Research Note

Evaluation of Frostgard as an Antifreeze, Inhibitor of Ice Nucleators, and Cryoprotectant on Pinot noir Leaf Tissue

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Laboratory procedures were developed to determine the effectiveness of Frostgard (FG). Specific experiments tested its antifreeze, anti-ice nucleation, and cryoprotection properties in grapes. The freezing point of water was related to the amount of impurities in the water and less with FG concentration. FG at 1% (v/v) depressed the ice nucleation temperature (INT) of water solutions by 1°C. Also, FG depressed INT of ice nucleation active (INA) bacterial suspensions in addition to kill the bacteria. The anti-ice nucleation properties were also tested on fluorophlogopite, an inorganic ice nucleator. FG required the presence of ice nucleators to effectively depress INT, possibly by binding to active nucleation sites. FG at 0.25% (v/v) reduced the freezing injury to grape leaf disks by 21% to 25% at -2°C. At lower temperatures there was no protection. Our results suggest that the practical significance of the cryoprotectant nature of FG is very limited.

KEY WORDS: antifreeze, cryoprotectant, freeze injury, INA, *Vitis vinifera*

Chemical methods for freeze protection of deciduous species were reviewed extensively (21). Growth regulators have been used to either increase directly plant hardiness (1,7,19), or indirectly to delay budbreak and avoid frosts (6). Antitranspirants have been unsuccessful (12,16,17), while effectiveness of cryoprotectants has varied among different products (11,17).

Frost injury of annuals was related to epiphytic INA-bacteria (14). Spring populations of INA-bacteria were reduced with antibiotics (14); but dead cells can retain ice-nucleating properties (2). Several bacterial inhibitors can inactivate INA-bacteria without killing the cells (14). Antagonistic microbes can suppress INA-bacteria, but they were ineffective in preventing frost injury to pear blossoms (5). Thus, a frost control strategy by reducing INA-bacteria populations in woody plants has been ineffective (3,9). Furthermore, intrinsic ice-nucleators within fruit trees were identified (2,3).

Frostgard (FG) is a commercial product advertised to have antifreeze, cryoprotectant, and anti-INA properties. Its composition is not disclosed, but the label claims the presence of cryoprotectant agents, as well as heavy metals and nitrogen. FG might protect grapes from unseasonable frost as it was suggested in a recent report (11). We developed a hypothesis to test each characteristic claimed by this product.

The purpose of this study was to develop laboratory procedures to evaluate the effectiveness of this type of product. We also wanted to determine whether FG was an effective antifreeze and cryoprotectant, and/or if it had sufficient anti-INA properties to confer frost protection of grape leaves.

Materials and Methods

The following conditions were common to the four experiments. Evaluations were controlled freezing tests in a programmable Endocal LT-50DD circulating bath (Neslab Instruments Inc, Newington, NH). Ethylene glycol (30% v/v) was used as a refrigerant. Treatments consisted of 1 mL of either chemical solutions or bacterial suspensions in 4-mL sterile disposable culture tubes (VWR Scientific, San Francisco, CA). The tubes were previously screened for INA at -14°C. The experimental designs were completely randomized with 10 replications, unless otherwise noted. Samples were equilibrated at 0°C for one hour before the temperature was reduced 0.5°C/hour. INT of individual replications was determined by visually examining the sample for ice formation. All experiments were done at least twice; given the similarities, only the results of the last runs are reported.

Experiment 1. Antifreeze properties: To evaluate FG as an antifreeze, FG at 0, 0.12%, 0.25%, 0.50%, and 1.00% (v/v) was applied to four water-impurity classes: *(1)* tap, *(2)* sterile (autoclaved) tap, *(3)* double-distilled, and *(4)* sterile double-distilled. A factorial arrangement was used for the five FG concentrations

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Frostgard is a trade name of Custom Chemicide, P.O.11216, Fresno, CA. 93772. No formulation is available.

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and the four water classes.

Experiment 2. Effect on INA-bacterial suspensions: The inhibitory property of FG was compared to the effect of various physical and chemical stresses on INA-bacteria. *Pseudomonas syringae* pv. *syringae*, strain B-15 (PssB-15) was obtained from the Bacterial Repository of the Department of Botany and Plant Pathology at Oregon State University. It was selected because of its aggressive INA properties (4). PssB-15 was grown on King's B media (10) with 1% glycerol for 60 hours at 27°C, and acclimated at 4°C for 12 hours before the freezing test (15). The bacterial suspension concentration was adjusted turbidimetrically in a Klett-Summerson photoelectric colorimeter model 800-3 (Klett Mfg. Co. Inc., NY) to a final density of 80×10^8 colony-forming units/mL (CFU/mL) and confirmed by a standard dilution plate assay. The stock suspension was divided equally, and the following five treatments were applied two hours before the freezing test: (1) control, *(2)* autoclaved at 110°C for 20 minutes, *(3)* FG at 0.25, *(4)* 0.50, and *(5)* 1.00% (v/v). Simultaneously, a sixth treatment consisted of another plate exposed to chloroform fumes for five hours. The suspension concentration prepared from the chloroform-exposed plate was adjusted to the same optical density as the stock suspension. Twenty replications were used with each of the six treatments. The bacterial populations were estimated before and after the freezing test (8).

Experiment 3. Effect on an inorganic ice-nucleator: Fluorophlogopite (mesh size -200 + 325, Mykroy Ceramics) is a synthetic mica with fluorine atoms replacing the hydroxyl ions of naturally occurring mica. It is a very effective ice nucleator, catalyzing ice formation at temperatures close to 0°C (20). This mica was used to compare the effect of FG on organic and inorganic ice nucleators. Five concentrations of fluorophlogopite (0, 0.3, 0.6, 1.2, and 2.4 mg/mL) and four FG concentrations (0, 0.25%, 0.50%, and 1.00% v/v) were evaluated in a factorial arrangement.

Experiment 4. Cryoprotectant effect on grape leaf tissue: Dormant single-node cuttings of *Vitis vinifera* L. cv. Pinot noir were potted and grown in a greenhouse. Day and night temperatures were 20°C and 12°C, respectively; the house was misted to produce 70% to 90% relative humidity. This artificial environment simulated natural spring conditions in the Willamete Valley, Oregon. Two weeks later, plants with growing shoots were separated into two groups and sprayed, either with a PssB-15 cell suspension of *ca.* 10^8 CFU/mL or with sterile, double-distilled water. These sprays were repeated a week later. Misting was regulated to prevent wetting the plants directly and washing off the inoculum. A week after the second inoculation, FG at 0, 0.25%, 0.50%, and 1% v/v was applied to the growing shoots with a hand sprayer, 36 hours before the freezing test. Bacterial populations were determined by a standard dilution plate assay, after the FG application and before the freeze test (8).

A preliminary trial using whole plants was done to establish the appropriate range of freezing temperatures and FG concentrations. Once those ranges were established, the evaluation was done on leaf tissue. Disks (5 mm diameter) from the terminal leaf of each plant were removed, pooled, and randomized for each FG treatment. Ten disks per treatment were wrapped on moist tissue paper between two sheets of aluminum foil and placed on a floating aluminum pan in a glycol bath. The control treatment was stored at 4°C. The bath temperature was equilibrated at 0°C for one hour, and the pan was covered with ice. Afterward, the bath temperature was lowered to -2°C, -3°C, and -4°C, at a rate of 1°C/h. Subsamples were removed at each temperature and stored at 4°C for 12 hours. The disks were placed on moist filter paper in petri dishes, and kept for 72 hours in an incubation chamber at 25°C, with a 12-hour photoperiod (13). Freezing injury to leaf disks was evaluated by tissue browning. Since a gradation was observed, we scored the injury as percentage of affected area and expressed it on a 0 to 10 scale. A factorial arrangement was used to evaluate the interaction between INA-bacteria, FG, and temperature on freeze injury to the leaf disks.

Results and Discussion

Experiment 1. Antifreeze properties: No interaction was found between the various classes of water and FG concentrations, but main effects were significant and are reported separately in Tables 1 and 2 (18). Despite FG concentration, the mean INT of water classes ranged from -13.9°C for tap water to -16.9°C for sterile, double-distilled water (Table 1). This showed that the higher the amount of impurities in water, the warmer the INT. It suggested that those impurities

Table 1. Mean ice nucleation temperature of different classes of water containing Frostgard at 0, 0.12%, 0.25%, 0.50%, and 1.00%.*

Type of water	Nucleation temperature (°C)
Tap	-13.96a
Sterile tap	-14.00a
Double-distilled	-14.42a
Sterile double distilled	-19.98b

*Mean separation by Fisher's protected LSD at 0.01 level. No significant interaction was found between water classes and Frostgard concentration. The values represent the mean ice nucleation temperature of water classes for the five Frostgard concentrations.

Table 2. Effect of Frostgard on the freezing temperature of tap, sterile tap, double-distilled, and sterile double distilled water.*

Frostgard (%)	Nucleation temperature (°C)
0.00	-11.64a
0.12	-12.06ab
0.25	-11.84a
0.50	-11.76a
1.00	-12.56b

*Mean separation by Fisher's protected LSD at 0.05 level. No significant interaction was found between water classes and Frostgard concentration. The values represent the mean ice nucleation temperature of Frostgard solutions for the four water classes described.

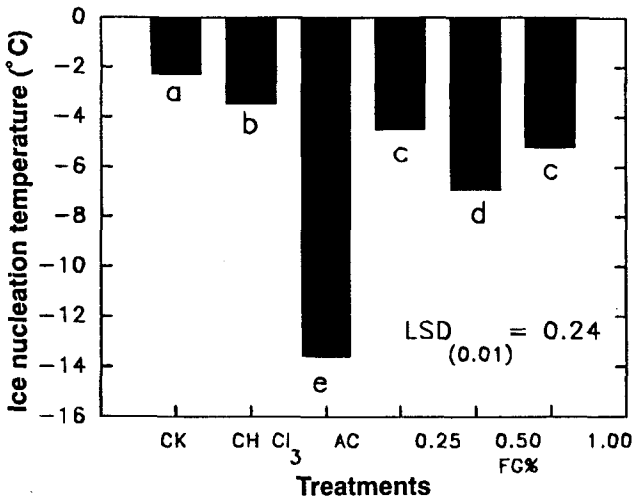

Fig. 1. Comparison of ice nucleating activity of *Pseudomonas syringae* pv. *syringae* strain B-15 in suspensions subjected to lethal treatments. A Pss-B15 suspension was divided in equal aliquots, and the following treatments were applied: CK, control; $CHCl_3$, chloroform fumes; heat, autoclaving at 110°C for 20 minutes; FG, Frostgard at 0.25%, 0.50%, and 1.00% (v/v).

were responsible for the ice-nucleation.

The INT of four water classes were depressed approximately 1°C by 1% FG, while other concentrations had no significant effect (Table 2). Therefore, FG at the concentrations tested was only a poor antifreeze, since an INT depression from -11.64°C to -12.56°C does not have a practical significance.

Experiment 2. Effect on INA-bacterial suspensions: There was a treatment effect on INT of bacterial suspensions (Fig. 1). The control suspension nucleated at -2.3°C, which was similar to other trials on PssB-15 (4,8). Bacterial cell walls contain the INA-Z protein responsible for the INA phenotype (14). Autoclaving denatured the proteins to deactivated the INA of the bacteria so no freezing occurred until -13.6°C. Cell wall integrity is preserved even after FG and chloroform fumes treatments, although the cells are killed (8). However, there was little change in INA from these treatments (2); for example, chloroform-treated bacteria nucleated at -3.5°C, in our trial and depressed INT only 1.2°C. FG at 0.25%, 0.50%, and 1.00% also depressed the INT of the bacterial suspensions to -4.5°C, -6.9°C, and -5.2°C, respectively. Besides acting as a bactericide (8), FG can also affect the INA of the dead bacterial cells and thereby depress the INT.

Experiment 3. Effect on inorganic ice-nucleator: A highly significant interaction was found between FG and fluorophlogopite, suggesting that FG requires the presence of ice nucleators to be effective (Table 3). In the absence of fluorophlogopite, sterile double-distilled water supercooled to *ca.* -15°C, despite FG concentrations as in Experiment 1. When no FG was present, all fluorophlogopite concentrations nucleated water at temperatures ranging from -2.3°C to -3.9°C, similar to a previous report (20). However, when both FG and fluorophlogopite were present, an increasing concentration of fluorophlogopite resulted in warmer INT at any FG level with a range of -6.7°C to -11.1°C. On the other hand, at constant fluorophlogopite concentration, increasing rates of FG produced an overall reduction of an otherwise effective ice nucleator, but this response did not follow a linear pattern. Therefore, FG did not act as an osmoticum, since increasing concentrations had no further depression in the INT. Although this report does not provide direct evidence, we hypothesize that at least one working mechanism of FG is the binding to active nucleation sites of the INA-agent. If true, increasing FG concentration may result in competition for binding sites in the nucleator, which in turn may affect FG performance.

Experiment 4. Cryoprotectant effect on grape leaf tissue: A significant interaction was found between PssB-15 inoculation, FG concentration, and temperature (Table 4). Bacterial inoculation increased injury slightly, and this may be due to limited INA of the established bacteria and/or low populations. Another possibility includes the presence of intrinsic nucleators within the leaf tissue limiting the capacity to supercool (2,3). Leaf disk injury depended chiefly on temperature, while no injury was noted at +4°C, injury was almost total at -4°C. At -2°C, more than 50% of the disk area was injured, while at -3°C, most of it was injured. FG performance depended on the concentration used, and the temperature at which they were tested. The best protection was found with 0.25% FG, but only at -2°C. The injury was reduced 21% in inoculated treatments and 25% in non-inoculated treatments. However, higher FG concentrations were associated with increasing injury in contrast to a previous report (11), where 1% FG

Table 3. Effect of Frostgard and fluorophlogopite (mica ice nucleator) at several concentrations on the ice nucleation temperature (°C) of double-distilled water.*

Fluorophlogopite	Frostgard (%)			
(mg/mL)	0	0.25	0.50	1.00
0	-15.00	-15.50	-13.80	-15.20
0.3	- 2.75	-10.40	-11.10	- 9.70
0.6	- 3.95	-10.10	- 9.50	- 9.20
1.2	- 2.35	- 9.10	-10.10	- 7.60
2.4	-2.45	-6.70	-8.50	-8.40

*Interaction significant at the 0.01 level. Standard error = 0.53°C.

Table 4. Injury to leaf disks as affected by the interaction of Pss-B15 inoculum, temperature, and Frostgard concentration. Leaf injury based on percentage of disk injury, 0 = none to 10 = 100% injury.*

	Mean disk injury (0-10)							
	Inoculated				Non-inoculated			
Temp.	+4°C	-2°C	-3°C	-4°C	+4°C	-2°C	-3°C	-4°C
Frostgard								
0%	0	6.6	9.8	10.0	0	7.2	9.5	10.0
0.25%	0	4.5	9.5	10.0	0.	3.7	9.7	10.0
0.50%	0	5.8	10.0	10.0	0	4.0	10.0	9.7
1.00%	0	7.4	9.6	10.0	0	4.5	9.9	9.7

*Interaction significant, $p = 0.018$. Standard error = 0.36.

decreased low temperature-exotherm of Concord leaf disks to -4.1°C. We observed foliage phytotoxicity, when 1% FG was sprayed at daylight, while night spray showed no injury.

Conclusions

Although FG had shown bactericide action (8), based on these results we conclude that FG is a poor antifreeze. Apparently, FG binds to active nucleation sites in both inorganic and bacterial ice nucleators and can depress INT. However, the cryoprotectant effect of FG on leaf tissue was limited to only -2°C. The practical significance of FG as a cryoprotectant against spring frost is questionable, since there was only a slight reduction in injury at a narrow temperature range.

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