Mefluidide Does Not Consistently Enhance the Freezing Tolerance of Cabbage

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Abstract. Mefluidide, a synthetic plant growth regulator, has been reported to protect chilling-sensitive plants from chilling damage and enhance the freezing tolerance of certain winter-hardy herbaceous plants. The potential of mefluidide to enhance the freezing tolerance of nonhardened and dehardening cabbage (*Brassica oleracea* L. Capitata Group) leaf tissue was investigated. Mefluidide at 0 to 60 mg·L⁻¹ was tested on 'Brunswick' and 'Golden Acre' cabbage in five experiments. Leaf tissue freezing tolerance was measured 3 to 9 days postapplication by electrolyte leakage assay. The interval between application and freeze testing had no effect on leaf freeze tolerance. The effect of mefluidide at low rates on leaf freeze tolerance was small and inconsistent. At 30 and 60 mg·L⁻¹, leaf freeze tolerance was decreased consistently. Chemical name used: *N*-(2,4-dimethyl-5-[[trifluoromethyl)sulfonyl]amino]phenyl)acetamide (mefluidide).

When grown for seed production, cabbage plants overwinter in the field and are subject to damage from severe freezes. In a field experiment, a freeze of -11.1 °C in February killed 38% of 'Brunswick' and all 'Golden Acre' plants (Hummel and Anderson, 1990). Application of a plant growth regulator (PGR) that enhances freeze tolerance may protect plants from freeze damage in the field (Howell and Dennis, 1981). The identification of a PGR that increases cabbage freeze tolerance would be beneficial to seed producers.

The PGR mefluidide protects chilling-sensitive plants from chilling injury (Li, 1991). Mefluidide induced a small and transient increase in freezing tolerance of spring and winter wheat (*Triticum aestivum* L.) seedlings (Li, 1991). In shoots of stem-cultured *Solanum tuberosum* L., mefluidide enhanced freezing tolerance only when applied at temperatures above 15 °C; moderate temperatures were required for the mefluidide-induced increase

in abscisic acid content, which preceded the de novo synthesis of proteins required for enhanced freezing tolerance (Costa et al., 1993; Ryu and Li, 1994a, 1994b).

From a practical standpoint, mefluidide is an attractive PGR because of its low toxicity and short half-life in plant tissues and in the soil (Li, 1991). The objective of this research was to determine the potential of mefluidide to enhance cabbage leaf-tissue freezing tolerance.

Materials and Methods

We used 'Golden Acre' and 'Brunswick' cabbage. To maintain a continuous supply of plants, seeds were sown in a greenhouse at weekly intervals in a peat-perlite medium (Fisons Sunshine Mix #2; Fisons Horticulture, Vancouver, B.C.) and 14-day-old seedlings were transplanted to 0.8-L plastic pots in the same medium amended with (per cubic meter) 1 kg Micromax micronutrients (Grace-Sierra, Milpitas, Calif.) and 7.1 kg Osmocote 17-7-12 (17N-3.1P-10K, Grace-Sierra). Plants for all experiments were grown in a greenhouse under automatic overhead irrigation with shallow plastic trays under the containers to prevent water stress. The mean daily maximum/minimum was 25.4 ± 1.9/12.0 ± 1.4 °C and the natural photoperiod was extended to 16 h with mercury vapor lamps to give a minimum photosynthetically active radiation (PAR) of 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Technical-grade mefluidide was prepared in stock solutions by first dissolving it in 2 to 3 mL acetone, then bringing the mixture to volume with glass-distilled water, followed by refrigeration until use. Aliquots of the stock solutions were diluted with glass-distilled water to make treatment solutions for immediate application. Just before application, two to three drops of Tween-20 were added to each

100 mL of treatment solution. Control solutions of glass-distilled water contained the appropriate amounts of acetone and Tween-20. Plants were sprayed to runoff in five separate experiments.

Freeze tolerance of the two youngest fully expanded leaves, typically the fifth and sixth leaves from the base of the plant, was measured in all experiments. Leaf disks (15-mm diameter) were cut from the apical margin of each leaf. A single plant was used as one replication of the treatment at all freeze test temperatures. Leaf disks from each plant were pooled and soaked for 1 h in cold (4 °C) deionized distilled (DID) water to eliminate solutes released from cut surfaces. Three disks were then placed in each glass test tube (20 × 2 cm) containing 1.5 mL cold DID water, and the capped tubes were placed randomly in an ethylene glycol freezing bath (model 2425 CH/P; Forma Scientific, Marietta, Ohio) set at 0 °C, while tubes containing nonfrozen control samples were put in a refrigerator at 4 °C. Additional tubes containing representative samples and fitted with 0.255-mm-diameter (30-gauge) copper-constantan thermocouples were placed randomly in the bath. The thermocouples were connected to a programmable datalogger (CR7X; Campbell Scientific, Logan, Utah) for constant monitoring of temperature.

The bath temperature was lowered to -2 °C at 1 °C·h⁻¹. When tissue temperature reached -2 °C, samples were nucleated with a small chip of DID ice and held overnight at -2 °C to ensure complete tissue freezing. The bath temperature was then lowered further at 1 °C·h⁻¹, the tissue temperature was allowed to come to equilibrium with the bath, and the tissue remained at each test temperature for 1 h before removal. The highest test temperature was -2 °C (except in Expt. 5 when it was -6 °C) and subsequent freeze test temperatures were at successively lower 1 or 2 °C intervals. The number of replications removed at each test temperature varied (Table 1).

After removal from the freezing bath, samples were slowly thawed at 4 °C, then 10 mL DID water and 1.28 mL 5% Tween-20 were added to each tube, resulting in a final Tween-20 concentration of 0.5%. Samples were then vacuum-infiltrated at 0.17 MPa for 15 min, shaken for 1 h, and incubated overnight at 4 °C. The following morning, samples were warmed to room temperature while shaking, and the initial conductivity (IC) of each sample was measured with an electrical conductivity meter (model CDN80; Radiometer, Copenhagen). Samples were autoclaved for 5 min, shaken at room temperature for 1 h, and the final conductivity (FC) measured. The relative conductivity (RC) of each sample was calculated by $RC = (IC/FC) \times 100$. The index of injury [i.e., percent injury (It)] for each sample was then calculated as follows: $It = (RC - CRC)/(100 - CRC)$, where CRC was the nonfrozen control relative conductivity for each sample (Flint et al., 1967). Linear interpolation of the It data gave an estimate of the sample T_{k50} , the temperature at which 50% of the leaf tissues were injured, considered to be

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the freeze-killing temperature (Calkins and Swanson, 1990).

Nonhardened plants. Plants used in the first four experiments were grown in a greenhouse, as described above before, during, and after mefluidide application. Temperature at the time of application averaged 20.9 ± 2.0 °C, while the postapplication mean daily maximum/minimum was $25.1 \pm 1.6/12.4 \pm 1.3$ °C for these experiments. Cultivars, mefluidide treatments, time intervals between treatment and freeze testing, plant ages, and number of replications for each experiment are detailed in Table 1.

'Golden Acre' and 'Brunswick' were included in Expt. 2 to evaluate cultivar differences in cold hardiness. Replications were increased to 12 in Expt. 3 to more precisely determine the effect of mefluidide on leaf $T_{k_{50}}$.

Dehardening plants. Experiment 5 was designed to evaluate the effect of mefluidide on leaf freezing tolerance of previously cold-hardened 'Brunswick' and 'Golden Acre' plants that were dehardening at the time of treatment. After growing for 4 weeks under greenhouse conditions, plants were moved to a controlled-environment chamber (model M-2; Environmental Growth Chambers, Chagrin Falls, Ohio) for cold hardening. Chamber temperatures were maintained at a constant 5, 2, and 0 °C during the first, second, and third weeks, respectively. The photoperiod was 12 h at $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of PAR and plants were subirrigated by placing water at the same temperature as the chamber air in shallow plastic trays under the containers. Plants of both cultivars, 49 days old, were removed from the cold-hardening chamber and placed in a 16 °C day/10 °C night growth chamber for 24 h before treatment with mefluidide at 0, 15, 30, and 60 $\text{mg}\cdot\text{L}^{-1}$. Treated plants were kept under dehardening conditions, 16 °C day/10 °C night, for 5 days, then freeze tolerance of the leaves was determined.

Separate analyses of variance (ANOVA) were performed on data collected for each experiment. Results of multifactor factorial ANOVAs indicated that there were no significant interactions in any of these experiments. The orthogonal polynomial trend comparisons procedure (Gomez and Gomez, 1984) was used to evaluate the effect of mefluidide rate and treatment interval, while cultivar comparisons were made using Student's *t* test.

Results and Discussion

Experiment 1. Leaf $T_{k_{50}}$ was not influenced by the number of days between mefluidide treatment and freeze tolerance evaluation (data not shown). Mefluidide rate had a significant linear effect on leaf $T_{k_{50}}$ (Table 2). Mefluidide at any rate tested decreased leaf $T_{k_{50}}$ relative to the control, thereby increasing freeze tolerance of the 58-day-old 'Golden Acre' cabbage plants by 1.1 to 1.2 °C.

Experiment 2. Neither the interval nor the mefluidide rate had a significant influence on leaf $T_{k_{50}}$ (data not shown). The difference in leaf $T_{k_{50}}$ between the cultivars was small but

Table 1. Treatments applied to nonhardened cabbage plants in Expts. 1–4.

Expt. no.	Cultivar(s)	Mefluidide rates ($\text{mg}\cdot\text{L}^{-1}$)	Interval(s) between treatment and freeze test (days)	Plant age (days)	No. replications
1	Golden Acre	0, 5, 10, or 20	3, 6, or 9	58	3
2	Brunswick	0, 2, 5, or 10	3 or 6	36	6
	Golden Acre	0, 2, 5, or 10	3 or 6	36	6
3	Brunswick	0, 5, or 10	3	40	12
	Golden Acre	0, 5, or 10	3	40	12
4	Brunswick	0, 15, 30, or 60	5	40	5

Table 2. Effect of mefluidide as a foliar spray on the freezing tolerance of cabbage leaf tissue. Freezing tolerance was measured by electrolyte leakage assays and the results expressed as mean $T_{k_{50}}$ values.

Mefluidide ($\text{mg}\cdot\text{L}^{-1}$)	$T_{k_{50}}$ (°C)		
	Expt.		
	1	3	4
0	−2.5	−3.8	−4.9
5	−3.7	−3.3	nt
10	−3.6	−2.9	nt
15	nt ²	nt	−3.8
20	−3.7	nt	nt
30	nt	nt	−2.8
60	nt	nt	−2.3
Significance ^y			
Linear	**	***	***
Quadratic	NS	nt	NS
Control vs. mefluidide	***	***	***

²nt = not tested.

^yComparison of mefluidide rate means within an experiment.

NS, **, ***Nonsignificant or significant at $P \leq 0.01$ or 0.001, respectively.

significant, with 'Brunswick' at −3.5 °C and 'Golden Acre' at −2.6 °C.

Experiment 3. There was no significant difference in $T_{k_{50}}$ between cultivars (data not shown). However, there was a significant linear main effect of mefluidide on leaf $T_{k_{50}}$ (Table 2). In contrast to results in Expt. 1, mefluidide at 5 and 10 $\text{mg}\cdot\text{L}^{-1}$ increased leaf $T_{k_{50}}$ by 0.5 to 0.9 °C, thus decreasing leaf freezing tolerance.

Experiment 4. The higher rates of mefluidide applied to 40-day-old 'Brunswick' leaves produced a significant decrease in leaf freeze tolerance compared to the controls, just as in Expt. 3.

The results of Expts. 1–4 indicate that mefluidide had no consistent beneficial effect on the freeze tolerance of nonhardened 'Brunswick' and 'Golden Acre' leaves. Even when statistically significant, the maximum response to mefluidide at 5 to 20 $\text{mg}\cdot\text{L}^{-1}$ or to cultivar was 1.2 °C or less.

Experiment 5. Leaf $T_{k_{50}}$ of 'Brunswick' and 'Golden Acre' plants at the end of the 3-week hardening period were −14.9 and −14.5 °C, respectively. The freeze test conducted after 6 days at 16 °C day/10 °C night, 5 days after treatment with mefluidide, indicated leaves had lost 8 to 10 °C of freeze tolerance. Many of these samples had *It* values >50% at the highest test temperature of −6 °C, making the calculation of treatment $T_{k_{50}}$ impossible. Cultivar differences for *It* at −6 °C were nonsignificant (data not shown). Mefluidide treatment resulted in a significant linear effect, with *It* at 43%, 56%, 50%, and 61% at 0, 15, 30, and 60 $\text{mg}\cdot\text{L}^{-1}$, respectively. Comparison of the control with all mefluidide-treated plants indicated mefluidide-treated plants had more leaf freeze injury.

Although not directly comparable, results of Expts. 4 and 5 indicate that the higher levels of mefluidide decreased cabbage leaf freezing tolerance. This negative effect of higher mefluidide dosage levels on freezing tolerance is consistent with results obtained with winter wheat (Li, 1991) and gloxinia [*Sinningia speciosa* (Lodd.) Hiern.] (Borochov and Shahar, 1989).

One necessary attribute for any plant growth regulator under consideration for ultimate use on a commercial basis is consistency in effect. In these experiments with nonhardened and hardened cabbage plants, significant enhancement of leaf freezing tolerance occurred only in one experiment out of five. In all others, leaf tissue freezing tolerance was either unaffected by mefluidide or it was rendered significantly less freeze tolerant, particularly at the higher rates. Thus, under our experimental conditions, mefluidide was not consistently useful in improving the freeze tolerance of cabbage.

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