of water-soluble fertilizer, the plants were irrigated with distilled water. Fertilization was stopped before cold preconditioning. The rooted plants were planted in plastic pots containing 40% peat moss, 20% perlite, and 40% coconut palm and kept in a greenhouse at 21±1°C air temperature and ambient light (4.82 mol $m^{-2}$ $day^{-1}$) until November.

### Cold Preconditioning Treatments

Three treatments were applied at the end of November to determine whether different periods of preconditioning could improve frost tolerance under non-cold acclimation conditions, as follows: (i) Non-cold acclimation temperature (20°C), (ii) 14 days of Cold Preconditioning ($CP_1$) after a non-cold acclimation temperature regime (20 °C), and (iii) 28 days of Cold Preconditioning ($CP_2$) after a non-cold acclimation temperature regime. Treatments consisted of two temperatures (cold preconditioning at 3-5 °C (CP) and non-cold preconditioning as control). Plants were kept at 5°C for nine days with short-day length of 8 h and at 3°C for five days. Hollyhocks were kept at 5°C for 18 days and 3°C for ten days, respectively, for eight hours at 500 mol $m^{-2}$ $s^{-1}$, during 28 days of cold preconditioning. Seedlings were randomly sampled for the determination of cold hardiness.

### Hardiness

The randomly selected plants were frozen in a thermogradient. Test temperatures ranged from 2 to -6°C in 2°C increments. At each test temperature, five plants per treatment were removed and thawed overnight at 3°C.

### Evaluation of Physiological Parameters

Two 5 cm leaf segments were placed in 100 mL of distilled water at freezing temperatures. As soon as the samples had been boiled, the conductance of the solution was measured. Electrolyte leakage was calculated according to Gusta *et al*. (2003). Asymmetric sigmoid functions were used to determine the lethal temperature, which is the temperature at which tissue reaches 50% of maximum damage during freezing. Electrolyte Leakage (EL) data were evaluated using the following model:

$$ELp = Ell + [(Elm - Ell)/(1 + e^{-B(T - Tm)})]$$

Where, ELp is the predicted value EL, Ell is the lower bound of the value EL, Elm is the upper bound of the value EL, e is 2.714, B is the rate of change of the curve slope, T is the absolute value of the treatment Temperature, and Tm is the inflection point of the curve (Lim *et al*. 1998).

Proline content was calculated based on the method of Bates *et al*. (1973). Proline was determined in 80% ethanol extracts of fresh weight using the ninhydrin method. Soluble-water sugar content was determined based on the method developed by DuBois (1956). Then, 80% ethanol extracts of dry weight with 5 mL of zinc sulfate, 5%, and 4.7 mL of barium hydroxide were centrifuged. About 1mL of 5% Phenol and 5 mL of sulfuric acid, 98%, were added to the supernatant. The absorbance was measured at a wavelength of 485 nm. The phenolic compound extraction was based on the method previously described by Singleton and Rossi (1965). The extracts were mixed with $Na_2CO_3$ (20%) and the Folin-Ciocalteu reagent in the correct ratios. The samples were then incubated at 45°C, and the absorbances of the mixtures were measured at 765 nm. Hydrogen peroxide ($H_2O_2$) extraction was performed according to Velikova *et al*. (2000) with some modifications. Then, 0.5 mL of supernatant (0.5 g fresh weight+5 mL 0.1% w/v trichloroacetic acid) was mixed with 0.5 mL potassium phosphate ($KHPO_4$) buffer and 1 mL potassium iodide. The upper phase was aliquoted to measure its absorbance at a wavelength of 390 nm. Lipid peroxidation was determined by quantification of MDA resulting from the thiobarbituric acid reaction, following the method of Heath and Packer (1968). Superoxide Dismutase (SOD) activity was measured according to the method

described by Beauchamp and Fridovich (1971). Then, 0.5 g of fresh leaves were homogenized in 5 mL K-phosphate buffer (50 mM, pH 7.0, and 0.1 mM EDTA). The homogenate was centrifuged in a refrigerated centrifuge at 4°C (12,000 rpm, 30 minutes). The spectrophotometric absorbance of the mixture was determined at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of Nitro Blue Tetrazolium (NBT) photoreduction. Catalase (CAT) activity was estimated according to Aebi (1984), and Ascorbate Peroxidase (APX) was determined by an accurate method by Asada (1992). Catalase activity (CAT) was determined in a reaction mixture containing 50 mM K-phosphate buffer (pH 7.0), 10 mM $H_2O_2$, and an aliquot of the enzyme. The decomposition of $H_2O_2$ was monitored at 240 nm. The reaction (50 mM K-phosphate buffer (pH 7.0), 0.5 mM Ascorbate (AsA), 0.1 mM $H_2O_2$, 0.1 mM EDTA, and enzyme extract in a final volume=700 mL) was initiated by the addition of $H_2O_2$. The activity was measured by observing the decrease in absorbance at 290 nm for 2 min. Chlorophyll was determined as previously described by Arnon (1949). A portable LCi photosynthesis system (ADC Bioscientific Ltd., Hoddesdon, England) was used to evaluate photosynthetic parameters including net Photosynthetic rate (Pn), stomatal conductance (gs), Transpiration rate (Tr) and intercellular $CO_2$ consideration *(Ci).* Endogenous ABA was determined using an HPLC system based on previously described methods with some modifications (Lang *et al.* 2019). The content of essential oils of individual samples of the studied plants was extracted separately by hydro distillation using a Clevenger-type apparatus (Letchamo 1993).

### Evaluation of Morphological Parameters

Plant survival and some morphological traits were evaluated after 21 days. Leaf area was measured by LI-3000C Portable Leaf Area Meter. After transferring the plant to the oven, the dry weight of aerial plant parts and roots was determined. Root volume was obtained by quantifying the volume of liquid a root displaces.

### Statistical Analysis

The experiment was designed with three temperature regimes×five freezing temperatures with three replicates. SAS statistical software was used to analyze the measured physiological parameters and regression analysis of the effective traits on survival percentage for the hollyhock (SAS Institute Inc, 2010). The analysis of variance and mean comparisons were achieved by the Least Significant Difference test (LSD) ($P < 0.05$).

## RESULTS

The electrolyte leakage results and hollyhock survival, presented in Figure 1, showed that temperature pretreatment and freezing temperatures significantly affected these traits ($P< 0.01$). Cold pretreatment reduced electrolyte leakage at freezing temperatures compared with the control. The $LT_{50}$ (lethal temperature 50 according to the electrolyte leakage percentage) value in the control was -3.2°C, while it was -4.8°C when cold preconditioning was applied (Table 1). Exposure of the plants to cold resulted in an additional increase in freeze hardiness. However, electrolyte leakage significantly increased at -6°C compared to 2°C in control plants and plants subjected to 14- or 28-day cold pretreatments. Changes in the phenotypes of *Alcea rosea* 'nigra' after freezing temperatures are indicated in supplementary Figure S2. Control plants and those pretreated with a cold could tolerate the reduced temperature to -4°C. Overall, Cold Pretreatment ($CP_1$ and $CP_2$) increased survival (84%) compared to the control condition at -4°C. Although the percentage of hollyhock survival under control

conditions was constant up to the temperature ranges of 2 to 0°C, there was a decreasing trend (by 11.1%) as the temperature decreased further and reached -2°C (Figure 1-b).

Hollyhock seedlings died entirely at -6°C, and freezing treatment reduced growth in all chilled plants, as indicated by the sharp decrease in the dry weight of aerial parts, roots, and leaf area (Figure 1). The 14- and

**Table 1**. The Lethal Temperature of 50% of plants ($LT_{50}$) in hollyhock plants after freezing stress under control conditions.

| Control | $CP_1$ | $CP_2$ |
|---|---|---|
| -3.2 °C | -4.6 °C | -4.8 °C |

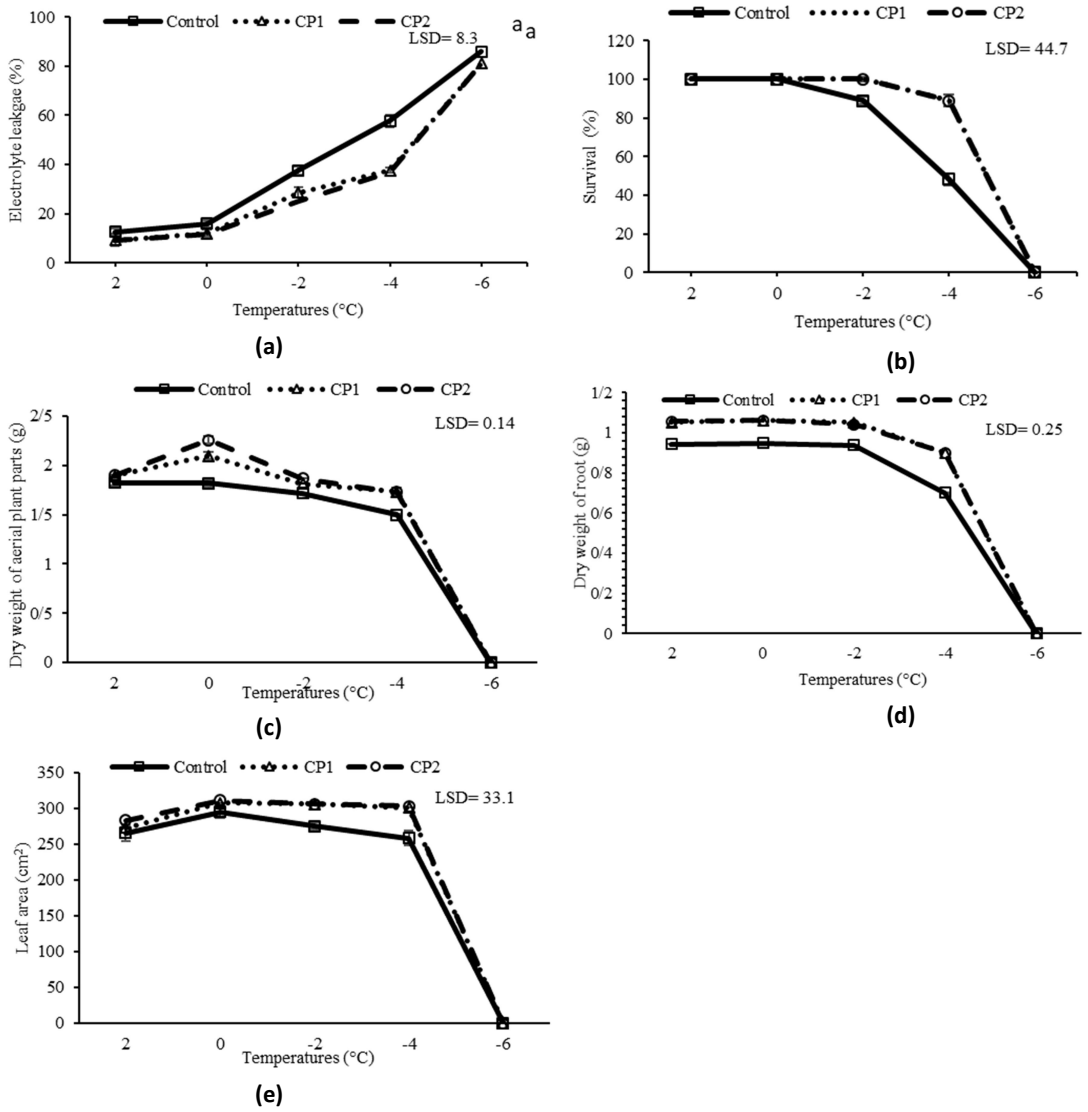



**Figure 1**. Effect of cold pre-conditioning on electrolyte leakage (a), survival (b), dry weight of aerial parts of plant (c), dry weight of root (d), and leaf area (e) of hollyhocks at different temperatures: $CP_1$: 14 days of cold preconditioning; $CP_2$: 28 days of cold preconditioning. Different letters show significant differences ($P \leq 0.05$) according to the LSD test (n= 3±SE).

28-day cold pretreatments significantly alleviated the decreases in the dry weights. Cold acclimation ($CP_1$ and $CP_2$) at 0°C resulted in a significant increase in the dry weight of aerial parts of plants, which increased by 15.4, and 24.2%, respectively, compared to the control. Freezing temperatures until -4°C resulted in a substantial decrease in the dry weights in the non-acclimated plants and was a 13.2% reduction compared to the $CP_1$ and $CP_2$ (Figure 1-c). The highest dry weight was recorded in $CP_1$ and $CP_2$ at 0°C. The dry weight of the root remained almost constant from 2 to -2°C. The root dry weight in $CP_1$ and $CP_2$ decreased (14.2%) significantly at -4°C compared to 2°C (Figure 1-d). The highest leaf area was recorded in the $CP_2$ plant at 0°C. The leaf area of the control plant decreased significantly from 0 to -4°C, but this index was constant in $CP_1$ and $CP_2$ by the temperature reduction (Figure 1-e). ). The data analysis ($P\leq 0.05$) (Table 1-S) revealed that the root volume of $CP_1$ and $CP_2$ increased (12%) significantly compared with the control, but decreased rapidly at -4°C (38.1%) compared to 0°C. Water-soluble sugar content increased significantly in all treatments under freezing temperatures ($P< 0.01$). Leaf water-soluble sugar of hollyhock increased from 9.3 mg $g^{-1}$ DW to 10.3 mg $g^{-1}$ DW in $CP_1$ and $CP_2$ plants in response to 0°C compared with 2°C. The highest water-soluble sugar content was observed at -2°C when preconditioned by cold. This increase was the highest in the $CP_2$ condition compared to the control. At a temperature less than -2°C, water-soluble sugar gradually increased to 10.4 mg $g^{-1}$ DW in $CP_2$, but it was not affected by lowering the temperature in $CP_1$ (Table 2). The proline content of plants from CP increased significantly under freezing temperature to -4°C ($P< 0.01$). The proline content was significantly higher in the plants of CP compared to the control plants with the lowest content. The lowest proline content was found at -4°C in the control plants as for the intensity of freezing stress, and the proline content at a temperature lower than -2°C gradually decreased to 7.25 mg $g^{-1}$ FW in the control plant compared to 2°C (Table 2).

The content of $H_2O_2$ increased rapidly in all plants at -4°C compared with those at 2°C, but no significant difference was observed in $CP_1$ and $CP_2$ plants (Table 2). The freezing temperature gradually increased MDA in cold-pretreated plants and the control ones. Lower amounts of MDA were observed in $CP_1$ and $CP_2$ plants at 2°C. MDA contents of $CP_1$ and $CP_2$ plants at different temperatures did not change, but significantly increased by 86% in the control plants at -4°C compared to 2°C. Freezing temperatures increased in phenolic content in the leaves of control plants and CP at -4°C compared to non-stressed plants at 2°C. No significant differences were found in phenolic content between $CP_1$ and $CP_2$ plants, while a 29.1% reduction in phenolic content was observed in control plants compared to CP plants at -4°C (Table 2).

The antioxidant activities (SOD, APX, and CAT) of the hollyhock were significantly affected ($P< 0.01$) by cold preconditioning and freezing temperatures. The SOD activity increased significantly (41.4 and 57.4%) in the control and $CP_2$ plants at -4°C compared with those at 0°C. The CAT activity in cold preconditioned leaves was significantly higher than that in the control plants. Furthermore, the activity of CAT in cold preconditioned leaves was 55.3% higher than that in the control plants at 2°C. The CAT activity of plants was not affected by lower temperatures (-4°C) compared to non-stressed plants (2°C). Freezing temperature (-2°C) significantly increased APX activity in $CP_1$ and $CP_2$ plant leaves by 33.3 and 95.2%, respectively, compared to non-stressed plants. The activities of APX in the control and CP were 148 and 76.9% higher at -4°C than at 2°C, respectively (Table 3).

Table 4 shows that preconditioning in the cold significantly increased *Pn* values (11%) of plants compared with those in the control condition at 2°C ($P< 0.01$) ), which was reduced in all treatments by lowering the temperature from 0°C to -4°C. The *gs* values

**Table 2**. Changes in water-soluble sugars, proline, $H_2O_2$, MDA, and phenol of cold preconditioned hollyhock by treatment with freezing temperatures.

| Treatments | Temperatures (°C) | Water soluble sugars (mg $g^{-1}$ DW) | Proline (mg $g^{-1}$ FW) | $H_2O_2$ (µmol $g^{-1}$ FW) | MDA (nmol $g^{-1}$ FW) | Phenol (mg $g^{-1}$ FW) |
|---|---|---|---|---|---|---|
| Control | 2 | 9.17±0.06 d | 8.05±0.01d | 9.07±0.13e | 4.00±0.29bc | 5.10±0.15f |
| | 0 | 9.40±0.03 c | 8.03±0.01d | 9.00±0.07bc | 4.00±0.33bc | 5.73±0.06ef |
| | -2 | 9.90±0.06b | 7.79±0.03e | 11.07±0.01c | 5.33±0.01b | 6.10±0.09de |
| | -4 | 9.50±0.03c | 7.25±0.01g | 12.13±0.03a | 7.47±0.58a | 6.80±0.06cd |
| $CP_1$ | 2 | 9.27±0.03f | 8.25±0.07c | 5.70±0.12ef | 2.80±0.15cd | 7.30±0.07bc |
| | 0 | 10.33±0.03a | 8.53±0.03b | 7.47±0.07de | 2.90±0.09cd | 7.71±0.15b |
| | -2 | 10.13±0.03a | 8.63±0.03a | 8.57±0.26cd | 3.23±0.25cd | 9.20±0.10a |
| | -4 | 10.13±0.03a | 8.37±0.03f | 9.57±0.15b | 3.80±0.42cd | 9.70±0.06a |
| $CP_2$ | 2 | 9.36±0.01ef | 8.30±0.01c | 5.00±0.01fg | 2.33±0.03d | 7.33±0.31bc |
| | 0 | 10.37±0.01ab | 8.61±0.01ab | 7.00±0.33def | 2.66±0.17cd | 8.00±0.36ab |
| | -2 | 10.40±0.03a | 8.70±0.01a | 8.33±0.58bc | 3.33±0.33cd | 9.28±0.01a |
| | -4 | 10.21±0.01bc | 8.60±0.03ab | 10.00±0.58bc | 4.03±0.33cd | 9.60±0.12a |

[a](a-g) Mean values at each site show interactions between cold preconditioning and freezing temperatures, means with the same letter(s) not significantly different at the 5% probability level (r= 3 for each observed value in the table; $CP_1$: 14 days of cold preconditioning; $CP_2$: 28 days of cold preconditioning).

**Table 3**. Changes in enzymes activities of cold preconditioned hollyhock by treatment with freezing temperatures.

| Treatments | Temperatures (°C) | SOD (U $g^{-1}$ FW) | CAT (µmol $min^{-1}$ $g^{-1}$ FW) | APX (µmol $min^{-1}$ $g^{-1}$ FW) |
|---|---|---|---|---|
| Control | 2 | 152±2.88d | 1.330±0.03g | 13.000±0.76e |
| | 0 | 186±2.84c | 1.900±0.03f | 14.0100±0.28de |
| | -2 | 192±1.52c | 2.470±0.06d | 21.020±0.28c |
| | -4 | 215±0.88b | 7.870±0.03b | 23.040±0.89c |
| $CP_1$ | 2 | 199±2.88bc | 2.033±0.03ef | 15.670±0.57de |
| | 0 | 211±0.01b | 2.200±0.03de | 20.330±1.73c |
| | -2 | 201±5.03b | 3.170±0.10c | 28.060±0.33b |
| | -4 | 315±0.88a | 8.030±0.03ab | 39.010±0.33a |
| $CP_2$ | 2 | 202±1.52cd | 2.070±0.12ef | 16.500±0.03d |
| | 0 | 209±1.66bc | 2.470±0.12d | 21.330±0.09c |
| | -2 | 211±3.18bc | 3.170±0.03c | 40.920±0.60a |
| | -4 | 318±1.01a | 8.170±0.03a | 40.830±0.76a |

[a](a-g) Mean values at each site show interactions between cold preconditioning and freezing temperatures, mean with the same letter(s) not significantly different at the 5% probability level (n= 3 for each observed value in the table; $CP_1$: 14 days of cold preconditioning; $CP_2$: 28 days of cold preconditioning; SOD: Superoxide Desmutase, CAT: Catalase; APX: Ascorbate Peroxidase).

of the control and $CP_2$ plants decreased by 90 and 62%, respectively, at -4°C compared to 2°C.

In all treatments, the *Tr* value decreased in seedlings at -4°C compared with 2°C. There was no significant difference in *Tr* of $CP_1$, $CP_2$, and the control plants at 2°C, but the *Tr* of the control seedlings decreased sharply with a decrease in temperature, and lower *Tr* (0.327 $H_2O_2$ mmol $m^{-2}$ $s^{-1}$) was found at –4°C. The Ci values remained almost constant for the control, $CP_1$, and $CP_2$ plants from 2-0°C. In this study, the *Ci* of seedlings showed a significant decrease with a

**Table 4**. Changes in photosynthesis parameters and ABA content of cold preconditioned hollyhock by treatment with freezing temperatures. [a]

| Treatments | Temperatures (°C) | Total chlorophyll (mg $g^{-1}$ FW) | *Pn* (µmol $m^{-2}$ $s^{-1}$) | *gs* (mol $m^{-2}$ $s^{-1}$) | *Tr* (mmol $m^{-2}$ $s^{-1}$) | *Ci* (ppm) | ABA (ng $g^{-1}$ DW) |
|---|---|---|---|---|---|---|---|
| Control | 2 | 8.047±0.07ef | 28.200±0.13b | 0.010±0.01bc | 2.167±0.07a | 370±0.03a | 2.100±0.04g |
| | 0 | 8.840±0.02de | 23.230±0.11de | 0.010±0.00d | 0.913±0.05cd | 350±0.07a | 3.100±0.01f |
| | -2 | 7.880±0.35ef | 22.600±0.15e | 0.010±0.00bc | 0.750±0.03d | 257±0.01c | 4.200±0.01bc |
| | -4 | 7.290±0.08f | 17.640±0.06f | 0.001±0.00d | 0.328±0.07f | 133±0.01e | 4.480±0.01a |
| $CP_1$ | 2 | 11.020±0.58ab | 30.330±0.33b | 0.020±0.00a | 2.133±0.01ab | 371±0.03a | 1.330±0.70h |
| | 0 | 8.840±0.28d | 24.670±0.33cd | 0.020±0.02a | 1.967±0.07b | 350±0.06a | 3.220±0.03f |
| | -2 | 7.500±0.33f | 22.670±0.33e | 0.018±0.02a | 0.916±0.03cd | 300±0.01b | 4.070±0.01d |
| | -4 | 7.200±0.01f | 18.330±0.33f | 0.008±0.01cd | 0.500±0.03ef | 173±0.09d | 4.300±0.03b |
| $CP_2$ | 2 | 11.580±0.42a | 31.280±0.33a | 0.023±0.01a | 2.233±0.06a | 377±0.06a | 1.230±0.20h |
| | 0 | 10.110±0.31bc | 24.730±0.27c | 0.201±0.03a | 2.050±0.02ab | 360±0.03a | 1.270±0.02h |
| | -2 | 8.370±0.05def | 22.730±0.27de | 0.167±0.03ab | 0.968±0.06c | 312±0.01b | 3.400±0.03e |
| | -4 | 7.830±0.21ef | 18.670±0.25f | 0.009±0.01c | 0.535±0.08e | 180±0.07d | 4.070±0.03cd |

[a](a-h) Mean values at each site show interactions between cold preconditioning and freezing temperatures, mean values with the same letter(s) not significantly different at the 5% probability level ($r$= 3 for each observed value in the table; $CP_1$: 14 days of cold preconditioning; $CP_2$: 28 days of cold preconditioning; *Pn*: Net Photosynthetic rate; *gs*: Stomatal conductance; *Tr*: Transpiration*; Ci:* Intercellular $CO_2$ consideration).

**Table 5** Survival percentage profile of cold and non-cold preconditioning according to the adjusted Gompertz nonlinear model.

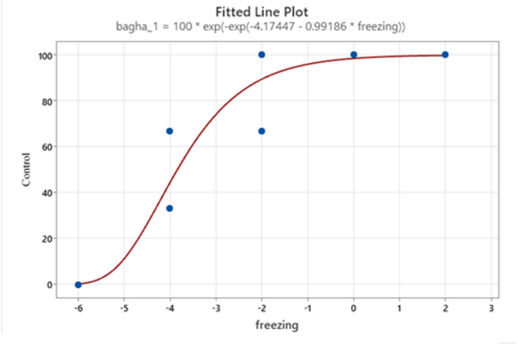
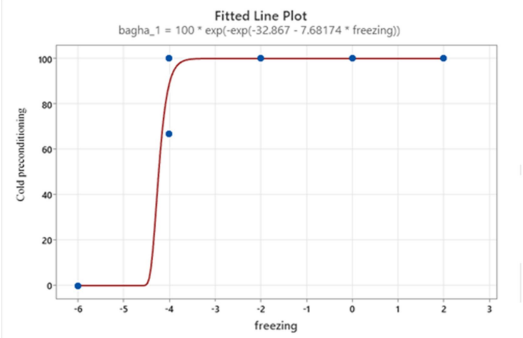
| Control | Cold preconditioning |
|---|---|
| $y = 100e^{-e^{-4.17447-0.99186x}}$ | $y = 100e^{-e^{-32.867-7.68174x}}$ |
|  |  |



**Table 6.** Variance Analysis of linear relationship of survival percentage with other traits of hollyhock

| Source | df | Mean Square |
|---|---|---|
| Model | 6 | 11796** |
| Error | 38 | 60.7 |
| Total | 44 | |

** Significant at 1% probability level.

decrease in temperature, and this reduction was more prominent at -4°C treatment. *Ci* of seedlings showed a significant decrease, and this reduction was more prominent at -4°C treatment. *Ci* of $CP_1$ and $CP_2$ showed 30% increase at -4°C compared with the control (Table 4). Freezing temperature resulted in a gradual decrease in chlorophyll up to -4 °C in all treatments. Plants from CP accumulated more chlorophyll in response to freezing temperatures than the control plants. Freezing temperature (-4°C) significantly decreased the chlorophyll content of $CP_2$ and the control plants (9.3 and 32%, respectively) compared to the plants at 2°C. There were no significant differences in chlorophyll content, *Pn*, and gs in $CP_1$ and $CP_2$ plants. The leaves of the control plants had significantly higher ABA content than those of the cold-pretreated plants at subzero temperatures. Acclimation resulted in a high ABA increase (18.4%) in the control plant at -4°C compared to $CP_2$ (Table 4). The essential oil was not significantly affected by the interaction of cold pretreatment and freezing temperatures (Table 1-S). The essential oil content ranged from 1.65% at 2°C to 0.73% at -4°C, which decreased (55.7%) when the plants were treated at -4°C compared to 2°C.

Table 5 shows that the response of survival percentage to the pre-cold conditioning follows a nonlinear equation called Gompertz, which was different for the samples treated with cold treatment than those without cold treatment. The survival percentage can be calculated at any value of cold treatment with this function. The table shows the nonlinear relationship of the regression model for the control and cold pre-conditioning. Figures S1 and S2 show that the survival percentage was the lowest (0) at low temperatures (-6°C) and the maximum survival rate was 100%, which does not follow a linear relationship. Control plants did not survive at -5°C due to cold pre-treatment, so, the survival percentage increased with cold pre-treatment. As the dependent variable, the survival percentage was analyzed against other independent

variables (Table 6). The linear relationship of the model was obtained as follows:

Y = -0.24+4.77 X1+21.46 X2-7.65 X3-9.50 X4-16.06 X5

Where, Y presents the survival percentage, X1 donates the root volume, X2 indicates the proline, X3 shows the MDA, and X4 is the *Tr*. The root, proline, MDA, and Tr volume were included in the model, responsible for 96.7% of the changes ($P< 0.01$). Based on the results of the correlation analysis among survival rate and other traits, the characteristics of electrolyte leakage showed a high negative and significant correlation with the characteristic of the survival rate. Therefore, the highest positive correlation was recorded between morphological traits (leaf area and dry weight) with survival percentage (Table 2-S).

## DISCUSSION

Although low temperatures cause cold acclimation (Li *et al*. 2020), very little is known about the effects of the duration of cold preconditioning on non-acclimated plants such as *Alcea rosea*. The electrolyte leakage (Figure 1) and $LT_{50}$ (Table 1) were evaluated after frost stress in all plants and the survival percentage after 21 days to determine $LT_{50}$ and frost tolerance threshold in hollyhock. However, traits such as secondary metabolites, photosynthetic parameters, and antioxidant activity were measured only in living cells until -4°C.

Electrolyte leakage, as an indicator of stress damage to plasma, and the survival percentage were determined after freezing the leaves of the control (non-acclimated plants) and cold-pretreated plants ($CP_1$ and $CP_2$) at different temperatures. The control plants and those from CP showed increased EL at lower temperatures. EL (%) increased with freezing to lower temperatures, indicating a direct effect of temperature on leaf plasma membranes (Elkelish *et al*., 2020). The percentage of survivors did not change in all treatments up to 0°C, but significantly decreased in the control plants at lower temperatures than CP plants. Therefore, the plasma membrane stability of CP plants and avoidance of extracellular ice formation of cells increased due to tissue desiccation (Ouyang *et al*. 2020). The $LT_{50}$ of non-acclimated plants increased considerably, and relatively lower frost tolerance was observed in these plants. Some cell walls modifications such as lignification and suberization are critical for the cold acclimation of plants (Ji *et al*., 2015). The lower $LT_{50}$ of the CP plants might be due to the cold-hardening-related modification of cell wall structure, which ultimately prevents the leakage of electrolytes from the cells into the extracellular water.

When temperatures decreased below -4°C, all plants suffered severe damage at -6°C, and plant death dramatically affected growth (Figure 1-b). Studies have shown that cold stress sometimes does not kill some plants. Growth of the plant does, however, suffered during this recovery period. Therefore, plants may not fully recover and resume growth properly under such conditions. Plant dry matter is related to physiological responses and decreases during recovery due to frost damage to plant physiological processes and the ability to regrow (Rastgo *et al*., 2022). Apart from maintaining survival, there is a potential for improving morphological traits such as dry matter accumulation after the recovery phase. The plants in $CP_1$ and $CP_2$ were better able to resume growth than those in control (Figures 1-c and -d).

The physiological traits were analyzed in live plants after freezing stress (until -4°C). In the present study, water-soluble sugars and proline concentrations increased significantly from 2 to -4°C. Carbohydrates can bind to the polar head groups of phospholipids and thus increase biomembrane stabilization by lowering the freezing point of free intercellular water. Increase in proline and water-soluble sugar concentration may also increase the osmotic potential and improve protoplasmic stability,

further contributing to cold acclimation (Beheshti Rooy *et al*., 2017). Water-soluble sugar and proline were accumulated during preconditioning by cold. These results confirmed the previous observations by Bertrand *et al*. (2019) and Fürtauer *et al*. (2019), who showed that the accumulation of soluble carbohydrates during hardening under field conditions resulted in high frost tolerance in several plants. The loss of cold tolerance of the control plants closely coincided with the reduction in water-soluble sugars (Ershadi *et al*., 2015). Thus, the lower water-soluble sugar content during warm periods could be due to increased respiration, but cold preconditioning before frost reduced respiration and accumulated carbohydrates. In the present study, frost increased the ABA content of hollyhock. The accumulation of soluble sugars under cold stress and ABA act synchronously (Hongtao *et al*., 2017). A cold-induced increase in endogenous ABA concentration is closely related to the subsequent frost tolerance of the plant (Wang *et al*., 2018).

Freezing temperatures increased $H_2O_2$ and MDA accumulation (Fu *et al*., 2017). CP conditions decreased the $H_2O_2$ concentration of hollyhock and effectively delayed the increase in MDA content. Pre-cold increased the tolerance of plants to freezing temperatures, and $H_2O_2$ levels in leaves often decreased (Arfan *et al*., 2019). Freezing in non-acclimated plants resulted in a continuous increase in $H_2O_2$ and MDA content. The results are consistent with those of previous studies (Fahimirad *et al*., 2013) in which maximum amounts of MDA and $H_2O_2$ were found in non-cold-acclimated plants. However, under extreme freezing conditions (-4°C), $H_2O_2$ and MDA production increased sharply, exceeding the ability of plants to intercept the excessive ROS (Zhang *et al*. 2020b) and resulting in plant death at this temperature. Previous studies have indicated a significant increase in phenolic compounds at freezing temperatures (Król *et al*., 2015; Wang *et al*., 2020). This study showed that phenolics in hollyhock increased at lower temperatures (Table 2). The explanation for these observations might be the antioxidant activity of phenols. Therefore, phenolic compounds, which could be effortlessly accumulated, reduced the harmful effects of oxidative stress (Wojdylo *et al*., 2007).

Alleviation of cold stress-induced oxidative damage is a crucial mechanism in cold stress tolerance. Plants have evolved antioxidant defense systems, including mobilizing non-enzymatic and enzymatic antioxidants, to overcome ROS -induced oxidative stress. SOD is the first enzyme to perform detoxification of ROS by converting the superoxide radical into an $H_2O_2$ molecule, which is then converted immediately to $H_2O$ by CAT and APX enzymes. Frost stress caused a significant increase in the activities of SOD, CAT, and APX at -4°C, which was accompanied by the accumulation of $H_2O_2$. Therefore, the increased activities of CAT and APX enzymes can effectively remove the overproduced $H_2O_2$. Although $H_2O_2$ and MDA concentrations were higher at -4 °C than in the control plants, the reduction in $H_2O_2$ and MDA accumulation in $CP_1$ and $CP_2$ plants from 65 to 15% and 70 to 19%, respectively, at -4°C reflected the effective enzymatic antioxidant system of CP hollyhock against frost stress and its role in protecting against cold stress-induced oxidative stress. Higher antioxidant activity corresponded with higher protection against cold damage in acclimated plants. These consequences emphasized that growth under freezing temperatures, especially without cold acclimation, significantly decreased (Turan and Ekmekci, 2011). The higher antioxidant activity at lower temperatures in the leaves of CP hollyhock is responsible for reducing MDA, $H_2O_2$, and membrane damage due to the reduction of oxidative damage during cold exposure. Various studies have used periods ranging from a few hours to several days for cold acclimation (Vyse *et al*., 2019). There was no difference between $CP_1$ and $CP_2$ for most traits, except water-soluble sugars and proline. Although proline and water-soluble

sugars were higher in the plants grown for 28 days before cold conditioning than in plants treated for 14 days, no significant difference was found in the survival. Therefore, secondary metabolites are necessary for cold acclimation, and enzyme activities are critical.

Photosynthesis is one of the features most rapidly affected by falling temperatures. The photosynthetic parameters in leaves decreased when plants were exposed to freezing temperatures (Zhang *et al*. 2020a). However, the leaves treated before cold treatment and after freezing showed higher *Pn*, *Tr*, and *gs* values than non-acclimated plants. The lower *Pn* values in non-cold-acclimated leaves were accompanied by lower *gs* values, confirming the role of stomatal limitation in lowering the photosynthetic rate in leaves after low temperatures (Karimi *et al*., 2016). Significant increases in *Pn* levels in cold-treated hollyhock's leaves were associated with higher ROS-purifying enzyme activities as well as higher levels of chlorophyll, ABA, and biomembrane stability (i.e., lower MDA levels), suggesting that ABA may have a signaling role in up-regulating these non-stomatal aspects of the photosynthetic apparatus. The high gs levels of CP hollyhock suggest reducing ABA and enhancing stomatal function and plant adaptation upon rewarming to normal temperatures. Stomatal and non-stomatal components of cold-induced carbon fixation depression have been detected in various plant leaves (Bhattacharya *et al*., 2022).

Environmental stresses can alter the quality of essential oil constituents in aromatic plants. Additionally, cold conditioning did not significantly affect the measured properties at the seedling stage, and essential oil levels decreased under lower temperatures. Reda *et al*. (1999) showed that the essential oil content increased when the seeds were treated at 6 °C for 15 days. Bagheri *et al*. (2020) studied four temperature levels and their effects on the properties of German chamomile genotypes. As cold stress increased from 20°C to 10°C, the essential oil content decreased. The results reported that the essential oil content decreased under low-stress conditions, consistent with the current study.

The results indicated that *Alcea rosea* 'nigra', has no morphological changes under freezing stress (2 and 0°C), but seedlings began to die under freezing stress (–4 and –6°C). Stepwise regression analysis was used to identify only those traits that justified a significant proportion of survival changes and remove ineffective or low-impact traits from the regression model. Moreover, the high correlation between morphological and physiological characteristics and survival percentage significantly influenced this critical index. Based on the results (Table 5), the increase in the survival percentage of hollyhock in pre-cold treatments was higher than in non-cold plants.

## CONCLUSIONS

The CP hollyhock showed a higher accumulation of compatible solutes and antioxidant activity and lower electrolyte leakage, MDA, and $H_2O_2$. Therefore, cold acclimation produced better physiological and biochemical responses than non-cold acclimation. Although proline and water-soluble sugar levels were higher in plants pre-acclimated for 28 days than in those pretreated for 14 days, no significant difference was observed in survival. In addition, this method significantly increased the tolerance of plants to cold stress, and physiological and morphological traits were affected by freezing temperatures. The resulted suggested that well-planned temperature regulation during the seedling stage is a remarkable strategy to control vegetative traits in a desired way and open new opportunities for horticulture.

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# کسب تحمل انجماد در ختمی سازگار نشده با قرار گرفتن در معرض دوره های مختلف پیش سرما

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## چکیده

کاهش دمای زمستان ممکن است، با جلوگیری از حداکثر سازگاری به سرما قبل از دمای انجماد بر بقای گیاهان تأثیر منفی بگذارد. سازگاری با سرما نیازمند سازگاری با ترکیبی از نور و دمای پایین است، شرایطی که به مدت زمان قرار گرفتن در معرض آن بستگی دارد. بر این اساس، تحقیقاتی برای شناسایی استراتژی هایی که می توانند سازگاری با سرما را تقویت کنند و تحمل یخ زدگی را افزایش دهند، لازم است. بنابراین، هدف از این آزمایش بررسی این موضوع بود که آیا یک تیمار سرد کوتاه‌تر یا طولانی‌تر می‌تواند مقاومت به سرما و آستانه تحمل به سرمای ختمی را افزایش دهد. نتایج نشان داد که هر دو ۱۴ (CP1) و ۲۸ روز پیش سرما (CP2) باعث کاهش نشت الکترولیت، افزایش فعالیت آنزیم های سوپراکسیددیسموتار، کاتالاز و آسکوربات پراکسیداز، مهار تجمع پراکسید هیدروژن و تاخیر در افزایش مالون دی آلدئید شد. در حالی که در گیاهان غیر سازگار محتوای مالون دی آلدئید، کاهش یافت. هیچ تفاوتی در فعالیت آنتی اکسیدانی و پارامترهای فتوسنتزی بین CP1 و CP2 مشاهده نشد. اگرچه محتوای پرولین و قند محلول در آب در گیاهانی که در معرض ۲۸ روز پیش سرما قرار گرفتند بیشتر از گیاهانی بود که به مدت ۱۴ روز تیمار شده بودند، تفاوت معنی داری در درصد بقا یافت نشد. دماهای پایین باعث کاهش پارامترهای فتوسنتزی و افزایش محتوای آبسیزیک اسید و محتوای فنولیک در برگ ها شد. نتایج نشان می‌دهد که پیش‌تنظیم سرمای ۱۴ روزه می‌تواند به عنوان تحمل سرما برای ختمی های سازگار نشده برای رشد در مزرعه در دمای ۴- درجه سانتی‌گراد استفاده شود.