

# Enhancing Freeze Tolerance of Field-grown Lettuce with Salicylic Acid, Ascorbic Acid, and Calcium Chloride

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**Keywords.** chemical-priming, electrolyte-leakage, freeze-injury, frost-damage, frost-protection, *Lactuca sativa*

**Abstract.** With the climate continuing to change, specialty crop growers are particularly at risk for economic loss caused by erratic weather conditions, excess heat and rain, and frost damage. Although tolerant to cold, lettuce (*Lactuca sativa*) is a major horticultural crop at risk for exposure to freezing temperatures in late fall or early spring in the Midwest. Exogenous applications of salicylic acid, ascorbic acid, and calcium chloride have been shown to improve abiotic stress in plants, particularly with freezing tolerance. This research investigated the effects of the exogenous application of salicylic acid, ascorbic acid, and calcium chloride, in varying concentrations, on field-grown lettuce. The study was conducted at the Iowa State University Horticulture Research Station, Ames, IA, USA. Weekly applications of salicylic acid (0.5 and 1.0 mM), ascorbic acid (0.5 and 1.0 mM), and calcium chloride (10 and 20 mM) were applied until plants were a marketable size. A control treatment (no spray) was also included. Data regarding plant size, yield, leaf area, leaf number, plant dry weight, plant nutrient analysis, and freeze tolerance were collected. Freeze tolerance assessments were conducted through laboratory-simulated freeze events and the evaluation of natural in-field freeze events. Freeze injury was quantified through the electrolyte leakage method. Marketable weights in 2020 and 2021 were statistically similar, except for 1.0 mM salicylic acid in 2020, which showed a significantly lower marketable weight and head diameter. Trends during both years showed that stress protectant applications had the most effective freeze protection at  $-12^{\circ}\text{C}$  in laboratory-simulated freeze events. Calcium chloride at 20 mM had the highest protection at  $-12^{\circ}\text{C}$ , with 13.3% and 24.0% less injury compared with the control in 2020 and 2021, respectively. Salicylic acid 1.0 mM at  $-12^{\circ}\text{C}$  had 4.7% and 23.7% less injury compared with control during these two years, respectively. Ascorbic acid 1.0 mM at  $-12^{\circ}\text{C}$  also showed 6.8% and 9.5% less injury than the control in 2020 and 2021, respectively. Similar trends were observed after in-field freezing events, with 20 mM calcium chloride providing the highest protection against frost in 2020 and 2021. These findings advance the understanding of the capabilities of stress protectants on lettuce as chemical primers after freezing events. This research highlights the benefits of ascorbic acid, salicylic acid, and calcium chloride as stress protectants and encourages future research and further exploration of concentrations, methods of application, and timing of application of products.

Lettuce (*Lactuca sativa*) is a major horticulture crop in the United States. In 2021,

more than 105,000 acres of head lettuce were grown, generating approximately \$965 million (US Department of Agriculture 2022). Although it is cold-tolerant, lettuce can still be exposed to freezing temperatures in early spring or late fall in the Midwest, reducing its quality. Midwest growers have a financial incentive to extend the growing season into early April and late October; however, this leads to higher freeze risks. Midwest temperature and weather conditions continue to become more erratic as the climate changes. Specialty crop growers are at substantial risk because crops are sensitive to weather and climate stress. Erratic freeze events have become prevalent in early

spring and late fall, causing crop damage and economic loss in the vegetable production industry (Kistner et al. 2018).

Vegetable producers have limited options when protecting against frost conditions, and those options are often energy-, labor-, and time-intensive. The utilization of row covers, planting in high or low tunnels, and irrigation are ways to mitigate frost; however, strategies that could be implemented on a larger scale are needed when considering commercial vegetable production (Snyder and de Melo-Abreu 2005). Growers of specialty crops have identified the need for crop management tools that mitigate the production risk with climate changes (Kistner et al. 2018). One potential solution to this problem is chemical priming with physiological stress protectants. Stress protectants such as salicylic acid (SA), ascorbic acid (AsA), and calcium (Ca) that are naturally found in the plant can regulate various physiological and biochemical processes. Furthermore, AsA, commonly known as vitamin C, is an abundant antioxidant in plants that actively regulates photosynthesis and defends against reactive oxygen species (Boubakri 2017). Additionally, SA, which is a precursor to aspirin, is a phytohormone that mediates endogenous signaling in the plant to defend against stresses (Hayat et al. 2010). Moreover, Ca is crucial to ensuring the cell wall and membrane structure in the plant cell and is an intracellular messenger that mediates hormone responses with stress signaling (Hepler 2005; Hepler and Winship 2010).

It has been reported that AsA, SA, and Ca respond to stress signaling and defend plants against pathogens, wounding, salinity, and heat stress (Boubakri 2017; Hayat et al. 2010; Hepler 2005). Recent studies involving spinach (*Spinacia oleracea*) under controlled environment settings and exogenous applications of AsA, SA, and calcium chloride ( $\text{CaCl}_2$ ) have demonstrated freeze protection (Min and Arora 2022; Min et al. 2020, 2021; Shin et al. 2018). However, little information regarding the performance of applications of these stress protectants in the field is available for the assessment of their applicability for growers. The primary objective of this study was to determine if exogenous foliar applications of AsA, SA, and  $\text{CaCl}_2$  decrease freeze injury on field-grown lettuce and affect lettuce yield and quality. The findings of this study could provide another option for extending the growing season and mitigating the economic losses of the spring and fall frosts.

## Materials and Methods

**Site description.** Trials were conducted at the Iowa State University Horticulture Research Station in Ames, IA, USA, during Fall 2020 and Fall 2021. Both trials previously had an oat cover crop incorporated into the soil. Before planting, soil tests were conducted at a local laboratory (Agsources, Ellsworth, IA, USA), which measured 2.6% and 3.4% organic matter in 2020 and 2021, respectively. In 2020, the soil pH was 5.4 and had a cation exchange capacity of 17.9. In

Received for publication 31 May 2023. Accepted for publication 2 Feb 2024.

Published online 8 Mar 2024.

We thank the Iowa Department of Agriculture and Land Stewardship Specialty Crop Block Grant Program and US Department of Agriculture Hatch IOW04201 for supporting this research project.

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Table 1. Field air and soil temperatures during the lettuce growing season at Iowa State University Horticulture Research Station, Ames, IA, USA, in 2020 and 2021.

	Air temp (°C) <sup>i</sup>		Soil temp (°C) <sup>ii</sup>	
	Mean	Min	Mean	Min
2020 <sup>iii</sup>				
Sep	20.1	6.2	18.6	12.3
Oct	10.8	−8.1	10.5	1.3
2021 <sup>iv</sup>				
Sep	24.9	9.7	22.5	19.7
Oct	14.3	−4.6	14.6	6.2
Nov	5.8	−10.3	6.6	0.9

<sup>i</sup> Mean air temperature determined using three sensors placed 30.5 cm above the lettuce bed.

<sup>ii</sup> Mean soil temperature determined using three sensors placed at a depth of 10.2 cm.

<sup>iii</sup> Data collected from 22 Sep to 31 Oct 2020.

<sup>iv</sup> Data collected from 29 Sep to 19 Nov 2021.

2021, the soil had a pH of 6.4 and a cation exchange capacity of 23. The soil in both fields was classified as a clarion loam. Average soil temperatures in 2020 were 18.6 °C in September and 10.5 °C in October. In 2021, 22.5 °C was the mean soil temperature in September, followed by 14.6 °C in October and 6.6 °C in November (Table 1).

The average air temperatures in Sep and Oct 2020 were 20.1 °C and 10.8 °C, respectively. In 2020, the minimum air temperature lows of 6.2 °C and −8.1 °C were recorded in September and October, respectively. In 2021, the average air temperature was 24.9 °C in September, followed by 14.3 °C in October and 5.8 °C in November. Minimum air temperatures in September, October, and November were 9.7 °C, −4.6 °C, and −10.3 °C, respectively.

**Experimental design.** The experimental design was a randomized complete block (RCBD) with five replications. The plot had a length of 12.8 m and width of 7.8 m. Four-week-old lettuce seedlings were transplanted on raised beds covered with embossed black plastic mulch (PolyExpert Inc. Canada, Laval, Quebec, Canada). Each treatment plot had a length of 1.8 m and width of 0.6 m, with two rows of eight butterhead lettuce (*Lactuca sativa* cv. Optima; High Mowing Organic Seeds, Wolcott, VT, USA) (16 plants/treatment). The seven treatments evaluated included one control (no spray) and two concentrations of the following: AsA (0.5 mM and 1.0 mM), SA (0.5 mM and 1.0 mM), and CaCl<sub>2</sub> (10 mM and 20 mM). Treatment concentrations were chosen based on previous research of stress-protectant supplementation for freeze protection in spinach (Min and Arora 2022; Min et al. 2020, 2021; Shin et al. 2018). Guard rows of lettuce (*Lactuca sativa* cv. Optima) were planted on field plot sides.

**Transplant production.** Lettuce seeds were sown into 72-cell propagation trays (TO Plastics, Clearwater, MN, USA) with soilless potting mix (Metro Mix 360; Sun Gro Horticulture, Agawam, MA, USA) on 5 Aug 2020 and 12 Aug 2021. Lettuce was watered uniformly across treatments as needed and fertilized with a water-soluble fertilizer (15N–2.2P–12.5K Peters Excel® Multi-Purpose and Cal-Mag;

Everris International, Geldermalsen, The Netherlands). The greenhouse was maintained at 22.2 °C, and 1000-W high-pressure sodium lamps (AgroMax, Summerdale, AL) provided supplemental light for 16 h each day. Abnormally hot conditions in Aug 2021 resulted in difficulties maintaining an optimal temperature for lettuce germination in a greenhouse. Therefore, plants were placed in the growth chamber (PGC 10; Percival Scientific, Perry, IA, USA) maintained at 18.3 °C with 16 h of light for the first 14 d before returning to the greenhouse. One week before transplanting, transplants were hardened outside the greenhouse for 7 d.

**Field management.** Lettuce was transplanted on 17 Sep 2020 and 15 Sep 2021. During year 1, Nutriculture 20N–20P–20K (Plant Marvel Laboratories LLC, Chicago Heights, IL, USA) was applied through drip irrigation. During year 2, urea 46N–0P–0K, monoammonium phosphate 11N–20.9P–0K, and potassium chloride (KCl) 0N–0P–49.8K were applied preplant. Fertilizer applications were determined according to soil test results and nutrient requirements provided in the *Midwest Vegetable Production Guide* (Midwest Vegetable Production Guide 2022).

A 76.2-cm-tall electric fence with a Hot-shock 600® controller (Horizont, Weldezauntechnik, Germany) was placed around the trial to prevent animals, mainly rabbits, from entering. Six HOBO pendant® sensors (Onset Computer Corporation, Bourne, MA, USA) were placed in the middle row of the field, equidistant, to capture soil and air temperatures. Of the six sensors, three collected air temperature data 30.5 cm above the plastic mulch layer, and three collected soil temperature data 10.2 cm below the soil under the plastic mulch. Each year, major pests included spotted cucumber beetles (*Diabrotica undecimpunctata*), aphids (*Aphidoidea*), cabbage loopers (*Trichoplusia ni*), and grasshoppers (*Caelifera*). In 2020, pests were managed using a tank mix of pyrethrins (Pyganic; McLaughlin Gormley King Company, Minneapolis, MN, USA) and *Bacillus thuringiensis* subspecies kurstaki, strain ABTS-351 (Dipel DF; Valent BioSciences LLC, Libertyville, IL, USA). In 2021, to manage pests, Acetamiprid (Omni Brand 30SG; Helena Chemical Company, Collierville, TN, USA) and *Bacillus thuringiensis* subspecies kurstaki, strain SA-11 solids, spores, and lepidopteran active toxins (Javelin WG; Certis USA, LLC, Columbia, MD, USA) were tank-mixed.

**Treatment applications.** Stress-protectant concentrations were applied weekly with 1 L of deionized water and 3 mL of Tween-20 (Thermo Fisher Scientific, Waltham, MA, USA) as a surfactant. The first application was applied as a drench the day before transplanting (Table 2). Thereafter, treatments were applied in the field on a per-plant basis using a 946.4-mL spray bottle set to release 2.3 mL with each pull. Each head of lettuce throughout the growing season received a uniform application each week. As plants grew, the amount of application received by individual plants increased to accommodate for growth.

**In-field plant growth measurements.** Three plants were chosen from each treatment to be monitored throughout the growth period and measured weekly over 3 weeks. The first measurement was taken 2 weeks after transplanting. Measurements of two equatorial diameters and one polar diameter were taken according to Jenni and Bourgeois (2008). Currence's equation was used to calculate the plant volume using the head volume ( $KD_c^2D_p$ ) (Jenni and Bourgeois 2008).

**Quality assessments.** Lettuce was harvested on 24 Oct 2020 and 15 Oct 2021, when heads were a marketable size. Harvested heads were classified into marketable and unmarketable categories, and the number of heads and weight were recorded. Unmarketable categories included heads with physiological disorders, tip burn, and insect and disease damage. Four marketable heads were randomly chosen to calculate the head diameter. The leaf area was measured using an LI-3100C area meter (LI-COR, Lincoln, NE, USA) on two marketable lettuce heads. Leaves of heads were counted and recorded. These heads were dried for 7 d at 67 °C (622; Hot-pack, Philadelphia, PA, USA), and dry biomass weights were collected. Samples were ground and sent for a plant nutrient analysis to assess macronutrients and micronutrients (Ward Laboratory, Kearney, NE, USA).

**Laboratory-simulated freezing.** Tolerance to freezing stress was determined using the electrolyte leakage-based laboratory protocol described by Min et al. (2014). After collecting the yield and quality data, heads were stored in a cooler under optimum storage temperature and humidity as outlined in the US Department of Agriculture Handbook No. 66 (*The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks*). Two marketable heads from each treatment of three replications were taken to the laboratory for simulated freezing events on 4 Nov 2020 and 17 Oct 2021. From each head, a mature leaf was collected one-third of the way in from the outer edge to the middle of the head. From these leaves, 2.5-cm × 7.6-cm sections were cut, dipped in deionized water three times, and blotted before being placed in a 2.5-cm × 20-cm test tube. Three replications of each treatment and temperature series were collected, creating a split-plot design. Samples were placed upright in 150 uL of deionized water within the test tube. Test tubes were placed in a glycol bath (Isotemp 3028; Fisher Scientific, Pittsburgh, PA, USA) starting at 0 °C for 1 h, followed by an additional 1 h at −1 °C. Thereafter, each sample was ice-nucleated −1 °C. Following a soak for 1 h, samples were slowly cooled (−0.5 °C every 30 min) to a series of sub-freezing temperatures from −4 to −12 °C. Upon reaching a specified sub-freezing temperature, samples were removed and thawed overnight on ice. Unfrozen controls were kept at 0 °C during this process. The following morning, all samples were moved to 4 °C for 1 h, followed by another 1 h at ~20 °C before electrolyte leakage readings.

Table 2. Weekly spray volume of stress protectants applied to butterhead lettuce (*Lactuca sativa* cv. Optima) at the Iowa State University Horticulture Research Station, Ames, IA, USA, in 2020 and 2021.

2020		2021	
Date	Spray volume per plant (mL)	Date	Spray volume per plant (mL)
16 Sep	41.7 <sup>1</sup>	14 Sep	41.7
26 Sep	13.8	26 Sep	13.8
11 Oct	23.0	4 Oct	18.4
18 Oct	27.6	9 Oct	27.6
23 Oct	32.2	17 Oct	32.2
4 Nov	32.2	1 Nov	36.8
11 Nov	36.8	10 Nov	36.8
		18 Nov	41.4

<sup>1</sup> The first stress protectant application was applied as a drench the day before transplanting in 2020 and 2021.

An electrical conductivity (EC) meter (model 3100; YSI Inc., Yellow Spring, OH, USA) collected two EC measurements of electrolyte leakage. Deionized water (20 mL) was added to each test tube; the test tubes were shaken for 90 min, followed by an electrolyte leakage measurement. Another reading was taken after samples were autoclaved, cooled to room temperature, and shaken. The percentage of injury was calculated from electrolyte leakage data as described by Lim et al. (1998). All percentage injury means of treatments and temperature series replications were pooled. This work does not present temperatures warmer than  $-8^{\circ}\text{C}$  because they were not significant. The unfrozen control is presented for comparison purposes.

**In-field freezing.** Freeze events at or below  $-6^{\circ}\text{C}$  occurred on 26 and 27 Oct 2020 ( $-7.1^{\circ}\text{C}$ ,  $-8.1^{\circ}\text{C}$ ) and 3 and 19 Nov 2021 ( $-7.1^{\circ}\text{C}$ ,  $-10.3^{\circ}\text{C}$ ). Preliminary trials indicated that damage to field-grown 'Optima' lettuce occurred after exposure to  $-6^{\circ}\text{C}$ . The morning after freezing events, two leaves from the same part of the plant were harvested from each treatment. Leaf samples were identified the same way they were identified for laboratory-simulated freezing. The leaves were taken to the laboratory, cut into  $2.54\text{-cm} \times 7.62\text{-cm}$  strips, and placed in respective test tubes. The, 20 mL of deionized water was added to each test tube, and the samples were shaken for 90 min. Then, EC measurements were taken using the same protocol as mentioned. An electrolyte leakage percentage was calculated as detailed by Lim et al. 1998.

**Statistical analysis.** The GLIMMIX procedure in SAS (version 9.4; SAS Institute, Cary, NC, USA) was used for the analysis of variance (ANOVA) and to determine the fixed effects of year and treatment on plant volume, yield, leaf number, leaf area, dry weight, plant nutrient analysis, and electrolyte leakage. The fixed effect temperature was added to the injury analysis (split-plot analysis). Blocks were random factors in all analyses. Injury data in 2020 and 2021 were square root-transformed for statistical inferences. Means and SEs presented for injury were back-transformed. Means separations were conducted using Fisher's least significant

different test ( $P \leq 0.05$ ). Stress-protectant treatments were compared with the control for all parameters.

## Results

Stress protectants demonstrated high quality and freeze protection in laboratory-

simulated freezing and in-field freeze event assessments, thus confirming our hypothesis of the beneficial effects of stress-protectant applications.

**In-field plant growth measurements.** The plant volume of each treatment grew consistently over time in 2020 and 2021 (Fig. 1). In 2020, at weeks 2 and 3, the plant volume of the control was statistically the same as that of all stress protectants. At week 4 in 2020, the plant volume with 20 mM  $\text{CaCl}_2$  was significantly smaller than that of the control, with a difference of  $473.51\text{ cm}^3$ . At 10 mM  $\text{CaCl}_2$ , the plant volume of lettuce was significantly larger than that of the control, with a difference of  $533.43\text{ cm}^3$ .

In 2021, there was no significant difference in the measurements during week 2 with all treatments. At week 3, the control lettuce was significantly larger than the lettuce under SA treatments, with  $456.09\text{ cm}^3$  and  $495.33\text{ cm}^3$  higher volumes than those achieved with 0.5 mM SA and 1.0 mM SA treatments, respectively. All lettuce under other stress protectants at week 3 were statistically the same

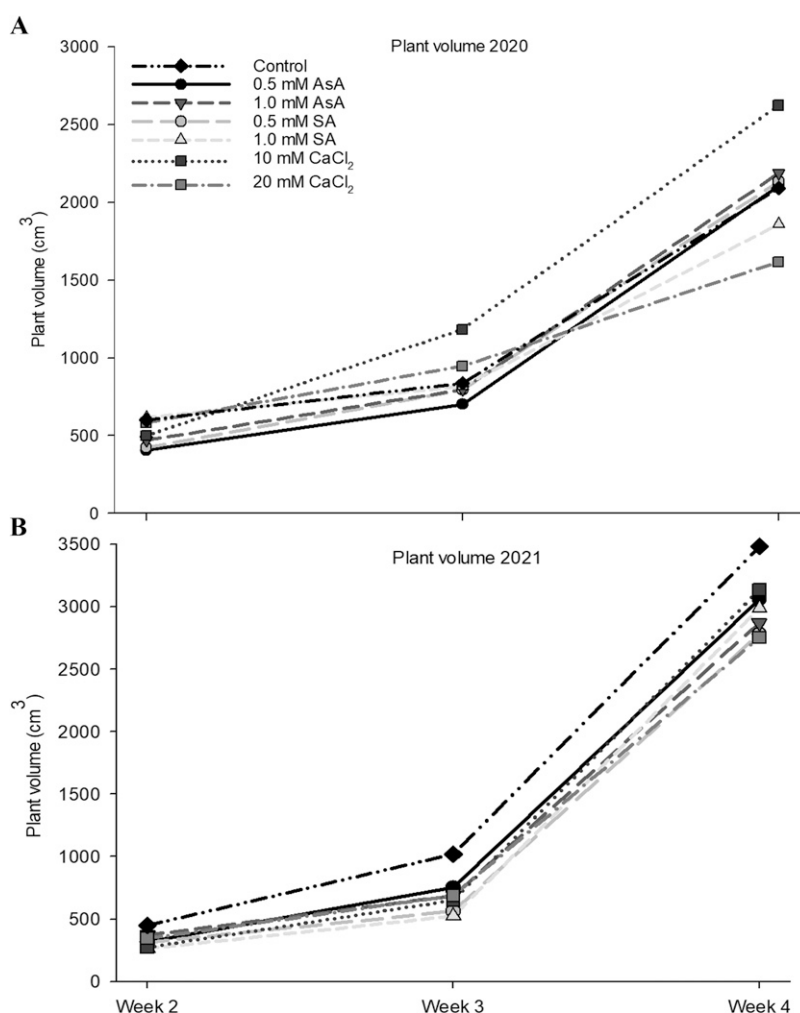


Fig. 1. Effects of stress protectants on butterhead lettuce (*Lactuca sativa* cv. Optima) plant volume grown at the Iowa State University Horticulture Research Station, Ames, IA, USA, in 2021 (A) and 2022 (B). Data were collected from three plants per treatment at 2, 3, and 4 weeks after transplanting. Two equatorial and one polar dimension of lettuce heads were measured. Volume was estimated using Currence's equation. AsA = ascorbic acid;  $\text{CaCl}_2$  = calcium chloride; mM = millimolar; SA = salicylic acid.

as the control. Measurements at week 4 showed significantly larger lettuce under the control treatment than under stress-protectant treatments. At week 4, lettuce under the 10 mM CaCl<sub>2</sub> treatment was statistically the same as lettuce under the control treatment. Lettuce under all other stress protectants were statistically smaller than the control lettuce at week 4. The lettuce under the 0.5 mM AsA treatment was smaller than the lettuce under the control treatment by 419.43 cm<sup>3</sup>, whereas the plant volume with the 1.0 mM AsA treatment was 609.42 cm<sup>3</sup> smaller than that under the control. With the SA treatments of 0.5 mM and 1.0 mM, the plant volumes were smaller by 699.35 cm<sup>3</sup> and 492.68 cm<sup>3</sup>, respectively, than of the control. The 20 mM CaCl<sub>2</sub> treatment resulted in a plant volume 727.96 cm<sup>3</sup> lower than that of the control.

**Quality assessments.** Lettuce sprayed with stress protectants showed high quality in 2020 and 2021, with limited adverse effects. In 2020, lettuce sprayed with stress protectants were statistically the same as the control lettuce in terms of plant dry weight, marketable weight, head diameter, leaf number, and leaf area (Table 3). An exception to this was the 1.0 mM SA treatment, which resulted in significantly lower plant dry weight, marketable weight, and head diameter than the control. In 2020, the lettuce under the 20 mM CaCl<sub>2</sub> treatment had a significantly higher leaf area that was 393.51 cm<sup>2</sup> more than that of the control. In 2021, there was no significant difference between treatments in terms of plant dry weight, marketable weight, and head diameter. The leaf area with the control treatment in 2021 was significantly larger than

that with 1.0 mM AsA, 0.5 mM SA, 1.0 mM SA, and 20 mM CaCl<sub>2</sub> treatments. Treatments comprising 0.5 mM AsA and 10 mM CaCl<sub>2</sub> resulted in the statistically same leaf area as that resulting from the control treatment.

The results of all stress-protectant treatments in 2020 showed no significant differences from those of the control in terms of macronutrient concentrations of nitrogen, phosphorus, potassium, sulfur, and calcium (Table 4). Magnesium with the 0.5 mM AsA treatment was significantly lower than that with the control treatment by 0.11%. The chlorine concentration was 0.5% higher with 20 mM CaCl<sub>2</sub> than with the control. Micronutrients zinc, iron, manganese, boron, and molybdenum showed no significant differences between treatments in 2020. Copper with the 0.5 mM SA treatment was 7.06 mg·kg<sup>-1</sup> higher than that with the control treatment in 2020.

All macronutrient concentrations in 2021 showed no statistical difference between the control and stress-protectant treatments. The control treatment had a statistically higher zinc concentration than stress-protectant treatments. The iron concentration with the 1.0 mM AsA treatment was statistically lower than that with the control treatment by 397.7 mg·kg<sup>-1</sup>. All other stress-protectant treatments and the control treatment had statistically similar iron concentrations. Copper concentrations with the 0.5 mM AsA and 1.0 mM AsA treatments were statistically lower than those with the control treatment by 2.8 mg·kg<sup>-1</sup> and 2.2 mg·kg<sup>-1</sup>, respectively. With SA at 1.0 mM the copper concentration was 2.9 mg·kg<sup>-1</sup> lower than that with the control treatment. Manganese, boron, and molybdenum concentrations with the stress-protectant

treatments were statistically the same as those achieved with the control treatment.

**Laboratory-simulated freezing.** In 2020, less injury was observed with stress-protectant treatments. At severe stress (−12 °C), although not always statistically significant, all stress-protectant treatments resulted in less injury than the control treatment (Fig. 2). The injury rates associated with the 0.5 mM AsA and 1.0 mM AsA treatments, respectively, were 2.4% and 6.8% less than that associated with the control treatment. The SA treatments of 0.5 mM and 1.0 mM resulted in 5.5% and 4.7% less injury, respectively, than the control treatment. The CaCl<sub>2</sub> treatments of 10 mM and 20 mM showed significantly lower injury values of 12.2% and 13.3%, respectively, than the control treatment.

At −10 °C in 2020, the 0.5 mM SA, 1.0 mM SA, 10 mM CaCl<sub>2</sub>, and 20 mM CaCl<sub>2</sub> treatments resulted in less injury than the control treatment; however, this was not significant. Both 0.5 mM and 1.0 mM SA treatments resulted in 4.3% less injury than the control treatment. The 10 mM and 20 mM CaCl<sub>2</sub> treatments resulted in 0.7% and 12.2% less injury, respectively, than the control treatment. The results of the 0.5 mM and 1.0 mM AsA treatments were also not significantly different from those of the control treatment at −10 °C. The results of all treatments and the unfrozen controls were statistically similar in 2020 at −8 °C.

In 2021, stress-protectant treatments resulted in less injury than the control treatment. At severe stress (−12 °C), all stress-protectant treatments resulted in less injury than the control treatment, even when not statistically significant. The 0.5 mM and 1.0 mM AsA treatments, respectively, resulted in 9.7% and 9.5% less injury than the control treatment. The 0.5 mM SA treatment resulted in 11.9% less injury than the control treatment. The 1.0 mM SA treatment resulted in a significantly lower injury level than the control treatment, with a reduction of 23.7%. The lowest amount of injury was observed with the 20 mM CaCl<sub>2</sub> treatment, with 24.0% less injury than the control treatment. The 10 mM CaCl<sub>2</sub> treatment resulted in 12.2% less injury than the control treatment. The 1.0 mM SA treatment resulted in 23.7% less injury than the control treatment. The 0.5 mM SA treatment resulted in 11.9% less injury than the control treatment. At −10 °C in 2021, stress-protectant treatments comprising 1.0 mM AsA, 0.5 mM SA, and 20 mM CaCl<sub>2</sub> resulted in 5.3%, 2.1%, and 4.6% injury reductions, respectively, compared to the control treatment. At moderate stress (−8 °C), the 0.5 mM AsA and 1.0 mM SA treatments resulted in 2.6% and 3.0% less injury, respectively, than the control treatment.

**In-field freezing.** The day after field freeze events at less than −6.0 °C on 26 Oct 2020 (−7.1 °C), 27 Oct 2020 (−8.0 °C), 3 Nov 2021 (−7.1 °C), and 19 Nov 2021 (−10.3 °C), lettuce leaves were harvested to estimate electrolyte leakage to quantify the damage. Lower electrolyte leakage with stress protectants was observed on multiple dates compared to that of the control (Fig. 3). On 26 Oct 2020, 0.5 mM

Table 3. Mean plant dry weight, marketable weight, head diameter, leaf number, and leaf area of butterhead lettuce (*Lactuca sativa* cv. Optima) grown at the Iowa State University Horticulture Research Station, Ames, IA, USA.

Treatment	Plant dry wt (g) <sup>i</sup>	Marketable wt (kg) <sup>ii</sup>	Head diam (cm) <sup>iii</sup>	Leaf number <sup>iv</sup>	Leaf area (cm <sup>2</sup> )
2020					
Control	10.32 abc <sup>v</sup>	1.10 a	16.55 a	15 bc	2007.6 bdc
0.5 mM AsA <sup>vi</sup>	9.82 abcd	1.06 a	16.60 a	14 c	1735.8 d
1.0 mM AsA	11.02 a	1.04 a	16.65 a	14 ab	2250.5 ab
0.5 mM SA	9.04 cd	0.92 ab	15.85 ab	16 abc	2206.5 abc
1.0 mM SA	8.59 d	0.82 b	14.55 b	15 bc	1850.0 dc
10 mM CaCl <sub>2</sub>	9.31 bcd	0.94 ab	15.65 ab	17 abc	2141.4 abc
20 mM CaCl <sub>2</sub>	10.82 ab	1.06 a	14.55 ab	18 a	2401.1 a
2021					
Control	12.44 <sup>NS</sup>	1.22 <sup>NS</sup>	17.40 ab	17 <sup>NS</sup>	3326.2 a
0.5 mM AsA	11.75	1.18	19.10 a	17	2982.1 ab
1.0 mM AsA	11.52	1.16	18.80 a	16	2772.3 b
0.5 mM SA	10.31	1.08	19.70 a	16	2733.7 b
1.0 mM SA	10.31	1.10	18.65 a	16	2626.9 b
10 mM CaCl <sub>2</sub>	11.79	1.28	20.10 a	17	3152.8 ab
20 mM CaCl <sub>2</sub>	11.28	1.26	14.25 b	17	2786.8 b

<sup>i</sup> Plant dry weight collected from two plants per treatment per replication.

<sup>ii</sup> Marketable weight and number collected on 24 Oct 2020 and 15 Oct 2021 from six lettuce heads per treatment per replication. Marketable heads include lettuce free of insect damage, disease, physiological disorders, and tip burn.

<sup>iii</sup> Mean head diameter collected from four marketable plants per plot treatment per replication.

<sup>iv</sup> Leaf number and leaf area collected from two marketable heads per treatment per replication.

<sup>v</sup> Mean separations are within columns and years based on Fisher's least significant differences at  $P \leq 0.05$ . Means with the same letters are not statistically different.

<sup>vi</sup> AsA = ascorbic acid; SA = salicylic acid; CaCl<sub>2</sub> = calcium chloride; mM = millimolar; NS = not significant.

Table 4. Macronutrient and micronutrient concentrations of lettuce (*Lactuca sativa* cv. Optima) grown at the Iowa State University Horticulture Research Station Ames, IA, USA, in Summer 2021 and Summer 2022.

Treatment	Macronutrient concentrations (%) <sup>1</sup>													
	2020							2021						
	N	P	K	S	Ca	Mg	Cl	N	P	K	S	Ca	Mg	Cl
Control	3.35 <sup>NS,ii</sup>	0.67 ab	5.79 <sup>NS</sup>	0.25 ab	2.10 ab	0.64 a	1.96 b	4.33 <sup>NS</sup>	0.60 <sup>NS</sup>	7.16 <sup>NS</sup>	0.20 <sup>NS</sup>	1.56 <sup>NS</sup>	0.57 ab	1.76 <sup>NS</sup>
0.5 mM AsA <sup>iii</sup>	3.65	0.61 b	5.48	0.23 b	1.82 b	0.53 b	1.69 b	4.55	0.58	7.78	0.20	1.58	0.58 ab	1.68
1.0 mM AsA	3.62	0.71 ab	6.35	0.26 a	2.11 ab	0.63 a	1.72 b	4.64	0.58	7.15	0.19	1.49	0.58 ab	1.66
0.5 mM SA	3.57	0.67 ab	6.17	0.24 ab	2.05 ab	0.61 ab	2.02 b	4.87	0.64	8.68	0.22	1.78	0.70 a	2.00
1.0 mM SA	3.56	0.69 ab	5.67	0.26 ab	2.05 ab	0.59 ab	1.71 b	4.82	0.56	7.23	0.19	1.48	0.54 b	1.62
10 mM CaCl <sub>2</sub>	3.35	0.70 ab	6.10	0.26 ab	2.12 ab	0.62 a	2.05 b	4.33	0.58	7.53	0.20	1.69	0.61 ab	1.63
20 mM CaCl <sub>2</sub>	3.51	0.75 a	6.22	0.26 ab	2.38 a	0.67 a	2.45 a	4.67	0.62	7.52	0.20	1.73	0.62 ab	2.05

Treatment	Micronutrient concentrations (mg·kg <sup>-1</sup> )													
	2020							2021						
	Zn	Fe	Mn	Cu	B	Mo		Zn	Fe	Mn	Cu	B	Mo	
Control	54.33 <sup>NS</sup>	2818.33 <sup>NS</sup>	256.33 <sup>NS</sup>	7.67 b	27.00 <sup>NS</sup>	0.76 <sup>NS</sup>		72.00 a	1628.67 ab	89.67 ab	20.27 a	30.23 <sup>NS</sup>	0.56 <sup>NS</sup>	
0.5 mM AsA	50.18	2587.29	236.86	6.22 b	23.75	0.82		60.67 b	1394.67 ab	98.33 ab	17.47 c	28.80	0.49	
1.0 mM AsA	53.67	3215.33	284.67	7.30 b	26.00	0.75		54.00 b	1231.00 c	80.33 b	18.03 bc	26.40	0.39	
0.5 mM SA	54.00	2747.00	263.00	14.73 a	26.70	0.82		61.67 b	1405.33 ab	105.67 a	19.17 abc	31.07	0.44	
1.0 mM SA	52.33	3216.33	284.33	9.93 ab	23.37	0.90		55.00 b	1130.00 bc	83.33 ab	17.37 c	26.33	0.42	
10 mM CaCl <sub>2</sub>	56.41	2927.60	278.82	12.92 ab	25.27	0.80		56.33 b	1628.67 a	100.33 ab	19.73 ab	29.63	0.53	
20 mM CaCl <sub>2</sub>	57.33	2709.67	281.00	10.10 ab	27.57	0.77		55.67 b	1222.67 abc	102.33 ab	18.27 abc	28.80	0.41	

<sup>1</sup> Data of the nutrient analysis collected from two marketable heads per treatment per replication that were dried and ground.

<sup>ii</sup> Mean separation based on Fisher's least significant differences at  $P \leq 0.05$ . Means followed by the same letter(s) within year are not significantly different. NS = not significant.

<sup>iii</sup> AsA = ascorbic acid; B = boron; Ca = calcium; CaCl<sub>2</sub> = calcium chloride; Cl = chlorine; Cu = copper; Fe = iron; K = potassium; Mg = magnesium; mM = millimolar; Mn = manganese; Mo = molybdenum; N = nitrogen; P = phosphorus; S = sulfur; SA = salicylic acid; Zn = zinc.

SA, 1.0 mM SA, 10 mM CaCl<sub>2</sub>, and 20 mM CaCl<sub>2</sub> treatments, although not significant, resulted in lower electrolyte leakages than the control. The AsA treatments had a higher percentage of electrolyte leakage than the control, with the 0.5 mM treatment resulting in significantly higher (7.41%) leakage on this date.

On 27 Oct 2020, most stress protectants showed less electrolyte leakage than the control, without statistical significance. On 3 Nov 2021, all stress protectants showed either equal or less electrolyte leakage than the control, without statistical significance. On 19 Nov 2021, all stress protectants showed lower percentages of electrolyte leakage than the control, with multiple occurrences of significance. The lowest electrolyte leakages were recorded for CaCl<sub>2</sub> treatments, with 20 mM showing 11.8% and 10 mM showing 8.6% less leakage than the control. The second lowest leakages were observed with SA treatments, with 1.0 mM showing 7.5% and 0.5 mM 6.3% less leakage than the control. Reductions in electrolyte leakage were also observed with AsA treatments, with 0.5 mM showing 3.8% and 1.0 mM showing 3.6% less leakage than the control.

## Discussion

Our research focused on foliar applications in the field setting and showed results consistent with previous studies of the protective effects of stress-protectant applications for freeze protection. Stress-protectant treatments resulted in high yields, with minimal negative impacts, and showed freeze protection during both laboratory-simulated and natural field freeze events.

**In-field plant growth measurements.** Growth patterns in the present research were similar between stress-protectant treatments and the control treatment, with few adverse effects at weeks 3 and 4 after transplanting. These adverse effects were not observed at the time of harvest when head diameter and marketability were measured, suggesting that growth patterns did not significantly impact yields. Adverse effects were not consistent between years, suggesting that an environmental effect may have influenced growth patterns in the field.

**Quality assessments.** Our study showed that stress-protectant treatments and the control treatment resulted in comparable lettuce quality. High quality and high yields are essential for specialty crop growers to earn high profits. In 2020 and 2021, plant dry weight, marketable weight, head diameter, leaf area, and leaf number all showed trends of similar high quality among treatments. An exception to this was the 1.0 mM SA treatment, which resulted in significantly lower plant dry weight and marketable weight than other treatments. Previous research has highlighted the possibility of high concentrations or continuous applications of SA causing reduced growth (Hara et al. 2012). A study conducted by Nazar et al. (2011) also reported restricted growth with 1.0 mM SA for mung bean. The present research may be linked to reductions in growth, although these reductions were observed only transiently; however, other quality factors associated with the SA treatment were statistically similar to those associated with the control treatment in 2020 and 2021.

Another exception was observed during the 2021 leaf area evaluation. The leaf area with the 1.0 mM AsA, 0.5 mM SA, 1.0 mM

SA, and 20 mM CaCl<sub>2</sub> treatments were significantly lower than that with the control treatment. However, this trend was not observed in 2020, and other quality parameters for 2020 and 2021 were statistically similar, showing that lettuce sprayed with stress protectants still had high quality. Environmental factors in 2021 could have influenced the decrease in leaf area observed with these treatments.

Beneficial effects of spray application of the 20 mM CaCl<sub>2</sub> treatment were observed in 2020, with a significantly larger leaf number and leaf area than those observed with the control. Other research of foliar applications of 20 mM CaCl<sub>2</sub> on lettuce found similar beneficial effects for leaf number and area (Youssef et al. 2017).

Macronutrient and micronutrient concentrations between stress-protectant treatments and the control treatment were the same in 2020 and 2021, with few differences. Research has demonstrated that environmental factors such as light, temperature, growing season (Mou 2009), and soil composition (Pinto et al. 2014) impact the nutritional quality of lettuce. Differences in the 2020 and 2021 growing season may have influenced some of the variabilities we observed. The mean air and soil temperatures in Sep and Oct 2020 were less than those in 2021, which could have influenced nutritional differences. Furthermore, soil characteristics such as organic matter, pH, and cation exchange capacity were all lower in 2020 than in 2021. The lower soil pH of 5.4 could be a reason why Fe and Mn levels were higher in 2020 than in 2021. These two micronutrients are more available to plants at lower soil pH (Neina 2019).

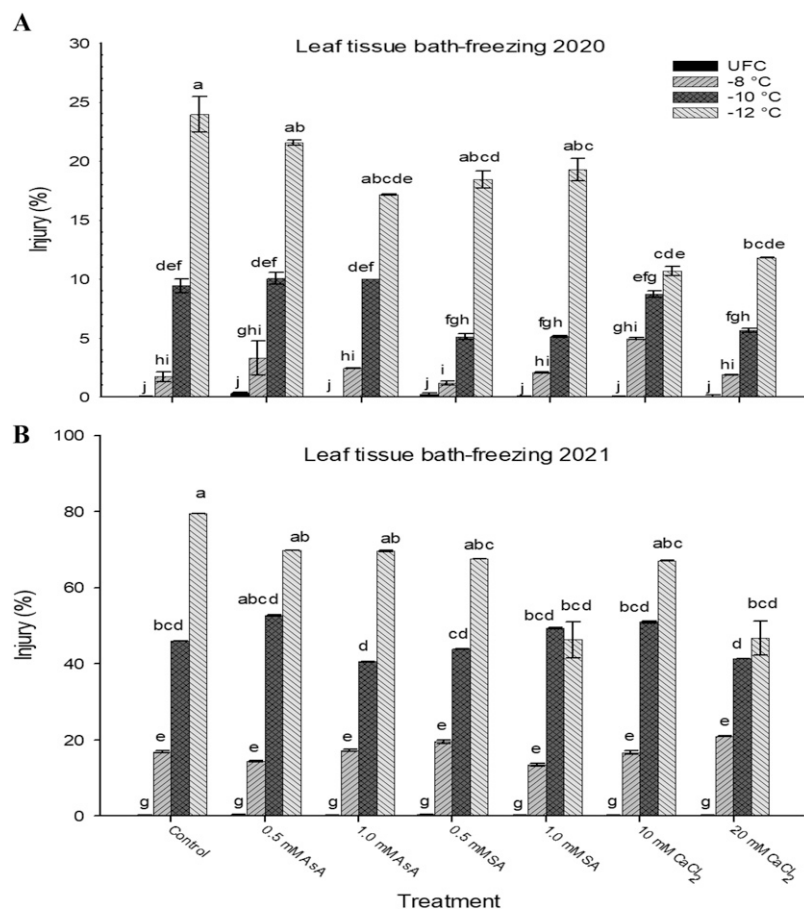


Fig. 2. Effects of stress protectants on freezing injury of butterhead lettuce (*Lactuca sativa* cv. Optima) leaves when exposed to temperature-controlled freezing in a glycol bath. Leaves were harvested 4 Nov 2020 (A) and 17 Oct 2021 (B) at the Iowa State University Horticulture Research Station, Ames, IA, USA. Freeze-thaw injury of leaves was assessed by electrolyte leakage and converted to the injury percentage. Mean separation was based on Fisher's least significant differences at  $P \leq 0.05$ . Means followed by the same letter(s) within years are not significantly different. AsA = ascorbic acid; CaCl<sub>2</sub> = calcium chloride; mM = millimolar; SA = salicylic acid; UFC = unfrozen control.

Overall, applications of stress protectants produced lettuce heads of high quality and did not show consistent adverse effects during our assessment. Research of SA, AsA, and Ca has shown that all three of these compounds assist plant growth and development by influencing internal plant metabolism (Boubakri 2017; Hayat et al. 2010; Hepler 2005; Hepler and Winship 2010).

**Laboratory-simulated freezing.** Research has shown that ice nucleation of plants during a natural freeze event occurs at temperatures of  $-0.5$  to  $-3.0^{\circ}\text{C}$  (Arora 2018) with exposure to cooling rates of  $-1.0$  to  $-3.0^{\circ}\text{C/h}$  (Ashworth et al. 1986; Steffen et al. 1989). To perform a temperature-controlled freeze, these parameters were simulated during our freeze protocol to conduct a realistic freeze assessment. As expected, the highest injury occurred at  $-12^{\circ}\text{C}$ , with colder temperatures increasing the damage. At this temperature, the control showed the most injury in 2020 and 2021, which indicated that the spray applications helped with frost tolerance. Higher concentrations of stress protectants resulted in lower injury. The CaCl<sub>2</sub> applications resulted in the most effective injury reduction,

with the 20 mM CaCl<sub>2</sub> treatment resulting in significantly less injury during both years at  $-12^{\circ}\text{C}$ . The SA treatments resulted in the second most effective protection during both years, with significantly less injury with the 1.0 mM SA treatment at  $-12^{\circ}\text{C}$  in 2021. The AsA treatment, while not as protective, still resulted in less injury than the control treatment during both years at  $-12^{\circ}\text{C}$ .

Although it is beyond the scope of this study, previous research provided further insight regarding how freeze protection during current research may occur at the cellular level. Min et al. (2020) assessed additional exogenous applications of AsA before a laboratory-simulated freeze event and found that these applications lowered the accumulation of reactive oxygen species, increased the amount of antioxidant metabolites and activity of antioxidant enzymes, and had the potential to augment cell walls with increases in branched amino acids. Research by Shin et al. 2018 indicated that exogenous pretreatments with SA before a freeze event lowered oxidative stress, increased antioxidants, and decreased membrane injury. Similar research of CaCl<sub>2</sub> found that pretreatments comprising

CaCl<sub>2</sub> before freeze events alleviated membrane damage, lowered reactive oxygen species accumulation, strengthened the cell wall, and improved the tolerance of photosystem II efficiency to freezing stress (Min et al. 2021). Fundamental research of stress protectants furthers our understanding of the mechanisms and capabilities of frost protection at the plant level and could explain the findings of our study.

It is worth noting that in 2020, the injury levels were much lower than those in 2021. This is because the lettuce was harvested later in the season in 2020, thus inducing cold acclimation in lettuce exposed to cold temperatures. Cold acclimation is the ability of the plant to improve freeze tolerance when primed with exposure to low temperatures. Cold acclimation is a complex response involving alterations of the physiological, biochemical, and molecular components of the plants. These include, but are not limited to, reducing growth, cell wall modifications, gene regulation, increasing antioxidants and osmolytes, and balancing hormones (Xin and Browse 2000). Cold acclimation may be another reason why laboratory simulations at  $-8^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$  resulted in relatively low injury levels with no significant differences between stress-protectant treatments. Future research should aim for consistent harvest times each year to circumvent any confounding effects of cold acclimation. Researchers interested in the effect of stress protectants on "constitutive" freeze tolerance, as assessed by laboratory-simulated freezing, should also consider harvesting the crop before the onset of consistent cold temperatures in the field that potentially induce cold acclimation.

**In-field freezing.** Because plant ice nucleation in nature typically occurs at temperatures of  $-0.5$  to  $-3.0^{\circ}\text{C}$ , plants harvested the night after temperatures below  $-6^{\circ}\text{C}$  were exposed to ice nucleation. Natural "in-field" freeze events occurred on 26 and 27 Oct 2020 at  $-7.1^{\circ}\text{C}$  and  $-8.0^{\circ}\text{C}$ , respectively. In 2021, "in-field" freeze events occurred on 3 Nov at  $-7.1^{\circ}\text{C}$  and on 19 Nov at  $-10.3^{\circ}\text{C}$ . Natural freeze event assessments are challenging because temperature fluctuations, the degree of stress, and stress duration influence the amount of injury/damage sustained by plant tissues (Arora 2018; Min et al. 2014). During laboratory-simulated freezing, a controlled freeze-thaw protocol avoided these variables. These factors influenced electrolyte leakage results and may provide a further understanding of fluctuations. On 19 Nov 2021, larger electrolyte leakage readings compared with those on other dates were observed, suggesting that the lower temperature and a longer duration of stress might have contributed to higher damage. During this freeze event, 20 mM CaCl<sub>2</sub> was the most protective treatment, followed by 10 mM CaCl<sub>2</sub> and the two SA treatments, which all provided significant protection. Although not statistically significant, AsA treatments also resulted in less freeze damage after this natural freeze. Although not all dates showed significance, a trend indicated that stress-protectant applications helped reduce

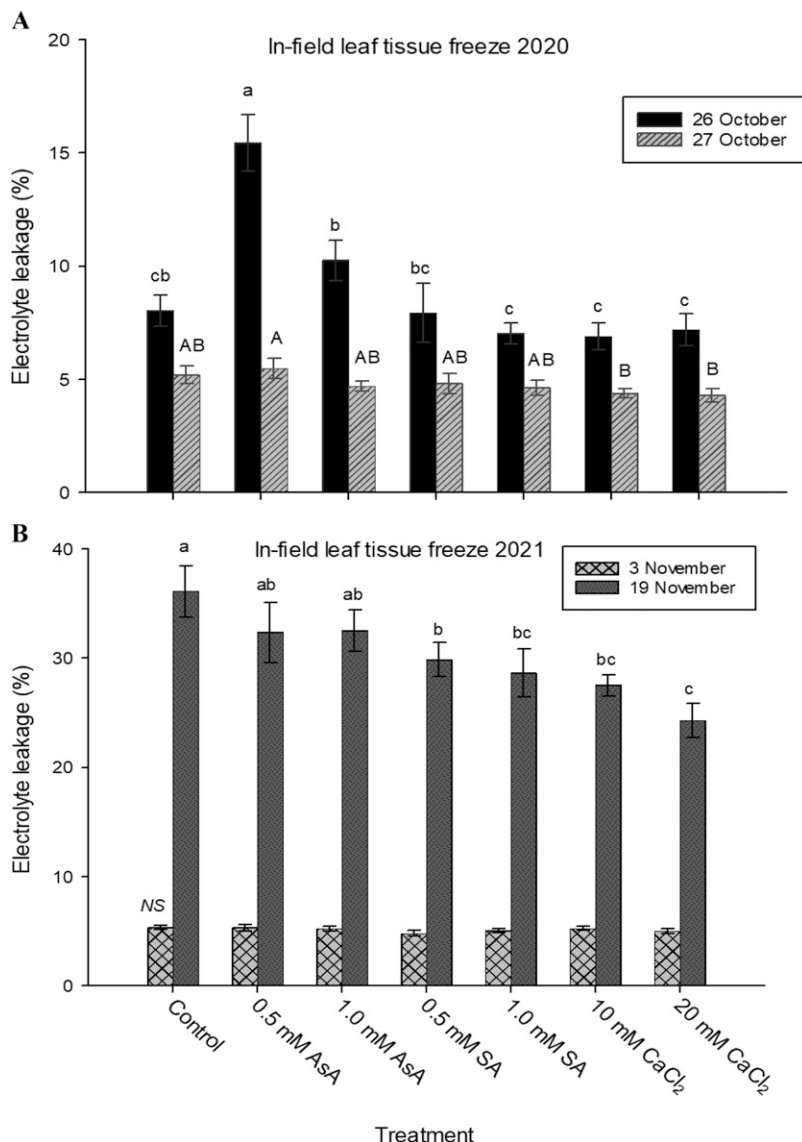


Fig. 3. Effects of stress protectants on electrolyte leakage of butterhead lettuce (*Lactuca sativa* cv. Optima) leaves exposed to outdoor freeze events in (A) 2020 and (B) 2021 at the Iowa State University Horticulture Research Station in Ames, IA, USA. Mean separation for individual dates based on Fisher's least significant differences at  $P \leq 0.05$ . Lowercase letters in 2020 (A) compare treatments for 26 Oct, and capital letters compare treatments for 27 Oct in 2021. Lowercase letters (B) compare treatments for 3 Nov, and capital letters compare treatments for 19 Nov. Means followed by the same letter(s) within years and dates are not significantly different. AsA = ascorbic acid; CaCl<sub>2</sub> = calcium chloride; mM = millimolar; NS = nonsignificant; SA = salicylic acid.

damage during natural freeze events on multiple occasions in 2020 and 2021.

Our study provides a further understanding of stress protectants as a frost mitigation tool, but there were limitations. Field testing is challenging because temperatures and durations of stressful temperatures fluctuate. During future natural freeze field testing, researchers should consider collecting leaf tissue temperatures and the duration of temperature exposure to further understand the overall magnitude of stress. The timing of sprays, nozzle size, and further quality analyses of stress-protectant applications on crops after natural freeze events are vital to assessing their applicability and developing recommendations for the future. Expanding research to less cold-tolerant species or perennial crops would also be interesting

because the economic impact of freeze injury is more detrimental for these species.

## Conclusion

This study is a first step to understanding how foliar exogenous stress-protectant applications work in a field setting under a natural freeze. Most studies of these stress protectants were conducted in controlled settings (Min et al. 2020, 2021; Shin et al. 2018). We must understand how AsA, CaCl<sub>2</sub>, and SA applications work in the field to leverage the benefits they provide regarding the mitigation of cold stress in specialty crop production. Our research highlighted that these applications have a place in frost protection for specialty crop growers; however, further research is needed to

develop crop-specific recommendations. Applications of stress protectants like the ones in this study have the potential to extend the growing season and protect specialty crop growers from economic losses in the Midwest and beyond.

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