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Factors Affecting Ice Nucleation in Plant Tissues

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ABSTRACT

Factors affecting the ice nucleation temperature of plants and plant tissues were examined. The mass of a sample had a marked effect on ice nucleation temperature. Small tissue samples supercooled to −10°C and were not accurate predictors of the nucleation temperature of intact plants in either laboratory or field experiments. This effect was not unique to plant tissues and was observed in autoclaved and control soil samples. Ice nucleation temperatures of bean, corn, cotton, and soybean seedlings were influenced by the length of subzero exposure, presence of ice nucleation active bacteria, and leaf surface wetness. The number of factors influencing ice nucleation temperature suggested that predicting the freezing behavior of plants in the field will be complex.

There has been recent interest in determining the temperature at which ice formation is initiated in plants. Several laboratories have reported that plants lack intrinsic ice nuclei active above −5 to −10°C (3, 8, 10, 13). These predictions were largely based on variations of a droplet freezing technique used by atmospheric scientists (17, 18). In this technique, a sample was subdivided into numerous small subsamples. These samples were cooled and based on the temperatures at which individual subsamples froze, an ice nucleus spectrum developed.

In previous studies we demonstrated that both sample mass and time had a significant effect on ice nucleation temperature in peach shoots (5, 6) and tomato plants (2). These observations led us to question the validity of using small tissue samples and rapid cooling rates for predicting ice nucleation temperature in intact plants. The purpose of this study was to determine whether our earlier observations were unique to peach and tomato or would be valid with a variety of herbaceous plants and nonliving systems. In addition, we hoped to further characterize the factors which influence ice nucleation within plant tissues.

MATERIALS AND METHODS

Corn (*Zea mays* L.), soybean (*Glycine max* [L.] Merrill), bean (*Phaseolus vulgaris* L.), and cotton (*Gossypium hirsutum* L.) seeds were planted in commercial potting mix and grown in a greenhouse. Plants were routinely screened for the presence of ice nucleation active bacteria as described previously (2). Soil for nucleation studies was collected from a peach orchard and stored in plastic bags at room temperature until used. A subset was autoclaved for 45 min. Kaolin (hydrated aluminum silicate) was purchased from Sigma Chemical Company.

Determination of Nucleation Temperature. Four methods were used to determine ice nucleation temperatures of plant and soil samples. A droplet freezing technique (18) was used to assess ice nucleation activity of plant homogenates and soil suspensions. Homogenates (2% w/v) were prepared by homogenizing the above ground portion of seedlings in sterile deionized H_2O for 1 min in a Waring Blendor. Soil suspensions (2% w/v) were prepared by vigorous mixing with deionized H_2O. Ten μl drops were applied to the surface of a thermoelectric cooling plate covered with a layer of vacuum grease and parafilm. Plate temperature was lowered between 12 to 15°C per h and the number of frozen drops determined visually. Values reported were based on 100 replicate drops of each plant species and 200 drops of both autoclaved and control soil. An equal number of drops of deionized H_2O were used as a control.

The ice nucleation activities of larger volumes of plant homogenates (2% w/v), soil suspensions (2% w/v), and saturated soil (1:1 w/v) were determined using a test tube method (5). There were 20 replicates of each treatment. The test tube method was also employed to determine nucleation temperature of submerged plants or homogenates made from the above ground portion of plants in 25 ml water. There were 10 replicates of each treatment.

The ice nucleation temperatures of leaf discs (6.5 mm diameter) were determined using differential thermal analysis (12). Samples were cooled at 2°C/h, and there were 15 replicates of each species.

The ice nucleation temperatures of intact seedlings (2.7 ± 1.2 g fresh weight) were determined using a computer interfaced method of thermal analysis (4–6). In laboratory experiments, plants were cooled in an incubator. The pots were placed in either boxes insulated with a layer of vermiculite or into Dewar flasks to prevent soil freezing. Samples were cooled at about 1.5°C/h by manually reducing incubator temperature in 0.5°C decrements. There were at least 40 replicates of each species.

In field experiments, greenhouse grown seedlings were transplanted into a tilled plot about 12 m from the laboratory building on the afternoon prior to an anticipated frost. Thermocouples were fixed to the plant stems with masking tape and data collected as described previously. In addition, the temperatures of small wooden stakes adjacent to the plants were monitored and served as references. Plant freezing was monitored on several evenings under variable conditions; between 9 to 15 replicates per species were examined.

The freezing of 100 g samples of saturated orchard soil was also monitored using computer interfaced thermal analysis. Thermocouples were attached to the inner wall of plastic beakers and 100 g of saturated soil was added. Beakers were transferred to a laboratory incubator and the temperature reduced to −2°C. The temperature and time of nucleation was determined as described above. Due to the mass of the soil sample and the unfavorable surface to volume ratio, sample temperature would not be uniform and the center of the soil sample would be expected to cool much slower than the outer portions. Therefore, we chose to monitor the temperature on the outside of the soil sample so that errors resulting from a lag in heat transfer would result in an overestimation of the extent of supercooling.

Influence of Time on Ice Nucleation. To assess the influence of time on ice nucleation, samples were held at constant temper-

Table I. *Effect of Assay Technique and Tissue Mass on Ice Nucleation Temperature*

The temperature at which plant samples nucleated ice formation was determined for a range of sample masses. Values for the 0.2 mg samples were determined by examining the freezing of 100 10 μl droplets of a 2% homogenate on a cooling plate. Values for the leaf discs were obtained from differential thermal analysis of 15 replicates of each species. The values for the 20 mg samples were obtained by freezing 20 1-ml samples of 2% homogenate in a circulating glycol bath. Nucleation temperatures for intact seedlings were obtained both under laboratory conditions (at least 40 replicates cooled at −1.5°C/h) and under natural frost conditions (between 9–15 replicates/species). The values reported are the temperatures at which the first sample froze and the median freezing temperature.

Treatment	Bean		Corn		Cotton		Soybean	
	1st	Median	1st	Median	1st	Median	1st	Median
	nucleation temperature, °C							
2% Homogenate, 10 μl (0.2 mg)	−9.8	−10.5	−10.0	−12.1	−7.3	−13.0	−9.0	−11.2
Leaf discs, 6.5 mm OD (4–5 mg)	−7.5	−11.0	−9.0	−10.7	−11.0	−13.5	−11.0	−13.5
2% Homogenate, 1 ml (20 mg)	−7.0	−8.0	−8.0	−9.0	−6.0	−7.0	−8.0	−8.0
Seedlings in laboratory (2.7 g)	−1.3	−3.9	−1.3	−4.1	−3.8	−5.6	−3.3	−5.5
Seedlings in field	−1.3	−2.7	−1.0	−2.5	−1.1	−2.5	−2.1	−2.7

Table II. *Effect of Sample Mass on the Temperature at Which Soil Samples Initiate Ice Formation*

The temperature at which soil samples nucleated ice formation was determined for a range of sample masses. Values for 0.2 mg samples were determined by examining the freezing of 10 μl droplets on a cooling plate. One ml suspensions of the 20 mg and 1 g samples were placed in test tubes and cooled in a circulating glycol bath. One hundred ml suspensions containing 100 g soil were cooled in an incubator. The values reported are the temperatures at which the first sample froze and the median freezing temperature. Values were based on 200 replicates for the 0.2 mg sample and 20 replicates for the other treatments.

Mass of Sample in Suspension	Soil		Autoclaved Soil	
	1st	Median	1st	Median
	nucleation temperature, °C			
0.2 mg	−5.2	−6.0	−6.6	−10.5
20 mg	−3.0	−5.0	−7.0	−7.6
1 g	−3.0	−4.0	−4.0	−6.0
100 g	−0.3	−1.0	−0.4	−1.9

atures and the number of individuals which froze over time noted. Intact seedlings were transplanted into plastic beakers, and the beakers were placed into Dewar flasks partially filled with warm tap water. The soil surface was approximately 2.5 cm below the top of the Dewar flasks. This zone was layered with dry vermiculite and sealed with parafilm. With this configuration, the stem and leaves were exposed to subzero temperatures for 24 h without the roots and soil freezing. Soil temperatures were monitored throughout to confirm this. Seedlings were held at −4 and −5°C for 24 h, and plant temperatures varied less than 0.2°C. The time and temperature of ice nucleation were determined as previously described (6). There were 10 replicates of each species at each temperature.

Experiments were also conducted using a 1% (w/v) suspension of kaolin. One ml samples were placed into glass test tubes and autoclaved for 20 min. Thermocouple junctions were secured to the outside of stoppered test tubes and the tubes transferred to a circulating glycol bath 1° above test temperature. After a 30 min incubation, tubes which had frozen were removed and noted. Bath temperature was then lowered to the test temperature and held constant for 24 h. Approximately 6 min were required to reach the test temperature, and once established, temperatures were stable (±0.1°C). Temperatures were monitored at 1 min intervals and the time and temperature of ice nucleation determined as described above. Experiments were conducted at −10, −11, and −12°C with 40 replicates at each temperature. Tubes of deionized H_2O were used as a control. Using this technique, the freezing of the tubes was monitored without the tubes being moved, thus avoiding the possibility of physically induced ice nucleation.

Effect of Leaf Surface Water and Ice Nucleation Active Bacteria on Plant Freezing. Sterile deionized H_2O was applied as a fine mist to seedlings using a chromatography sprayer. Plants were then frozen in an incubator and ice nucleation temperature determined as described above. There were at least 11 replicates per treatment. An equal number of dry seedlings of comparable age and size were used as a control.

To determine the effect of ice nucleation active bacteria on seedling freezing, plants were sprayed with a suspension of 10^8 cells/ml of *Pseudomonas viridiflava* Burkholder. The bacteria had been grown in a medium containing 1% Bacto-peptone, 1% dextrose, and 0.1% Bacto casamino acids at 22°C on a gyrotory shaker. Cultures were centrifuged at 2000*g* for 10 min, resuspended in sterile deionized H_2O, and adjusted to 0.3 *A* at 600 nm (approximately 10^8 cells/ml). Suspensions were applied with a chromatography sprayer. Control plants were sprayed with sterile deionized H_2O. Seedlings were incubated at 22 ± 2°C in a plastic enclosure with elevated humidity for 48 h to facilitate bacterial establishment. Plant nucleation temperature was determined as described above. Immediately after the experiment, half the plants were homogenized in sterile deionized H_2O for 1 min in a Waring Blendor and the populations of total and fluorescent bacteria determined by dilution plating on *Pseudomonas* Agar F (Difco, Detroit, MI). The ice nucleation activities of the cultures were determined using a plate harvesting technique (2). There were between 11 and 15 replicates of each treatment for each species.

RESULTS

Sample mass had a marked effect on ice nucleation temperature. Experiments with bean, corn, cotton, and soybean tissues and intact seedlings were conducted with tissue masses varying from 0.2 mg in the plant homogenates to approximately 2.7 g with the intact seedlings. In each of the species examined, the

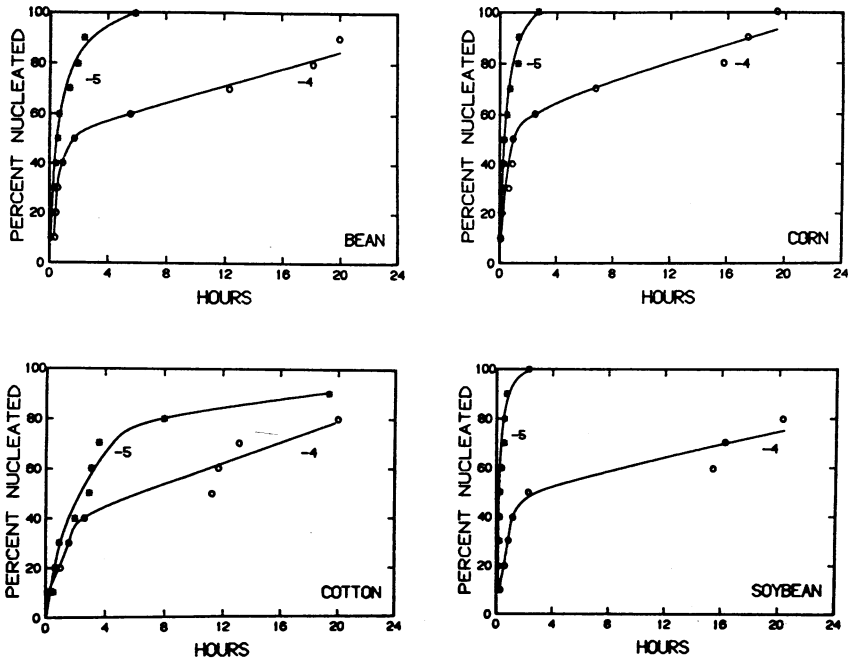

FIG. 1. Effect of time on the nucleation of bean, corn, cotton, and soybean seedlings. Seedlings were held at either −4 or −5°C for 24 h, and the proportion of seedlings frozen over time was determined using computer interfaced thermal analysis.

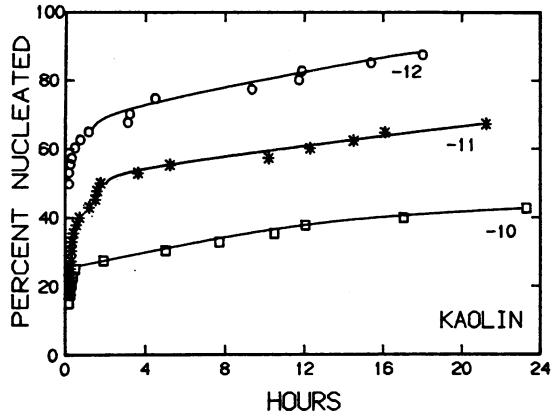

FIG. 2. Effect of time on the nucleation of 1% (w/v) suspensions of kaolin. Test tubes containing 1 ml of suspension were held at constant temperatures of −10, −11, and −12°C for 24 h. The proportion of samples frozen over time was determined using computer interfaced thermal analysis.

Table III. *Influence of Ice Nucleation Active Bacteria on the Temperature at which Plants are Nucleated*

Greenhouse grown seedlings (2.0 ± 0.7 g fresh weight) were sprayed with either a suspension of 10^8 cells/ml of *P. viridiflava* or with sterile deionized H_2O. Plants were incubated at 22°C ± 2 and high humidity for 48 h. Intact seedlings were cooled at −1.5°C/h in an incubator and nucleation temperature was determined by computer interfaced thermal analysis. There were between 11 and 15 replicates per treatment.

	Control	Inoculated
	mean nucleation temperature, °C ± SD	
Bean	−3.7 ± 0.8	−3.1 ± 0.2
Corn	−4.7 ± 0.9	−3.1 ± 0.4
Cotton	−5.2 ± 0.6	−4.0 ± 0.8
Soybean	−4.8 ± 0.6	−3.3 ± 0.5

smaller the tissue mass tested, the lower the ice nucleation temperature (Table I). Small droplets of homogenized tissues and leaf discs supercooled to −10°C, whereas larger volumes of tissue homogenate and intact plants froze at warmer temperatures.

The effect of sample mass was also observed when varying amounts of soil suspensions were frozen. The median ice nucleation temperature of 10 μl droplets containing 0.2 mg soil suspension was −6°C and the warmest drop of the 200 examined froze at −5.2°C (Table II). As the amount of soil in the sample increased, the median nucleation temperature rose and was −1°C for 100 g samples. Autoclaved soils were less efficient ice nucleators than control soil, but this difference diminished as sample mass rose (Table II). It was observed with both the soil and the herbaceous plants that the nucleation temperature of small samples did not accurately predict the nucleation temperature of larger samples (Tables I and II).

When bean, corn, cotton, and soybean seedlings were held constant at −4 or −5°C, the number of plants which froze increased with the length of exposure (Fig. 1). Plants were routinely observed to freeze after being held for 5 h at a constant temperature (Fig. 1). The nucleation rate was faster at −5 than at −4°C. The increase in the number of samples frozen over time did not appear to result from slow temperature equilibration, fluctuations in sample temperature, or the slow propagation of ice. Plant temperatures were monitored throughout the 24 h period and plants rapidly equilibrated to the test temperatures and deviated little (±0.1°C). In addition, once freezing was initiated ice rapidly spread throughout the tissue.

Time dependent ice nucleation was also observed with sterilized suspensions of kaolin. When samples were incubated at constant temperatures of −10, −11, and −12°C, additional nucleation events were observed with time (Fig. 2). As with the plant tissues, the nucleation rate was faster at lower temperatures. These observations demonstrated that time dependent ice nucleation was not just a property of biological systems which may have adapted in response to low temperatures. In addition, the phenomenon was observed under different experimental conditions so that the observations would be less likely to be artifacts of experimental design.

The effect of inoculation with ice nucleation active bacteria

Table IV. *Effect of Surface Moisture on the Temperature at which Ice Formation was Initiated in Seedlings*

Greenhouse grown seedlings (2.1 ± 0.8 g fresh weight) were used. Intact plants were cooled at −1.5°C/h in an incubator. The soil and root systems were placed in Dewar flasks to prevent freezing. In the other treatments, the above ground portions of the plants were excised and either placed in test tubes containing 25 ml sterile deionized H_2O or homogenized in the same volume. Nucleation temperature was determined by computer interfaced thermal analysis. There were at least 10 replicates/treatment.

Treatment	Bean	Corn	Cotton	Soybean
	mean ice nucleation temperature, °C ± SD			
Control	−4.3 ± 0.7	−4.5 ± 1.8	−5.2 ± 0.8	−5.0 ± 1.0
Misted	−1.2 ± 0.4	−3.8 ± 1.9	−1.4 ± 0.1	−1.8 ± 0.8
Submerged	−5.7 ± 0.3	−5.5 ± 1.3	−5.5 ± 0.8	−7.0 ± 0.1
Homogenized	−6.1 ± 0.2	−6.6 ± 0.5	−5.9 ± 0.6	−6.0 ± 0.4

on plant nucleation temperature was also examined in bean, corn, cotton, and soybean seedlings. In each species, plants inoculated with INA[1] bacteria froze at warmer temperatures than control plants (Table III). Cultures from a subsample of half the plants examined demonstrated that 80% of the inoculated seedlings harbored populations of INA bacteria active at −3°C and all inoculated seedlings harbored bacteria active as ice nuclei above −5°C. In contrast, none of the control plants harbored detectable populations (600 cells/plant) active above −5°C and 70% did not harbor detectable populations active above −9°C (data not presented).

Seedlings covered with a fine mist of sterile deionized H_2O froze at warmer temperatures than control seedlings (Table IV). Differences of approximately 3°C were observed with cotton, bean, and soybean seedlings relative to the controls. The effect on corn seedlings was not as great. Temperature measurements confirmed that the effect of leaf surface water was not a reflection of the leaves cooling well below air temperature via evaporative cooling (data not presented). The effect of leaf wetness was greater than that observed by inoculating tissues with INA bacteria (Tables III and IV). The effect of misting could not be duplicated by putting the entire above ground portion of a seedling under water or by homogenizing the tissue in deionized H_2O (Table IV).

In the field, the temperature of plant stems was between −1 and −3.7°C when ice formation was initiated (Table I). Each of the four species behaved similarly and a mean nucleation temperature of −2.6°C was observed. A subset of seedlings was examined for INA bacteria and none active above −9°C were detected on the plants used for field experiments. Even though seedlings were quite small, plant temperatures were not uniform under field conditions. Leaves tended to be cooler than both the stem and the surrounding air temperature except under cloudy or windy conditions. These differences were variable throughout the evening. Differences of 0.5 to 1°C were routinely observed and differences of 2°C were observed over short intervals. We observed that on calm, clear evenings, plant temperatures fluctuated rapidly and there were numerous instances when plant temperature dropped below the final nucleation temperature prior to freezing.

DISCUSSION

The mass of a sample had a marked effect on ice nucleation temperature. Small tissue samples such as drops of homogenized leaf tissue or leaf discs supercooled to much lower temperatures than intact plants prior to freezing. The current results corroborated our earlier observations with peach shoots (5, 6) and tomato plants (2). The combined data clearly demonstrated that small tissue samples do not accurately predict the freezing behavior of intact plants. The only exception occurred when samples harbored significant populations of INA bacteria (2). The observed effect of sample mass on ice nucleation was not unique to plant tissues. Small soil samples did not freeze at the same temperature at which larger samples nucleated ice. Experiments with 200 10 μl droplets of soil suspension demonstrated a median nucleation temperature of −6°C and detected no nuclei active at temperatures above −5°C. However, 100 g soil samples initiated ice formation just below 0°C.

Two explanations for the effect of size on nucleation temperature exist. One, ice nuclei were infrequent and unevenly distributed within samples. Therefore, the probability of a large sample containing an ice nucleus active at warm temperatures was much greater than that of a small sample. The second possibility was that ice nucleation was a function of probability and that the larger the sample mass, the greater the probability that a nucleation event would occur. Regardless of which explanation accounted for these observations, it is clear that predictions of plant nucleation temperature based on small tissue samples would not be valid and would overestimate the extent of supercooling. Therefore, reports that certain plant species lacked intrinsic ice nuclei active above −5 to −10°C which were based on small tissue samples may need to be reexamined.

Much of the recent work on ice nucleation in plants has been based on Vali's treatment of the singular hypothesis (16–18). That is, ice nucleation temperatures were characteristic of the single most effective ice nucleus present within a sample. Determinations of plant nucleation temperature have been made without regard to cooling rate, and time has not been considered to be of critical importance in the expression of ice nuclei. Vali (16–18) noted that temperature had a far greater role than time on the ice nucleation of water droplets. He did observe, however, an increased number of samples freezing with time when held at a constant temperature and an influence of cooling rate on droplet nucleation (16, 18).

In the present study, a time dependency for ice nucleation of bean, corn, cotton, and soybean seedlings was observed. When seedlings were maintained at either −4 or −5°C, the number of seedlings which froze increased with time. The later freezing events were not due to ice forming in potting soil and spreading into the roots. Precautions were taken to maintain soil temperatures above 0°C and soil temperatures were monitored throughout the experiment to verify this. The effect of time on nucleation in biological samples had been noted previously. Studies with bean seedlings (7), citrus (19), tomato (2), and peach shoots (6) all demonstrated an increased proportion of samples freezing when tissues were maintained at constant subzero temperatures. Salt (14) made similar observations with insects and noted that nucleation rate resembled the compound interest law.

Experiments were conducted with suspensions of kaolin to determine whether the time dependency of ice nucleation was the result of a biological adaptation of the ice nucleating site with time or was a general feature of ice nucleation. Our results clearly

[1] Abbreviation: INA, ice nucleation active.

demonstrated the effect of time on ice nucleation of kaolin suspensions. Therefore, although temperature had a greater effect on ice nucleation than the length of exposure, time was significant. Therefore, it seems appropriate that both cooling rate and the length of exposure to subzero temperatures be considered when designing experiments to determine plant freezing temperatures.

These results support a modified singular hypothesis to describe ice nucleation (18). Ice nuclei have a characteristic temperature range over which they are active. However, the occurrence of a nucleation event within that range is stochastic. The probability of ice nucleation would be very low at the warm end of the range but would increase as temperature decreased. Therefore, ice nucleation would be primarily a function of temperature. However, at temperatures where the probability of nucleation was low, increased exposure time increased the chance of a nucleation event occurring. Large sample masses would increase both the probability of a nucleation event, and the probability that ice nuclei active at warmer temperatures would be present.

Further evidence that plant freezing fits a modified singular hypothesis comes from field observations. We observed fluctuating air and plant temperatures under natural conditions and noted on several occasions that plants became colder than the ultimate nucleation temperature prior to freezing. If nucleation fits a strictly singular hypothesis, ice formation would have been initiated at the first instance when the critical temperature was reached. Instead, plants were observed to become cooler, warm up, and then freeze at a later time.

The temperature at which plants freeze can be affected by other biotic and abiotic factors. Inoculation of seedlings with populations of INA bacteria raised the nucleation temperature. Similar results have been reported with a variety of herbaceous plants (1, 3, 8, 9). The effectiveness of bacteria as ice nucleators can be influenced by the species (1, 8, 11), bacterial strain (8, 11), and preconditioning environment (1, 9).

Plants covered with a fine mist of water froze at warmer temperatures than did controls. This treatment was thought to be analogous to the condensation of dew on leaf surfaces during a radiative frost. The effect of misting was greater than that observed by inoculating plants with INA bacteria. Thomas and Barber (15) have noted similar effects of leaf surface wetness on the freezing of *Eucalyptus urnigera*. Surprisingly, submerged seedlings and homogenates of entire seedlings did not freeze as warm as the misted plants. The reason for this was not readily apparent.

In conclusion, the complexity of factors influencing ice formation in plant tissues under field conditions make it difficult for laboratory experiments to predict field performance. In the laboratory, temperatures are stable, cooling rates controlled, and plants are cooled by advective cooling so that the entire plant is the same temperature. In contrast, under field conditions temperatures are variable and can change rapidly. Depending on exposure, wind speed, and cloud cover, the temperatures of tissues within the same plant can differ by as much as 1 to 2°C. Since cooling rate, exposure time, dew, and plant temperature all affect nucleation temperature, simulating frost conditions will be difficult.

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