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PROTECTIVE ROLE OF SAFFLOWER (*Carthamus tinctorius* L.) FLOWER EXTRACT-BASED NATURAL ANTIFREEZE SPRAY AGAINST FREEZING INJURY IN COMMON BEAN (*Phaseolus vulgaris* L.) TISSUES

Elif Özbey*

Malatya Turgut Ozal University, Battalgazi Vocational School, Department of Park and Horticultural Crops, Malatya (Türkiye)
*****e-mail: *elif.ozbey@ozal.edu.tr*

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ABSTRACT

Late spring frost is major abiotic stress factor causing significant yield losses in vegetable and fruit crops. Low temperatures induce intracellular ice formation, leading to the membrane damage, increased electrolyte leakage, and reduced tissue viability. Therefore, developing ecofriendly strategies to mitigate frost injury are essential. In this study, a natural antifreeze spray was developed using glycerol and sorbitol as osmoprotectants, safflower (*Carthamus tinctorius* L.) flower-extract as a natural antioxidant source, and sodium alginate as a film-forming biopolymer. The protective effect of the formulation was evaluated in buds, flowers, and young fruit tissues of common bean (*Phaseolus vulgaris* L.) under three temperature conditions (22-24°C, -4°C, and -10°C). Results revealed that the antifreeze treatment significantly improved tissue viability and reduced freezing-induced damage. At -10°C, cellular damage in seed tissues decreased from 82.8% to 30.0%, representing approximately 52% reduction in cell damage. Electrolyte leakage was also reduced from 97.3% to 75.0%, indicating enhanced membrane stability. Overall, the developed formulation effectively mitigated freezing damage and unveiled potential to protect cold-sensitive crops.

Keywords: Electrolyte leakage, freezing stress, natural antifreeze spray, osmoprotectants, safflower extract, TTC assay

INTRODUCTION

Low temperature and freezing events represent one of the most important abiotic stress factors that adversely affect plant growth and development, and crop productivity. In particular, late spring frosts can cause severe yield losses in vegetable and fruit production, especially during flowering and early fruit development stages. Under freezing conditions, the formation of intracellular ice crystals causes mechanical damage to cell membranes, leading to increased membrane permeability, electrolyte leakage, and disruption of cellular metabolism. These physiological disturbances ultimately reduce tissue viability and lead to irreversible cellular damage (Ding *et al.,* 2019; Guan *et al.*, 2023). In addition, exposure to low temperatures adversely affects photosynthesis, respiration, and other metabolic processes (Roychowdhury *et al.*, 2025; Wu *et al.*, 2026).

Plants have evolved several physiological and biochemical mechanisms to cope with cold stress conditions. These mechanisms include stabilization of cellular membranes, activation of antioxidant defense systems, and accumulation of small molecules known as osmoprotectants. Osmoprotective compounds such as glycerol, sorbitol, proline, and sugars play key role in maintaining cellular osmotic balance, reducing ice crystal formation, and protecting membrane integrity (Crowe *et al.*,

1998; Elbein *et al.*, 2003; Pattnaik *et al.*, 2021). In addition, sugar alcohols and related compounds are known to stabilize proteins and lipid membranes under stress conditions (Govrin *et al.*, 2019; Wingler *et al.*, 2020). Another important consequence of cold stress is the excessive production of reactive oxygen species (ROS), which can cause oxidative damage to lipids, proteins, and nucleic acids. Therefore, antioxidant defense systems are crucial in determining plant tolerance to freezing stress (Gill and Tuteja, 2010; Sharma *et al.*, 2012; Hasanuzzaman *et al.*, 2020). In this context, plant-derived extracts rich in phenolic and flavonoid compounds have gained considerable attention due to their ability to enhance antioxidant capacity and mitigate stress-induced damage (Qian *et al.*, 2024).

Safflower (*Carthamus tinctorius* L.) flowers are rich in phenolic compounds, flavonoids, and other bioactive metabolites with strong antioxidant properties. These compounds have been widely reported to exhibit protective effects against oxidative stress (Li *et al.*, 2019; Chang *et al.*, 2025; Clemente *et al.*, 2026). Therefore, safflower flower extract represents a promising natural source for improving plant tolerance to environmental stresses.

In addition to biochemical protection, the use of biopolymer-based coatings has emerged as an effective strategy for protecting plant tissues against environmental stress. Sodium alginate can form a semi-permeable protective layer that reduces water loss, regulates gas exchange, and enhances resistance to external stress factors (Krochta and De Mulder-Johnston, 1997; Dhall, 2013). Previous studies have demonstrated that edible coatings can mitigate both physical and physiological stress damage in plant tissues (Rojas-Graü *et al.*, 2009).

Although several studies have investigated the role of osmoprotectants, antioxidants, and edible coatings individually, limited studies have focused on the development of integrated natural antifreeze formulation, combining these components for effective plant protection under freezing stress (Griffith and Yaish, 2004; Ashraf and Foolad, 2007; Yang *et al.*, 2020). Therefore, the present study was aimed to develop an effective ecofriendly antifreeze strategy that can be applied directly to plant tissues, using a low temperature-sensitive common bean as a model system.

MATERIALS AND METHODS

Plant material

Common bean (*Phaseolus vulgaris* L.) plants were used as experimental material in this study. The experiment was conducted in 2025 at Malatya Turgut Ozal University, Battalgazi Vocational School (Malatya, Türkiye) under greenhouse conditions. Bean seeds were sown in plastic pots (20 cm diameter) containing approximately 2 kg sterile potting soil (peat: perlite, 2:1, v/v). Plants were grown under natural photoperiod conditions at 22–24°C and irrigated regularly until they reached the bud, flowering, and early fruit development stages.

Preparation of safflower flower extract

Safflower (*Carthamus tinctorius* L.) flowers were air-dried at room temperature and ground into powder. The extract was prepared using an aqueous extraction method. Briefly, 10 g dried flower powder was mixed with 100 mL distilled water and heated at 60°C for 2 h under continuous stirring. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was collected as crude extract and stored at 4°C until use.

Preparation of natural antifreeze spray formulation

In the present study, a natural antifreeze spray was developed using glycerol and sorbitol as osmo-protectants, safflower flower extract as an antioxidant source, and sodium alginate as a film-forming biopolymer. The protective effect of this formulation against freezing stress was evaluated in common bean tissues under different temperature conditions. The antifreeze formulation consisted

of sorbitol (3%, w/v), glycerol (3%, v/v), sodium alginate (0.3%, w/v), and safflower flower extract (1%, v/v). Initially, 900 mL distilled water was heated to 60-70°C, and sodium alginate was gradually added under continuous stirring using a magnetic stirrer until its complete dissolution. After cooling to room temperature, glycerol and sorbitol were added and mixed thoroughly. Finally, safflower extract was incorporated into the mixture. The pH of solution was adjusted to 6.2-6.8, and the final volume adjusted to 1 L. The formulation was stored under dark and cool conditions until use.

Experimental design and treatments

The experiment was arranged in a completely randomized design (CRD) with six treatment groups as: 1) control at room temperature, 2) antifreeze treatment at room temperature, 3) control at -4°C; 4) antifreeze treatment at -4°C, 5) control at -10°C, and 6) antifreeze treatment at -10°C. The antifreeze solution was applied using a hand-held atomizer sprayer (100 mL capacity) until all aerial plant tissues (buds, flowers, and young fruits) were uniformly wetted (10 mL plant^{-1}). Each treatment consisted of three biological replicates, with at least five plants per replicate.

Freezing stress treatment

After application of antifreeze formulation, plants were exposed to different temperature regimes. Control plants were maintained at room temperature (22-24°C), while freezing treatments were performed at -4°C and -10°C. Plants were placed in a programmable cooling chamber (or laboratory freezer/refrigerator) and incubated for 24 h. Following the treatment, plant tissues were immediately subjected to physiological and biochemical analyses.

TTC viability assay

TTC (2,3,5-triphenyl tetrazolium chloride) assay was performed as per the method of Steponkus and Lanphear (1967), with minor modifications. Approximately 0.2-0.3 g plant tissue was incubated in 5 mL of 0.5% TTC solution at 30°C for 4 h in dark. After incubation, the TTC solution was replaced with 95% ethanol, and the samples incubated at 60°C for 30 min to extract the formazan. Absorbance was measured at 485 nm using a UV–vis spectrophotometer (Shimadzu UV-1800, Japan). Tissue viability was calculated as under:

$$\text{Viability (\%)} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Visual damage assessment

Morphological damage was evaluated using a visual scoring system based on tissue discolouration, wilting, and structural deformation, as described by Wisniewski *et al.* (2014). Damage was scored on 0 to 4 scale, where 0 indicated no damage and 4 indicated complete tissue necrosis.

Electrolyte leakage analysis

Electrolyte leakage was determined according to the method of Lutts *et al.* (1996). Approximately 0.2-0.3 g plant tissue was placed in tubes containing 10 mL distilled water and incubated at 25°C for 4 h. Initial electrical conductivity (C1) was measured using a conductivity meter (WTW Cond 3110, Germany). The samples were then boiled for 15 min, cooled to room temperature, and final conductivity (C2) was measured. Relative electrolyte leakage (REL) was calculated as under:

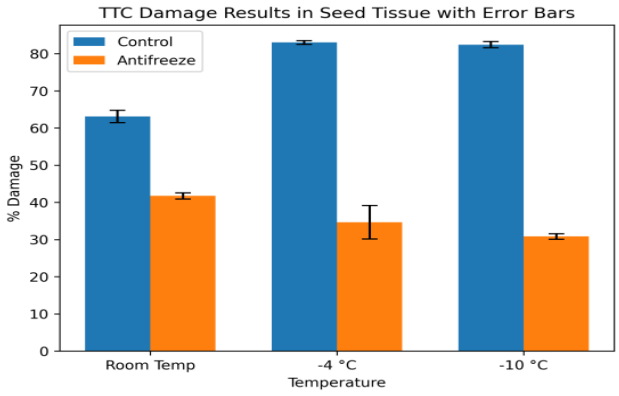
$$\text{Relative electrolyte leakage (\%)} = \frac{\text{Initial electrical conductivity}}{\text{Final electrical conductivity}} \times 100$$

Statistical analysis

All experiments were conducted with three independent biological replicates. Data were expressed as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine significant differences among treatments at $p < 0.05$ (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The results of TTC viability assay revealed that the developed natural antifreeze sprays significantly preserved cellular viability in plant tissues exposed to freezing stress (Fig. 1). The protective effect of antifreeze formulation became more evident under low temperature conditions. In seed tissues, the highest level of cellular damage was observed in the control group at -10°C, where the damage rate reached 82.8%. In contrast, antifreeze-treated samples exhibited a substantially lower damage rate of 30.0%, corresponding to an approximate reduction of 52% in cellular damage. A similar protective effect was observed at -4°C, indicating that the antifreeze formulation effectively maintained cellular metabolic activity under freezing stress.



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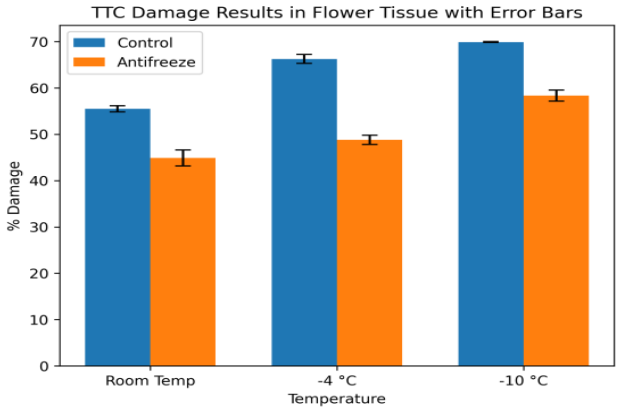

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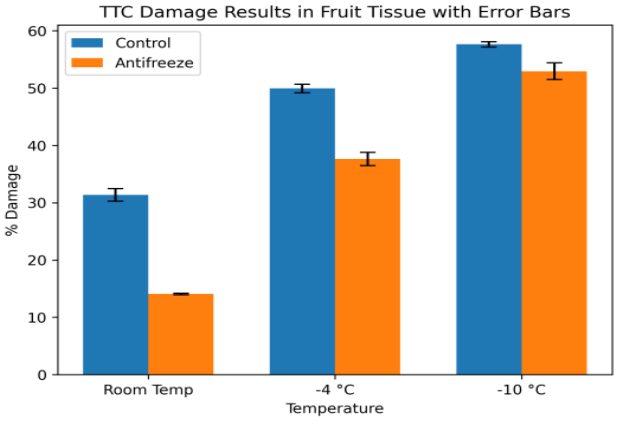


(C)

Fig. 1: Effect of antifreeze treatment on freezing damage in bean tissues determined by TTC viability assay under varying temperature conditions. Panels show the percentage cellular damage in A) seed tissues, B) flower tissues, and C) fruit tissues. Error bars represent standard deviation (±SD) of three biological replicates.

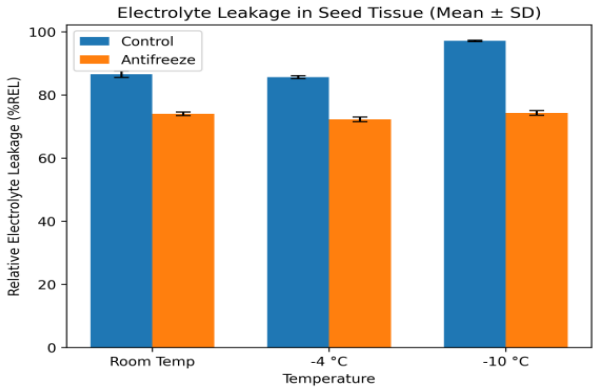
The TTC viability assay is based on the reduction of TTC to red-colored formazan by dehydrogenase enzymes present in metabolically active cells. Therefore, higher TTC reduction indicates higher cellular metabolic activity and tissue viability. Previous studies have reported that freezing stress suppresses cellular metabolism and significantly reduces TTC reduction in plant tissues (Ding *et al.*, 2019; Roychowdhury *et al.*, 2025). The higher TTC reduction observed in antifreeze-treated samples in the present study suggests that the developed formulation helped maintain cellular metabolic activity under freezing conditions.

Electrolyte leakage analysis

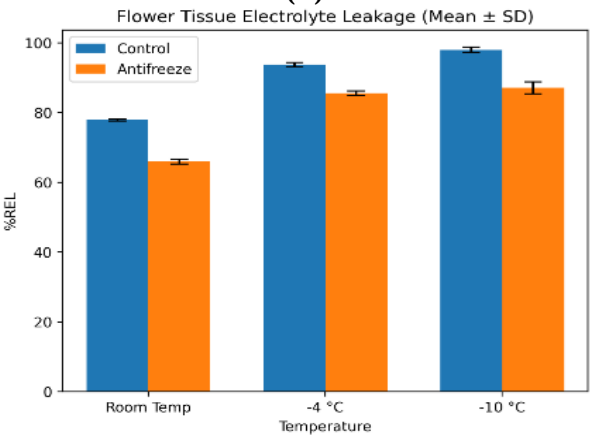
Electrolyte leakage analysis revealed that the antifreeze treatment played an important role in maintaining membrane integrity under freezing stress. Increasing freezing stress resulted in significantly higher electrolyte leakage in the control groups. The highest electrolyte leakage value was recorded in the control group at -10°C (97.31%), while antifreeze-treated samples showed a considerably lower value of 75% (Fig. 2). Similarly, antifreeze application also reduced electrolyte leakage at -4°C compared with the untreated control group.

Electrolyte leakage is widely used as an indicator of cell membrane integrity. Damage to cellular membranes caused by freezing

stress leads to ion leakage from cells, thereby increase the electrical conductivity of the surrounding solution (Gill and Tuteja, 2010; Hasanuzzaman *et al.*, 2020). The lower electrolyte leakage values observed in antifreeze-treated samples indicated that the formulation contributed to the membrane stabilization, and reduced freezing-induced membrane damage.



(A)



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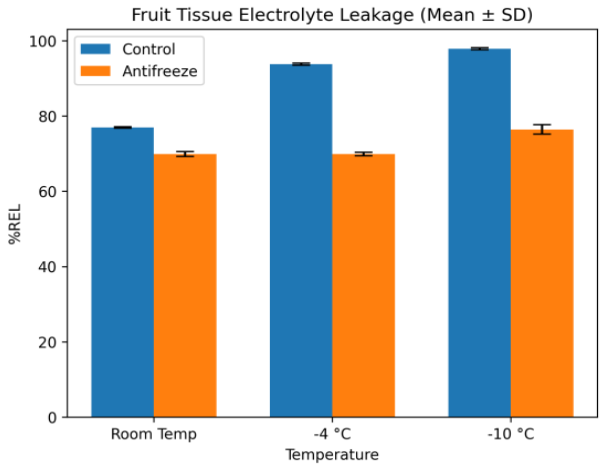


(C)

Fig. 2: Effect of antifreeze treatment on relative electrolyte leakage (%REL) in bean tissues under varying temperature conditions. Panels represent A) seed tissues, B) flower tissues, and C) fruit tissues. Error bars represent standard deviation (±SD) of three biological replicates.

The protective effect observed in antifreeze-treated tissues can be attributed to the combined action of osmoprotectants and antioxidant compounds present in the formulation. Glycerol and sorbitol are known to reduce ice crystal formation and maintain cellular osmotic balance under freezing conditions (Crowe *et al.*, 1998; Pattnaik *et al.*, 2021). The lower electrolyte leakage observed in treated samples indicates improved membrane stability, suggesting that the formulation effectively protected cellular structures against freezing-induced damage. In addition, safflower flower extract, rich in phenolic and flavonoid compounds, may have contributed to reducing oxidative stress by scavenging reactive oxygen species generated under low temperature conditions (Gill and Tuteja, 2010; Hasanuzzaman *et al.*, 2020). These combined effects likely enhanced the overall tolerance of plant tissues to freezing stress.

Visual damage assessment

The visual damage assessment results were consistent with the biochemical analyses. No visible damage was observed in plant tissues at room temperature. However, under freezing conditions, clear morphological differences were detected between control and antifreeze-treated samples. At -10°C, control samples exhibited severe morphological damage, including tissue discoloration, shrinkage, and structural deformation, particularly in flower and fruit tissues. In contrast, antifreeze-treated samples maintained better tissue integrity and exhibited considerably less visible damage.

Freezing stress is known to stimulate morphological changes such as loss of turgor, discoloration, and tissue deformation in plant tissues (Wisniewski *et al.*, 2014; Guan *et al.*, 2023). The lower visual damage observed in antifreeze-treated tissues further confirms the protective effect of developed antifreeze formulation.

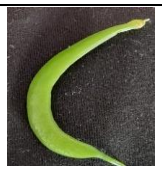
Table 1: Visual damage assessment of seed tissues under different temperature conditions

Treatments	Visual criteria (macroscopic)	Photo Example	Damage level	Damage score
Room temperature (control)	Tissue colour natural (light green), turgor intact, no darkening or collapse		No damage	0
Room temperature (antifreeze)	Tissue colour natural (light green), turgor intact, no darkening or collapse		No damage	0
-4°C (control)	Slight wilting, slight discoloration, tissue integrity preserved		Slight damage	1
-4°C (antifreeze)	Slight wilting, slight discoloration, tissue integrity preserved		Slight damage	1
-10°C (control)	Partial colour change (yellowing), slight softening, no severe darkening		Moderate damage	2
-10°C (antifreeze)	Partial colour change (light yellow-pale green), slight softening, no severe darkening		Moderate damage	2

Table 2: Visual damage assessment of flower tissues under different temperature conditions

Treatments	Visual criteria (macroscopic)	Photo example	Damage level	Damage score
Room temperature (control)	Tissue colour completely natural (white/light green), turgor intact, no darkening or collapse		No damage	0
Room temperature (antifreeze)	Tissue colour completely natural (white/light green), turgor intact, no darkening or collapse		No damage	0
-4°C (control)	Slight wilting, slight pink discoloration, tissue integrity preserved		Very slight damage	1
-4°C (antifreeze)	Slight wilting, slight pink discoloration, tissue integrity preserved		Very slight damage	1
-10°C (control)	Pronounced colour change, tissue softening and shrinkage		Severe damage	3
-10°C (antifreeze)	Slight wilting, partial discoloration, slight tissue softening, no darkening		Moderate damage	2

Table 3: Visual damage assessment of fruit tissues under different temperature conditions

Treatments	Visual criteria (macroscopic)	Photo Example	Damage level	Damage score
Room temperature (control)	Tissue colour completely natural (green/light green), turgor intact, no darkening or collapse		No damage	0
Room temperature (antifreeze)	Tissue colour completely natural (green/light green), turgor intact, no darkening or collapse		No damage	0
-4°C (control)	Slight wilting, partial discoloration, slight tissue softening		Moderate damage	2
-4°C (antifreeze)	Slight discoloration, tissue integrity preserved		Very slight damage	1
-10°C (control)	Partial colour change, tissue softening and shrinkage		Moderate damage	2
-10°C (antifreeze)	Slight discoloration, tissue integrity preserved		Slight damage	1

Overall, the results of the TTC viability assay, electrolyte leakage analysis, and visual damage assessment consistently indicate that the developed natural antifreeze spray significantly reduced freezing-induced physiological and cellular damage in bean tissues. These findings suggest that the combined use of osmoprotectants, antioxidant-rich plant extracts, and film-forming biopolymers may represent a promising strategy for reducing freezing injury in cold-sensitive crops.

Conclusion: The results of this study demonstrated that the developed natural antifreeze spray significantly reduced freezing-induced damage in common bean tissues. The formulation improved cellular viability, decreased electrolyte leakage, and preserved tissue integrity under freezing conditions. These effects can be attributed to the combined action of osmoprotectants, antioxidant-rich plant extract, and film-forming biopolymer, which together contributed to membrane stabilization and enhanced stress tolerance. The findings suggest that this eco-friendly antifreeze formulation has strong potential for practical applications in protecting cold-sensitive crops from frost damage. Future studies may focus on field-scale applications and detailed biochemical investigations to further elucidate the underlying mechanisms.

Conflict of interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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