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Factors Influencing Freezing of Supercooled Water in Tender Plants[1]

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ABSTRACT

Seedlings of beans (*Phaseolus vulgaris*), corn (*Zea mays*), and tomatoes (*Lycopersicon esculentum*) were grown in the greenhouse and then exposed to controlled freezing conditions in a growth chamber. Variables were adjusted to determine the influence of plant water potential, freezing time, and external dew formation on the seedlings' susceptibility to frost injury. Freezing, detected visually and by release of latent heat, progressed rapidly throughout plants with high water potential and was always lethal. Spreading of the ice phase was impeded in plants with low water potential. In this case, the freezing injury appeared as spots on the leaves which gradually enlarged to encompass the entire leaf as the exposure continued. In general, the plant water supercooled before freezing. Supercooled water within the plant appeared to be internally nucleated if the leaf temperature remained above th atmospheric dewpoint temperature. Under these conditions root temperature, plant water energy, and duration of the freezing period all influenced the stability of the supercooled water. On the other hand, external inoculation prevailed when the freezing temperatures were accompanied by condensation of water from the air and subsequent formation of ice crystals on the leaves. One important exception was noted when ice on corn seedling leaves failed to nucleate the supercooled internal plant water with a potential of —18 bars.

Additional Key Words: **Microclimate, Plant survival, Freezing temperatures, Plant water potential.**

WHILE freezing damage to plants has been studied extensively during the past 50 years, most of the emphasis has been directed toward plants that have the ability to cold-harden, particularly biennials and perennials which survive low winter temperatures in a dormant stage (12). An equally important problem is frost damage to growing, nonhardened plants. Frost damage to seedlings or immature crops results in serious economic losses. This phase of the frost problem has received relatively little research aside from air temperature control which may be attempted for orchards and other specialty crops during periods of unseasonably cold weather (10).

Growing plants may survive freezing temperatures in two ways. The water in the plant may supercool and not form ice crystals, or the plant may tolerate some ice crystals without having a significant number of cell membranes ruptured (6). Single (15) reported that wheat plants could, under certain conditions, be kept at temperatures of —3 to —5C "almost indefinitely" without having ice form in the tissue, provided there were no ice crystals in the external environment. When ice crystals do form in the tender, growing tissue of many plants, the cells rupture and die. However, Olien (13) has postulated that some plants contain polysaccharides which impede the growth of ice crystals so that the plant cells are not all killed. It has been reported that the application of some chemicals may increase the tolerance of tender plants to frost (9). There is essentially no information on how protective mechanisms interact with the natural physical environment around plants, particularly with respect to the stability of supercooled water in the plant. There is an immediate need for a basic understanding of what does occur during periods of light frost.

METHODS

'Pinto' beans *(Phaseolus vulgaris)*, 'Green Giant code 20' sweet corn *(Zea mays)*, and 'Sioux' tomatoes *(Lycopersicon esculentum)* were grown in the greenhouse (9- to 12-hour day length) in 4-liter pots of silt loam soil. Eight to 12 corn or bean plants and about 20 tomato plants were grown per pot. The corn and bean plants were allowed to reach a height of 15 to 20 cm, and tomatoes a height of 8 cm before they were exposed to various cold stress conditions in the growth chamber.

Prior to the freezing experiments, plants were preconditioned in the growth chamber for a minimum of 40 min in the dark at 20C to induce closing of stomata. The air temperature was then lowered at a rate of 0.5C/min to various specified points from —2 to —5C. Air temperatures in the growth chamber and leaf surface temperatures were measured with 127 μm copper-constantan thermocouples connected to a multipoint strip chart recorder. Thermocouples were kept in contact with the leaves by inserting the tip of the thermocouple into the leaf tissue. Air temperatures in the darkened growth chamber cycled $\pm$ 0.7C and plant leaf temperatures closely followed this cycling. Humidity in the chamber was not controlled, though it was normally about 80% relative himidity when temperatures were lowered to the freezing range. The low water-vapor pressure was a result of the condensation and freezing of vapor on the cooling fins as the air was recirculated in the chamber. A frost layer less than 1 mm thick formed on the cooling fins within a few minutes after the temperature reached zero and thereafter no change was observed, indicating there was not a gradual buildup of ice crystals which could break loose and be circulated in the air flow.

Following exposure to freezing temperatures, plants were allowed to warm to 20C and were returned to the greenhouse. The percentage of fatally frozen plants in each pot was recorded the following day. Each treatment was replicated in at least five pots with each pot treated as one random replicate and the results were statistically analyzed with the Duncan multiple range test. Differences between treatment means of 20 to 25% and 26 to 30% were generally required for significance at the 5% and 1% levels, respectively.

When needed, two identical growth chambers were used simultaneously and temperatures were monitored continuously with the same multipoint recorder. In some experiments, the pots were insulated by wrapping the outside in aluminum foil and by covering the soil surface with approximately 2 cm of dry vermiculite. Plant water potential was measured (2) just prior to initiating the freezing period. The soil moisture treatments were grouped into two general categories, wet and dry. Dry indicated that the surface appeared dry and pots had not been watered for at least 2 days. Wet indicated a moist soil surface which had been irrigated within the previous 12- to 24-hour period.

The preceding description of methods applies to all of the experiments in which the survival of corn, beans, and tomatoes are reported. The individual treatments in each experiment are pointed out as the results are discussed. Observations of ice formation in sugarbeets *(Beta vulgaris)*, peas *(Pisum sativum)*, alfalfa *(Medicago sativa)*, and lettuce *(Lactuca sativa)* were made in the growth chamber on individual potted plants rather than on plants in complete replicated designs. However, the observations were repeated from three to five times to give confidence in their validity.

Separate experiments were also conducted to measure supercooling and the spontaneous freezing point of the intact plant leaves. Thermocouples were inserted in the leaves and the

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chamber temperature lowered from 20C at a rate of about 0.25 degree per minute until freezing was detected by latent heat release in the leaves.

RESULTS AND DISCUSSION

Supercooling and Internal Nucleation of Plant Water

Preliminary observations suggested that beans with leaf temperatures of —2 to —3C could be kept in the chamber for at least 2 hours before any ice formed. For periods longer than 3 hours, the number of plants having the ice phase slowly increased. After 24 hours, most plants were frozen. The difference in color intensity between frozen and unfrozen leaves made it possible to detect the initiation and progress of freezing. This method of detection was verified by measurements of latent heat release and by the immediate death of frozen tissue upon thawing.

It was noted on recently irrigated bean plants that once freezing began somewhere in the plant, it progressed through the entire plant top within a few seconds. On the other hand, if the soil was dry and the leaves were visually suffering from water stress, small frozen spots appeared on the leaves and the spread of ice through the plant was slowed. If the plant was removed from the chamber within a few minutes after spots of frozen tissue were observed, the plant could survive but the injured cells became watery, necrotic, and died. A similar spotting of citrus leaves has been reported by Young and Peynado (17).

These observations suggested that when bean plants with a high water potential survived freezing temperatures, their water supercooled and ice crystals did not form. Consequently, the question arose as to what conditions caused ice nucleation in supercooled plant water. The stability of supercooled water is a phenomenon which is not presently understood. It is known that pure water may be stable in the liquid phase below a temperature of —20C (3). When ice nucleation occurs at temepratures above —10C, it is generally believed to be associated with the catalytic-type surface reaction of some dissolved or suspended particle (1, 3).

Internal Nucleation or External Inoculation

Because bean plants with a high water potential froze randomly, it appeared that environmental factors such as airborne dust particles or ice crystals might occasionally come in contact with the leaves and inoculate the supercooled plant water. If this were the case, the degree of stomatal opening could affect the chances of inoculation. Gates (5) reported that temperatures near freezing may prevent opening of stomates in light. If stomates do not respond rapidly to changes in light at low temperatures, they could remain open at the onset of a freeze period and allow greater opportunity for inoculation of supercooled plant-water than if stomates were closed. To test this possibility, two growth chambers were set to precondition the plant stomates so that they would be open (lighted chamber) or closed (darkened chamber) for 20 minutes before imposing the freezing temperatures. Cellulose acetate negatives were made of leaves following this conditioning period and examined with a microscope. After the onset of freezing in the dark, there was no evidence to indicate that the stomatal opening in leaves of the beans, corn, or tomatoes was affected differently by the pretreatments; nor did either pretreatment cause a different response to freeze damage at the 1% significance level.

To test the hypothesis that nucleation might occur randomly from external contact with airborne particles, experiments were conducted simultaneously with two growth chambers. In one chamber, plants were subjected to a continuous freeze lasting 10.5 hours. Plants in the second chamber were also subjected to a total freezing time of 10.5 hours, except that every 1.5 hours the temperature was raised to + 2C under full light for a 5- to 7-min period. Short breaks in the freezing cycle generally favored survival of the plants (Table 1). This was also true when the treatments were reversed in the two growth chambers. Since the frost accumulation on the heat exchanger was constant after the first few minutes of cooling, the density of airborne ice particles, if present, should have been similar in the two chambers. Thus, if ice nucleation was from an external source, the survival in the two chambers should have been the same. It appears that, under these conditions where the relative humidity was always less than 100% so that no water condensed on the leaves, nucleation was occurring inside the plant and the plants' susceptibility to nucleate was affected by the intermittent warming. This concept of internal nucleation of supercooled plant water is in agreement with studies reported by Kitaura (8), Modlibowska (11), and Kaku and Salt (7).

The distribution of the data summarized for beans in Table 1 also suggests that there was some internal control over nucleation. While the plants with a high water potential seemed to freeze randomly with time, the plants with low potential froze pot by pot, i.e. in general either all the plants or none of the plants in a pot would freeze (Table 2). This would not be expected if nucleation were occurring from random contact with particles in the air unless the low-water potential plants were more resistant to external nucleation. If they were more resistant, they should have had the highest survival.

Table 1. Plant fatalities caused by 10.5 hours of intermittent or continuous exposure to freezing temperatures with the relative humidity less than 100%.

Plants	Soil treatment	Temp.	Plant water potential	Fatalities* Intermittent	Fatalities* Continuous
		C	bars	%	
Beans	Dry	-2.5	-10.0	57 ef	100 f
	Wet	-2.5	- 6.5	45 e	75 ef
Corn	Dry	-4.0	-13.5	93 c	95 c
	Wet	-4.0	-11.4	5 a	29 b
Tomatoes	Dry	-4.0	-12.2	29 b	33 b
	Wet	-4.0	-11.0	31 b	94 c

* Corn and tomato data followed by the same letter are not significantly different at the 1% significance level. Because of different temperatures, the bean data are not comparable to the corn and tomatoes.

Table 2. Details of bean plant fatalities summarized in Table 1, showing the number of bean plants killed in each of seven pots with eight plants in each pot.

Treatment	Plant water potential	Plants killed in each pot I	II	III	IV	V	VI	VII	Mean
	bars								%
Intermittent	- 6.5	3	5	1	5	3	3	4	45
Intermittent	-10.0	8	0	8	0	0	8	8	57
Continuous	- 6.5	7	8	7	4	4	6	7	75
Continuous	-10.0	8	8	8	8	8	8	8	100

Plant water potential also affected the time of freezing. Three hours after the onset of freezing, the bean plants with high water potential were beginning to show the first instances of ice formation, and from then on, the number of frozen plants slowly increased with time. On the other hand, the low-water potential plants did not show any nucleation until 5 hours after the onset of freezing temperatures. This type of differences was also observed during other trials. Consequently, when a freeze was terminated after 3 to 5 hours, the effect of plant water potential tended to be opposite to that found in freezing times greater than 6 hours, provided the relative humidity was less than 100% so that ice crystals did not form in the air or on the leaf surface.

It is possible that the effects attributed to plant water potential are more directly a result of water content. This could presumably be studied by growing the test plants under different salinity levels.

In 1948, Dorsey (3) reported his studies of the supercooling of various solutions. He found that solutions had widely varying but relatively stable supercooling temperatures. The supercooling temperature appeared to depend upon the type of impurities dissolved or suspended in the solution. Nucleation was not caused by shock or movement of the solution, though slippage of two surfaces lubricated by a film of supercooled solution would cause ice formation. In general, he noted that decreases in spontaneous freezing temperatures were approximately proportional to increases in freezing point depression when salts were added to the solution. The freezing point of a solution decreases approximately 1C per 12 bars of osmotic potential. Anderson (1), using clay, also showed that the supercooling increased with decreasing water potential, however our preliminary studies did not show any reproducible correlation between moderate plant moisture potential (—5 to —15 bars) and supercooling temperatures. There was also no apparent relation between spontaneous freezing points and the differences between freezing tolerance of the plant species studied. This can be seen from the results in Table 3. Comparison of Tables 1 and 3 shows that the supercooling stability is time-dependent because some plants did eventually freeze at temperatures higher than those shown in Table 3. Dropping ice crystals on the leaves decreased the stability, while dropping sand grains on the leaves had no effect.

There is an important difference between the effect of pressure and the effect of solutes on the freezing point depression of a solution. Increasing the salt concentration in a solution lowers both the chemical potential of the water and the freezing point. On the other hand, increasing the pressure on the same solution decreases the freezing point but increases the water's chemical potential (4). This may be important in plants, since the pressure inside a turgid cell may be several bars positive, while the pressure in the conductive tissue may be several bars negative. If the supercooling stability depends upon the freezing point of the solution, one would expect the water in the vascular system to be more subject to nucleation than the water in the cell. It has been observed that ice crystals first form in the extracellular spaces (12) and then probably spread through the vascular system (6).

Table 3. Minimum supercooling temperatures of plants when cooled at a rate of 0.2 to 0.3C/min with the relative humidity less than 100%.

Plant	Number of samples	Temperature: Mean supercooling	Temperature: Variance of mean
Beans	26	-6.7	±0.2
Beans (iced at -2C)	22	-3.3	±0.2
Beans (sanded at -2C)	6	-6.7	±0.3
Corn	11	-9.3	±0.2
Tomatoes	16	-5.1	±0.2

One would expect that increasing the water pressure in the extracellular spaces might increase the stability for supercooling. Bean plants with their cut-stem ends submerged in water in Dewar flasks were much more resistant to freezing than the check high-water potential plants growing in soil. However, when checking for stem temperature effects, a similar resistance to nucleation occurred in corn and beans when the pots and soil surface were insulated (Table 4). It took about 2 hours longer for the base of the stem to reach 0C than it did for the air and leaves when the pot was not insulated. When the pot was insulated, it took about 4 hours longer. The wetter soils also took longer to cool than the drier ones. Thus, one explanation for the increased liquid phase stability in the plant could be that heat was conducted from the soil up the plant stem to the leaves. Temperature measurements did show that there was a thermal gradient in this direction; however, it was dissipated in the first 7- to 10 cm of stem above the soil surface. Leaf temperatures were not different from air temperature and thus were not measurably different for any plants, regardless of pot insulation. Another possible explanation could be that frozen soil at the base of the plants induced ice nucleation in the plant. No evidence was found that this occurred; in fact, water could be frozen around bean stems at the soil surface without inoculating the water in the remainder of the plant. It appears, then, that the temperature of the root system or the lower part of the stem somehow influenced the susceptibility to ice formation in the leaves.

External Inoculation of Plant Water

The growth chambers with relative humidities below 100% were not representative of all field conditions. While it is possible, at least in arid regions, to have leaf temperatures drop below freezing without reaching the dewpoint of the air, it is also possible to have a heavy condensation of dew and subsequent frost formation as plant temperatures drop below 0C. A number of trials were run in which dew or ice crystals were formed on plant leaves in the growth chamber (Table 5). In general, when water was sprayed

Table 4. Effect of pot insulation on frost damage when the relative humidity is less than 100%.

Treatment	Plant water potential	Fatalities*
	bars	%
Beans - 5.5 hours at -2.5C		
Not insulated, dry	-11.8	100 c
Not insulated, wet	- 8.0	64 b
Insulated, wet	- 8.0	0 a
Corn - 6 hours at -2.5C		
Not insulated, dry	- 6.5	76 b
Not insulated, wet	- 6.4	38 a
Not insulated, dry	-14.3	94 b
Insulated, dry	-14.3	17 a

* Data followed by the same letter are not significantly different at the 1% significance level.

Table 5. Plant fatalities caused by 5 hours at —4.5C. Water droplets or snow crystals were placed on the treated leaves 2 hours before the end of the freeze.

Plant	Treatment	Soil appearance	Fatalities*
			%
Tomatoes	Water drops	Dry	96 a
	Water drops	Wet	100 a
	No inoculation	Wet	31 b
Corn	Snow	Wet	32 a
	No inoculation	Wet	8 b

* Data within each crop set followed by the same letter are not significantly different at the 1% significance level.

Table 6. Plant fatalities caused by external nucleation with snow.

Plants	Soil appearance	Plant water potential	Fatalities*	
			Insulated	Not insulated
		bars	%	
Beans	Wet	- 8.8	100 d	100 d
	Dry	-12.0	90 cd	50 b
Corn	Wet	- 8.5	86 cd	72 bc
	Dry	-19.0	0 a	0 a
Tomatoes	Wet	-11.8	--	93 c
	Dry	-13.2	--	68 bc

* Data followed by the same letter are not significantly different at the 1% significance level.

with an atomizer on high water potential leaves at temperatures below 0C, there was a sharp decrease in survival when compared to nonsprayed plants.

Because water droplets sprayed on plant leaves sometimes supercooled without freezing or evaporated before freezing, more consistent results could be achieved by sprinkling snow or other types of frost crystals directly on the leaves. The results of one such experiment are summarized in Table 6. These plants were held at —3C for 4.5 hours. One hour before the freeze ended, a light covering of snow was sprinkled over the leaves and pots.

The beans at a plant moisture potential of —8.8 bars were all killed, while the —19-bar corn was not damaged. All other plants in this experiment showed some damage, so that the data given for percent fatalities were somewhat more subjective than in the experiments on internal nucleation. For example, the difference between the insulated "wet" beans and insulated "dry" beans was striking. While all of the "wet" beans were completely killed, many of the beans growing in "dry" soil would have eventually produced regrowth and matured if given sufficient time. However, the extent of damage to these plants was so high that the potential commercial production was greatly reduced and so treatment was rated as 90% lethal.

In general, insulating the pots did not have much effect on survival of the plants when they were nucleated externally. However, the water potential in the plant at the time of nucleation made a large difference because, in the case of beans and tomatoes with low water potential, the ice spread so slowly through the tissues that after an hour much of the plants water was unfrozen. The effect of water potential (or water content) on the internal spreading of ice crystals may be related to the work of Single and Olien (16) showing morphological characteristics in the nodal region of some plants which tend to slow the spread of ice. A low water potential may increase the effectiveness of any ice growth barriers in the vascular system (6).

The corn, under a water potential of —19 bars, was not inoculated at all even though snow lay on the leaves for 1 hour at a temperature of —3C. In separate studies using corn with low water potential, it was noted that even when water droplets were placed on the leaves and frozen, the water inside the leaves did not nucleate. On the other hand, if the leaf was cut and ice brought into contact with the wounded area, nucleation occurred readily. The low water potential corn leaves were also quite hydrophobic to liquid phase water as well as being effective barriers to ice nucleation. Salt (14) has discussed some of the possible mechanisms involved in biological barriers to ice nucleation.

Ice Formation in More Resistant Plants

In all cases with the corn, beans, and tomatoes, when ice crystals did form in the growing tissue, the tissue was killed, resulting in either death of the entire plant or spotting and burning of leaves and growth tips. This was not true in the case of other growing plants observed in the freezing chamber. For example, alfalfa, sugarbeets, lettuce, and peas tolerated extensive ice crystal formation inside the tissue without showing any immediate damage, as long as temperatures did not drop below —5C, and as long as no mechanical pressure was applied to the tissue while it was frozen. The spreading of ice in the tissue in these plants was observed by cooling the plant below its freezing point, then inserting a small ice crystal through the leaf. Visible changes in the leaf then followed as the ice phase spread through the tissue. Spreading of ice through the tissue in these more tolerant plants was always slower than the almost explosive reaction which occurred in the turgid bean leaves, possibly because of the existence of some polysaccharide (13) which modified the crystals so that they did not rupture adjacent cells.

CONCLUSIONS

Some kinds of growing plants, such as lettuce, peas, alfalfa, and sugarbeets, will tolerate some ice crystals without cell damage, while other plants, such as corn and beans, suffer cell death wherever ice has formed. The temperature of the plant in relation to the atmospheric dew point becomes very important when studying plants which cannot tolerate internal ice (Table 5).

Plant Temperatures Above the Atmospheric Dew Point

If air temperatures are below the plant water freezing point and the dew point of the atmosphere has not been reached, ice nucleation appears to occur internally in the plant. Under these conditions, warm root temperatures reduce the chance of nucleation. Periodically raising the temperature above freezing for a brief period also reduces the incidence of internal nucleation. The plant water potential influences the pattern of internal nucleation. In general, when the temperatures are between —2 and —5C for 6 hours or more, the plants with high water potential in wet soil tend to show a higher rate of survival than drier plants. When the freezing period is 4 hours or less, the reverse may be true.

Plant Temperatures Below the Atmospheric Dew Point

When the atmospheric dew point has been reached so that water and/or frost is condensed on the plant leaves, responses to freezing temperatures are quite different. Water in growing plants will be readily inoculated from the exterior, and the higher their water potential, the more quickly ice will form throughout the tissue. Because of a slower internal ice spreading rate, the plants with lower water potentials show greater survival provided ice crystals are not in contact with the plant's exterior surface for more than 1 or 2 hours. After this length of time, the ice crystals will have spread through the plants, resulting in death of susceptible species. One exception is high water-stressed corn seedlings, which have some leaf surface property which prevents inoculation by ice from the exterior surface.

Even though low water potential favors plant survival under some conditions, irrigation for frost control in the field should still be utilized because of its effectiveness in buffering air temperatures.

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