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Ice Nucleation in Tomato Plants

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Abstract. **Tomato plants (*Lycopersicon esculentum* Mill. 'US 28') and plant parts ranging in fresh weight from 6 mg to 180 g were frozen in the presence and absence of epiphytic ice nucleation active (INA) bacteria. As weight increased, freezing temperatures rose from −10.5° to −2°C in the absence of INA bacteria but varied from −3° to −2° in inoculated samples. The freezing behavior of entire plants could only be estimated using small plant parts when INA bacteria were present. INA bacteria were detected by a plate harvesting method. The fraction of tomato stem sections frozen increased with increasing exposure duration at constant temperature.**

Tomato plants are frost susceptible and cannot tolerate ice formation within the tissue. Plants exposed to short periods below 0°C in the absence of freezing are not injured. Therefore, survival is contingent upon the absence of ice initiators or nucleators active at the exposure temperature.

The finding that certain epiphytic bacteria are active ice nucleators has raised the possibility that bacteria limit supercooling of plants. However, the capacity of plants to supercool is a requisite for external ice nucleating agents (such as epiphytic bacteria) to play a causal role in frost damage of tender plants. Studies using plant homogenates (4), leaf disks (9), and shoots (3) have demonstrated supercooling of tomato plant parts and small plants appreciably below the melting point depression of the tissue solution. Thermal analysis experiments demonstrated that sections of peach shoots also supercool below −6°C (5). However, when sample size was greater than 5 g fresh weight, all of the samples froze at about −2.5°. Apparently, once a critical mass was reached, the probability of freezing above −3° approached unity.

The primary objective of this study was to determine whether a similar relationship between freezing temperature and sample weight exists in tomato. Secondarily, the effect of exposure duration at constant temperature on number of samples frozen was examined.

Materials and Methods

Tomato seeds were planted in a commercial potting mix in peat pots. Seedlings were transplanted to 15 cm pots and maintained in a greenhouse under ambient humidity and light intensity. Temperature was maintained above 18°C. Plants were fertilized biweekly with 20–20–20 and staked as needed.

Thermal analyses. Plants were exposed to low temperature

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Table 1. Freezing temperature (°C) of resuspended bacteria, total and fluorescent colonies per plate of 10-fold dilutions from various proportions of a fluorescent, ice nucleation active (INA) bacterial suspension and a nonfluorescent, nonINA bacterial suspension.

	Ratio of INA: non-INA																				
	1:0			1:1			1:10			$1:10^2$			$1:10^3$			$1:10^4$			0:1		
Dilu-	Freeze	Colonies		Freeze	Colonies		Freeze	Colonies		Freeze	Colonies		Freeze	Colonies		Freeze	Colonies		Freeze	Colonies	
tion	temp[z]	Total	Fluor[y]	temp	Total	Fluor	temp	Total	Fluor	temp	Total	Fluor	temp	Total	Fluor	temp	Total	Fluor	temp	Total	Fluor
0	−3	**[x]	*	−3	*	*	−3	*	*	−3	*	*	−3	*	*	−3	*	*	−9	*	0
1	−3	*	*	−3	*	*	−3	*	*	−3	*	*	−3	*	*	−4	*	4	−9	*	0
2	−3	*	*	−3	*	*	−3	*	*	−3	*	4	−3	*	1	−9	*	0	−9	*	0
3	−3	*	*	−3	*	*	−3	*	23	−3	*	3	−7	*	0	−9	*	0	−9	*	0
4	−3	31	31	−3	38	19	−3	39	3	−3	45	1	−9	46	0	−9	38	0	−9	50	0
5	−3	1	1	−3	5	2	−9	2	0	−9	5	0	−9	1	0	−9	3	0	−9	8	0

[z]Freezing temperature (°C).
[y]Fluorescent.
[x]**Too many to count.

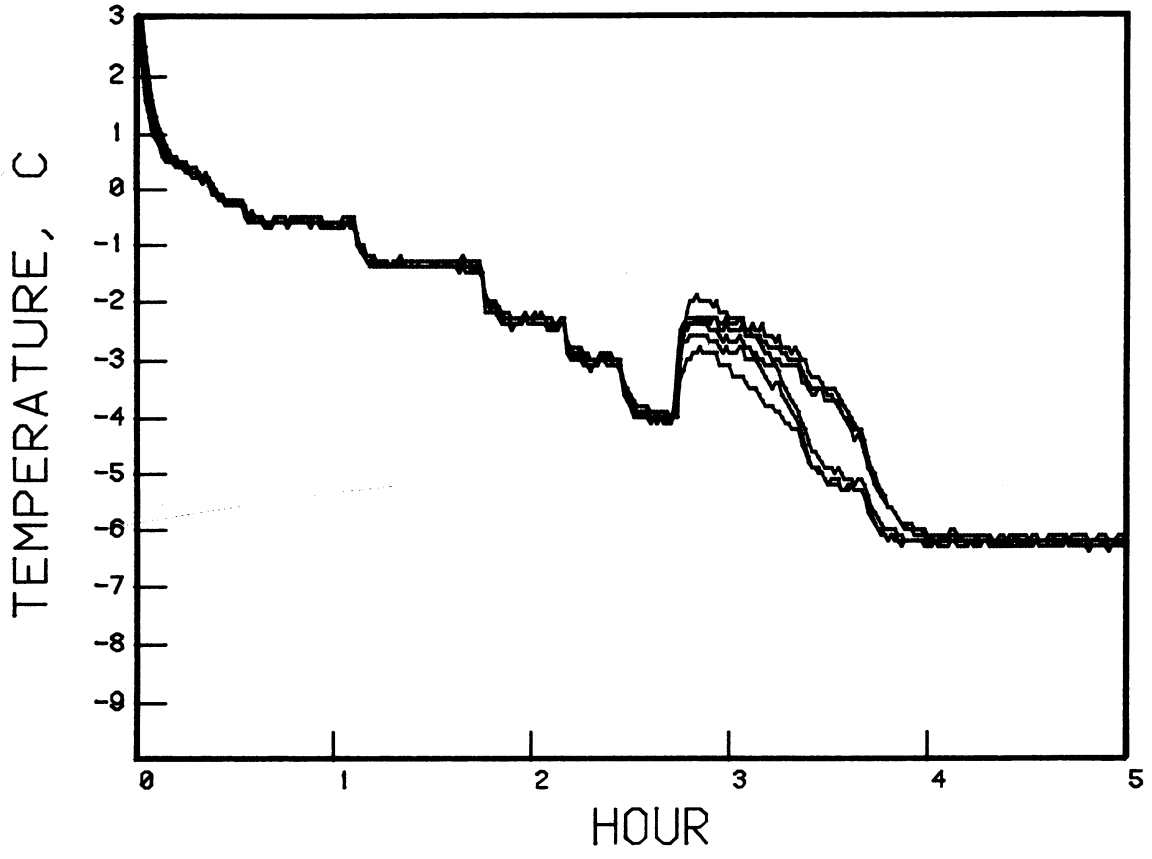

Fig. 1. Thermal analysis of a tomato plant exposed to low temperature stress in a laboratory freeze chamber. Thermocouples were attached at 3 locations on the stem and on 3 petioles.

stress in a refrigerated incubator equipped with a fan to minimize temperature gradients. Pots were placed in insulated boxes to prevent the soil from freezing. Temperature was measured by attaching 36 gauge copper-constantan thermocouples to plants with masking tape. Thermocouple output was monitored by a data logger (Fluke 2200B, John Fluke Man. Co., Everett, Wash.) at 1 min intervals and transferred to a computer (7). Freezing temperatures were determined from thermal analyses displayed on a graphics computer. Most freezing events were evident as "exotherms" (released heat of fusion). In some instances, particularly when freezing occurred near the melting point depression of the tissue solution, distinct exotherms were not detected, although the plants had frozen. However, freezing could be detected by comparing the temperature data from an adjacent wooden stake to those of the plant. The plant and stake remained at essentially the same temperature except when the plant was warmed by the heat of fusion. In this manner, freezing was detected as a period of divergence in thermal analyses.

Since plants ranging in size (0.4 to 180 g fresh weight) were used, thermocouple placement varied accordingly. One thermocouple was fastened to the stem of very small plants while intermediate-sized plants had thermocouples placed at alternate internodes between the 2nd and 7th nodes, and on petioles at nodes 3, 5, and 7 (numbered acropetally). Large plants also had

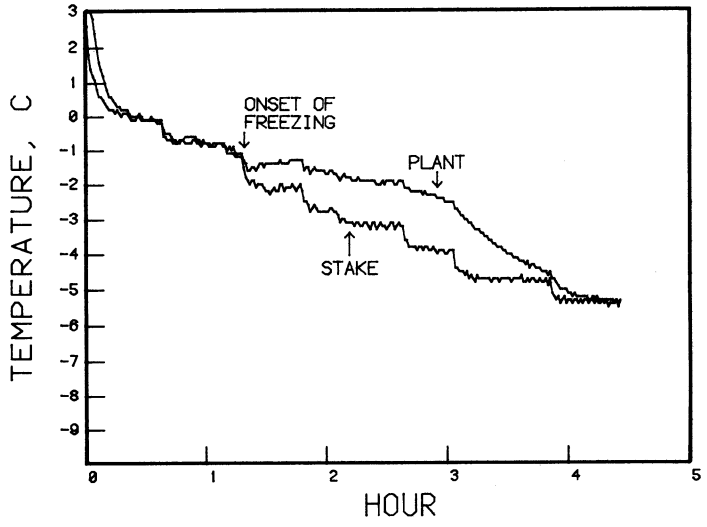

Fig. 2. Thermal analyses of a wooden dowel and tomato plant exposed to low temperature stress in a laboratory freeze chamber.

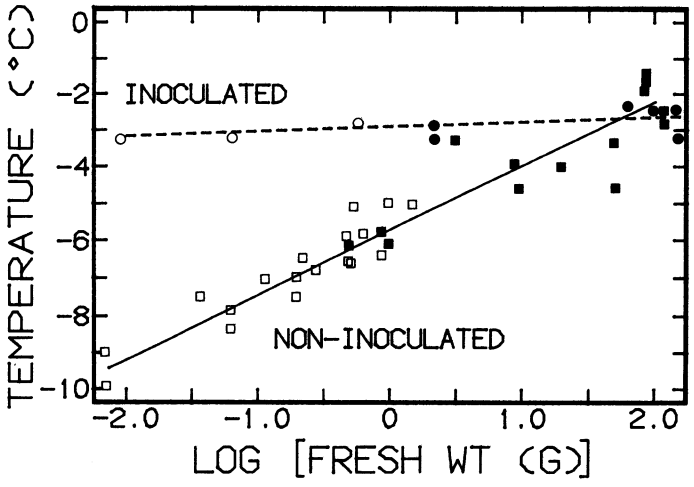

Fig. 3. Relationship of sample fresh weight and freezing temperature in plants sprayed with water or 10^8 cells/ml *Pseudomonas syringae*. Circles represent inoculated samples ($y = -2.97 + 0.16x$, $R^2 = 0.40$), while squares correspond to controls ($y = -5.66 + 1.68x$, $R^2 = 0.89$). Closed symbols represent entire plants whereas open symbols correspond to plant parts.

thermocouples placed at internodes farther up the plant. Plants weighing 1, 10, and 100 g average about 11, 17, and 78 cm in height, respectively. Leaf temperature also was monitored in the 1st few plants but not subsequently, since the system was essentially isothermal. Leaves, petioles, stem, and chamber walls came to equilibrium with air temperature with no radiative cooling observed. Temperature was lowered in steps of 0.5° to 1.0°C, resulting in an overall cooling rate of about 1.5° per hour.

Plant parts were freeze-tested in test tubes containing 5 ml sterile deionized water. Tubes were immersed in a refrigerated bath at −3°C. Samples were checked for freezing after 30 min, then the bath was lowered 1°. Thirty min after temperature equilibration, the remaining tubes were checked for freezing. This procedure was repeated until all replicates were frozen. Care was taken to standardize the procedure, since excessive mechanical agitation increased the percentage of frozen samples. Data were reported as T_{50}, the temperature at which 50% of the replicates had frozen.

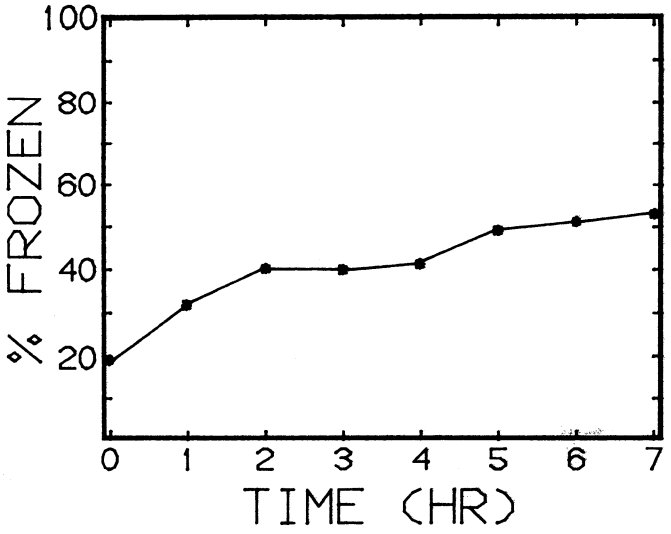

Fig. 4. Effect of exposure duration at −4°C on freezing of tomato stem sections cooled to −5° for 30 min prior to 7 hr at −4°.

The time dependency of ice nucleation in tomato stem sections was examined. Thirty 2 cm stem sections (630 ± 88 mg) were placed in test tubes with 5 ml sterile deionized water. Ten test tubes contained only water as a control. The tubes were cooled to −5°C, held for 30 min, then warmed to −4° for 7 hr. The number of frozen tubes was determined just after reaching −4° and each subsequent hour. Bath temperature remained constant, not varying more than 0.2° from the set-point. This experiment was repeated 3 times.

Preliminary field experiments were carried out by transplanting plants of various sizes immediately prior to an anticipated frost. Thermal analyses were conducted as previously described.

Culture and recovery of bacteria. An INA strain of *Pseudomonas syringae* (isolate 1080) was used for plant inoculation. Bacteria were grown at 22°C in a medium containing 1% Bactopeptone, 1% dextrose, and 0.1% Bacto-casamino acids. After 24 hr on a revolving shaker (125 rpm) cultures were centrifuged at 2000 g for 10 min. The pellet was suspended in sterile deionized water and adjusted to 0.3 absorbance at 600 nm (about 10^8 cells/ml). Suspensions were applied to plants with a chromatography mister. Plants were held in the laboratory at ambient temperature and humidity for 24 hr prior to thermal analyses.

Following freezing, each plant was assayed for INA bacteria to ensure against INA bacterial contamination on control plants. Plants were severed at the soil line, weighed, and homogenized in 300 ml sterile deionized water. Entire plants were assayed to eliminate sampling error. Serial dilutions of plant homogenates were made on Pseudomonas Agar F (Difco, Detroit, Mich.). Total and fluorescent colonies were counted directly, whereas ice nucleation active colonies were detected by a plate harvesting method. Five ml of sterile liquid medium were poured over each plate, and colonies were suspended using a wire loop. The turbid suspensions were transferred to test tubes and placed in a refrigerated bath at −3°C. Dense plates required 2 separate "harvests". Tubes were held at −3° for 30 min, then examined visually for freezing. Bath temperature was lowered and tubes subsequently examined at −5°, −7°, and −9°. The reliability of this method was verified by plating various proportions (v:v)

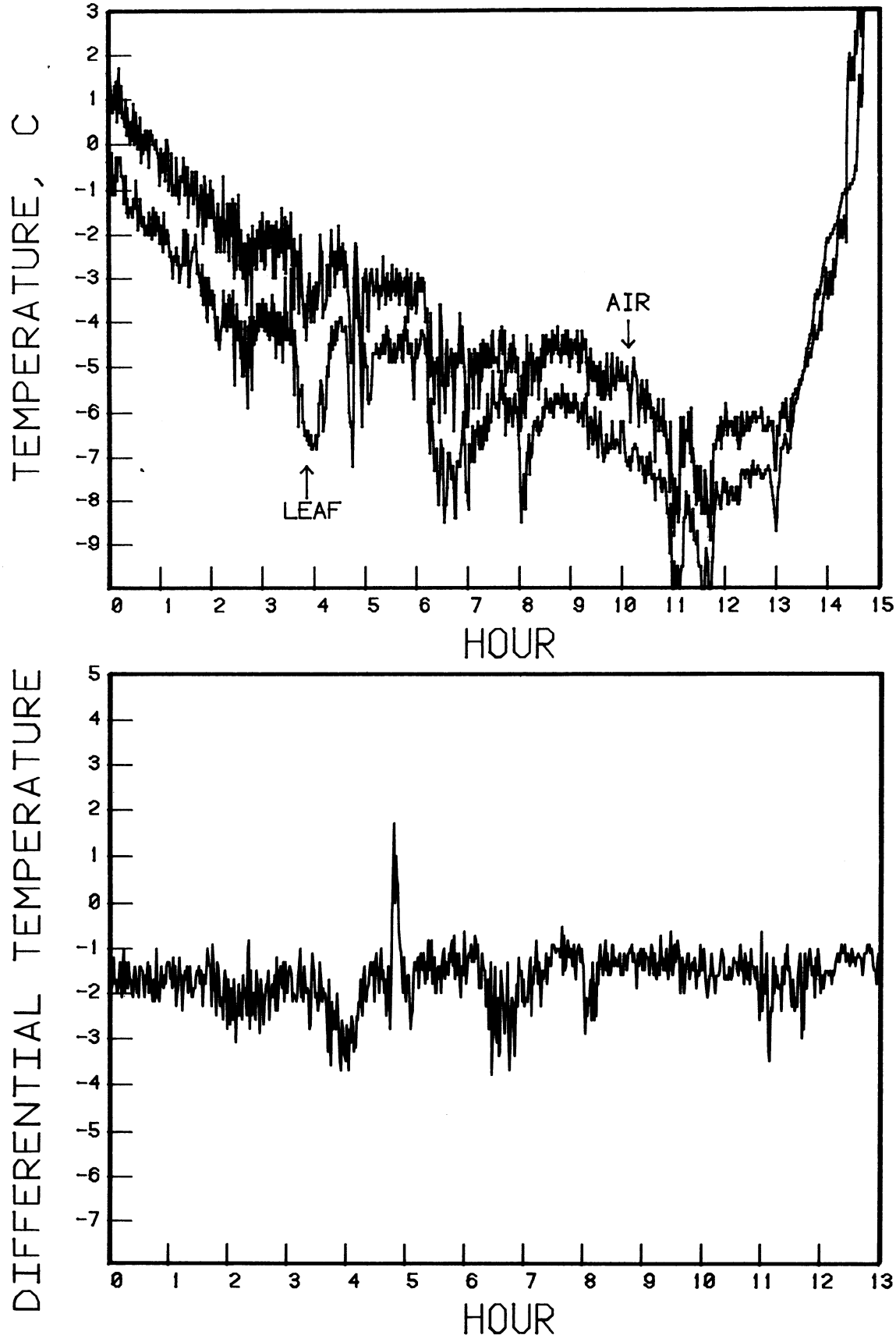

Fig. 5. Air and tomato leaf temperature plotted separately vs. time **(a)** and as the difference between leaf and air temperature **(b)** during a natural frost.

of suspensions of a fluorescent, INA bacterium and a nonfluorescent, nonINA bacterium.

Results and Discussion

Detection of INA bacteria. Epiphytic INA bacteria were detected by a plate harvesting method. Tubes containing suspensions from plates with only the INA bacterium all froze by −3°C, even when only 1 colony was present (Table 1). Tubes containing bacterial suspensions harvested from plates with only the nonINA bacterium were not frozen until −9°, except for 1 dilution which was frozen at −7°. When the INA and nonINA bacteria were mixed in proportions from 1:1 to $1:10^4$ (v/v), all tubes from plates containing fluorescent colonies were frozen by −4°. INA colonies were detected even when they constituted only 0.01% of the total. Inter-colony competition probably would make detection of INA bacteria comprising less than 0.01% of the total unreliable.

This unreliability also would be true for the replica-plating method (8), since the procedures differ only in detection of ice nucleation activity. The plate harvesting technique was developed to reduce ambiguity in detecting freezing. The authors found it difficult to determine frozen areas reliably using replica plating, even when ambiguous areas were prodded with toothpicks as suggested (8). The plate harvesting technique, which involves the freezing of 5 ml of liquid, simplified the detection of freezing. The plate harvesting technique was useful for detecting the presence of epiphytic INA bacteria and providing an order-of-magnitude estimate of the concentration.

Greenhouse-grown tomato plants harbored from 2.6×10^3 to 3.3×10^7 bacterial cells per gram fresh weight. Fluorescent bacteria were detected on about half of the plants, with a mean population of 2.6×10^3 cells per gram fresh weight. Fluorescent bacteria comprised 0.06% to 9.2% of the total cells when present. The source of inoculum was probably the potting soil, which contained 3.0×10^7 cells per gram (87% fluorescent, no INA bacteria detected). None of the noninoculated plants used in this study contained detectable levels (5×10^2 cells/g fresh weight) of INA bacteria. This level was deemed sufficient, since not every cell is active in ice nucleation (4, 9, 10). The fraction that is active decreases with increasing temperature, comprising less than 1 in 10^7 at −2.3°C (9). Plants sprayed with INA bacteria harbored from 1.9×10^4 to 8.2×10^6 cells per gram fresh weight (5% to 93% fluorescent). Ice nucleation active bacteria were detected on all inoculated plants.

Thermal analyses. Ice formation in tomato plants was detected readily. In most instances, a distinct exotherm clearly indicated freezing (Fig. 1). However, some of the plants that froze just below 0°C did not display distinct exotherms. In these instances freezing was detected as a divergence in plant and reference temperature (Fig. 2). Freezing was detected throughout a plant in a 3–4 min span in nearly all trials. However, the onset of freezing varied 19 min in 1 plant. No barriers to the spread of ice were observed. It appeared that ice nucleation in 1 part of a plant triggered freezing in an entire supercooled plant (Fig. 1).

The warmest temperature determined upon the release of the heat of fusion was not considered an accurate estimation of the melting point depression of the tissue solution. Heat was dissipated from the plant too rapidly for the melting point to be reached. As a result, the exotherm maxima decreased with decreasing freezing temperatures (slope of regression line = 0.52, $r = 0.93$). Similar findings were reported with *Citrus* (1).

Sample weight had a marked effect on freezing temperature of tomato plants and plant parts not inoculated with INA bacteria. Leaf disks (6 mg) had a median freezing temperature of −10.5°C, whereas petiole sections (0.1 g) froze at −8 (Fig. 3). Small seedlings and stem sections (1 g) froze at about −6°. As plant size increased to about 100 g, freezing temperatures rose to about −2°. It is apparent that the supercooling capacity of entire plants can be overestimated by freezing small plant parts.

In contrast, plants and plant parts inoculated with INA bacteria froze between −2° and −3°C (Fig. 3). Since freezing was probably initiated by the INA bacteria, the mass of the tissue had no effect on freezing temperature.

The results indicated that reducing the number of INA bacteria residing on/in tomato plants larger than about 50 g would not affect the freezing temperature. The potential for this method of frost control is therefore limited to small plants. Tomato plants weighing about 1 g could supercool about 2° to 3°C lower if INA bacteria were not present. This level of freeze avoidance would provide substantial frost protection. Methods of frost control based on reducing INA bacterial populations are probably best suited for seedling protection in the spring. It does not seem likely that this strategy would be productive for prolonging mature plant survival in the fall.

The ice nucleation activity of plant material has been quantified as percentage samples frozen vs. temperature (2) and by variations of Vali's cumulative nucleus spectrum (CNS) equation (14). This equation calculates the number of nuclei active at and above a particular temperature in a unit volume of water. Lindow et al. replaced the volume term with tissue weight (9), whereas Rajashekar et al. eliminated the volume term entirely (12). Both of these approaches have drawbacks. Quantifying freezing behavior as fractional freezing or cumulative number of nuclei vs. temperature provides useful information. However, the results are valid for a particular sample size and exposure duration (or cooling rate). Expressing the cumulative number of nuclei on a unit tissue weight basis does not eliminate the limitation. As sample weight increases, the threshold freezing temperature increases, not merely the concentration of nuclei at a particular temperature.

Vali's equation is based on a singular hypothesis that assumes freezing is the result of an impurity with a characteristic freezing temperature. This assumption is valid for glass distilled water which behaves in accordance with a modified singular hypothesis (15). Singular theory predicts that nucleation is only a function of temperature, and no further freezing events would occur under isothermal conditions. In contrast, stochastic theory assumes that all samples have the same chance of freezing at any particular moment and predicts continued freezing events over time at constant temperature (15). Although the CNS input (fractional freezing) is fundamental, it has not been demonstrated that a singular hypothesis is valid for plant material. In fact, the definite time dependency of ice nucleation in citrus plants (16) and peach shoots (6) indicate a stochastic nature.

A direct test of whether tomato stem sections freeze in accordance with a singular or stochastic model was conducted. When samples were cooled to −5°C and then warmed to −4°, singular theory predicted that all ice nucleation sites active at −5° and above would have been expressed and no further freezing would occur at a warmer temperature or over time. However, if samples behave according to a stochastic model, freezing events would have occurred while the temperature was held constant at −4°. The freezing behavior of the tomato stem sections was best explained in terms of a stochastic model since

an additional one-third of the samples froze while held at −4° (Fig. 4). After cooling to −5°, then warming to −4°, 20% of the samples had frozen. An additional 7 hr at −4° resulted in 53% frozen. An increase in exposure duration significantly increased the fraction of samples frozen (t test, 5% level).

Natural frost. Tomato plants exposed to a natural frost exhibited the same relationship between weight and freezing temperatures as plants exposed to freezing temperatures in the laboratory when both were free of detectable populations of INA bacteria. Plants weighing 9.5, 33.7, 82.7, and 124.0 g froze at stem temperatures of −4.9°, −3.2°, −2.4°, and −1.2°C, respectively. These preliminary data corroborate previous reports with *Citrus* and *Prunus* (1, 5).

Measurement of leaf and air temperature confirmed previous reports that leaves cool below air temperature under radiative conditions (11, 13). Although the temperature difference between leaf and air averaged about 2°C, transient differences of up to nearly 4° were observed (Fig. 5a). This resulted in leaf temperatures 0.8° to 2.3° colder than stems at the moment of freezing. Differences in leaf, stem, and air temperature probably fluctuate in response to windspeed and extent of cloud cover. The overall cooling rate of the air was about 1° per hour. However, fluctuations of up to 0.5° per minute for short duration were not uncommon (Fig. 5a). Although stems were damped against rapid fluctuations in temperature by their relatively large thermal mass and volume to surface area ratio, leaves paralleled changes in air temperature very closely (Fig. 5b). Freezing was initiated rapidly throughout the plants, so it was not possible to determine whether freezing started in stems or leaves. Leaves were not found to be poorer ice nucleators than stems, when equal masses were frozen by the test tube method (data not presented).

Low temperature stress in a laboratory freeze chamber differed from a natural frost in that the former is essentially an isothermal system. Leaves cool below air temperature under radiative conditions in the field.

The results of this work indicate that future experimental designs should take into account the relationship between freezing temperature and sample size as well as exposure duration. Small samples freeze at a lower temperature than large samples. A previous report (9) that plant injury is predictable on the basis of leaf disk freezing is valid only for plant material harboring sufficient populations of INA bacteria. Freezing leaf disks overestimates the supercooling ability of entire plants when INA bacteria are not present.

An increased fraction of samples freeze at constant temperature when the exposure period is lengthened. It follows that the probability of freezing for an individual plant increases as exposure duration increases. Generalizations on the supercooling ability of plants during a frost can not be made from relatively short periods of exposure.

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