Freezing Patterns in Twigs of Evergreen Azalea[1]

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Abstract. An electrophoretic mobility technique utilizing molecular probes was employed to study the freezing patterns in apical stems of 2 azalea cultivars, *Rhododendron yedoense* var. *poukhanense* (Levl.) Nakai (unnamed clone termed 'Poukhanense' herein for convenience) and *R.* x 'Maryann' [*R. indicum* x *(R. yedoense* var. *poukhanense* x *kaempferi)*]. Excised twigs were frozen at 2°C per hr during which current and voltage were measured, and tissue resistance was calculated. As freezing occurred there was a dramatic increase in tissue resistance, indicating that ice quickly filled the extracellular space. This rapid ice formation is a nonequilibrium process independent of temperature change once nucleation has begun. Injury due to the rapid formation of extracellular ice, at temperatures well below the freezing point, may be a factor influencing winter survival.

Low temperature injury to plants is by no means a clear cut phenomenon. Injury can be mechanical and/or physiological. Mechanical injury results when ice crystals form intracellularly or when the quantity of extracellular ice is sufficient to cause splitting or other physical disruption (5, 14). Most explanations of injury to hardy plants are based on physiological phenomena which are the direct result of extracellular ice crystal formation (6, 9).

Many techniques have been used to study injury in plant tissues prior to, during and after freezing. Some workers have observed the formation, location and effect of ice in an effort to explain nucleation and propagation patterns (15, 16), particular types of injury symptoms or tissue responses (4, 5, 8) and generic differences (1). Measurements of tissue bioelectrical properties have been used to determine intact plant viability (19), physiological changes induced by low temperatures (18), degree of injury (2), occurrence of physical changes (3) as well as the relationship between temperature and the content of extracellular liquid (11, 12, 13, 17).

Previous work showed that survival of azalea twigs was a function of tissue moisture content and resulting ice formation (8). Since physical stress during freezing can be directly affected by tissue moisture content and rate of ice formation, this investigation was designed to study the freezing process and ice formation in twigs of 2 azalea clones differing in hardiness.

To specifically evaluate stresses in plant tissues during freezing a technique utilizing molecular probes has been developed by Olien (11, 12). This technique is based on electrophoretic displacement of indicator molecules in the extracellular continuous liquid system of plant tissue. The mobility of indicator molecules is a function of film thickness, since it is inversely dependent on the effective viscosity and indicates the state of water in the electrophoretic pathway. Electrophoretic mobility, which correlates with electrical conductivity within lower limits of voltage and frequency when contact resistance is subtracted, can be continuously evaluated from electrical measurements in intact tissues during freezing and thawing. This technique assesses only physical response to freezing. Physiological phenomena leading to tissue injury are not directly evaluated.

Materials and Methods

A series of experiments was performed to evaluate Olien's method for azalea. There were no previous reports of this type of study with azaleas so the following facts were determined: 1) ability of twigs to withstand weak electric current; 2) effects of the measurement apparatus on the freezing pattern; 3) the path of current flow in the twig; and 4) the correlation between current flow and observed molecular mobility. These facts, in addition to freezing patterns, must be determined in order for the electrophoretic mobility technique to be a valid evaluation of freezing stress.

Freezing pattern experiments. Stems of 'Maryann' and 'Poukhanense', 10 cm long all with flower buds, were cut from acclimated plants held in a 1.5°C growth chamber. A test apparatus that would accommodate 2 twigs consisted of a hollow plexiglass cylinder which could be placed over a plasticized paper container fitted with a moist sponge. Each electrical contact consisted of a flattened platinum wire circularly shaped to 2 mm in diameter that was supported and pressed against the plant tissue by a coiled copper wire spring (Fig. 1). At the point of contact placement on the twigs a small slice of peridermal tissue 2 mm in diameter was removed and carbon-water paste was worked into these cuts with a small brush. The platinum contacts were moistened with the carbon paste and pressed

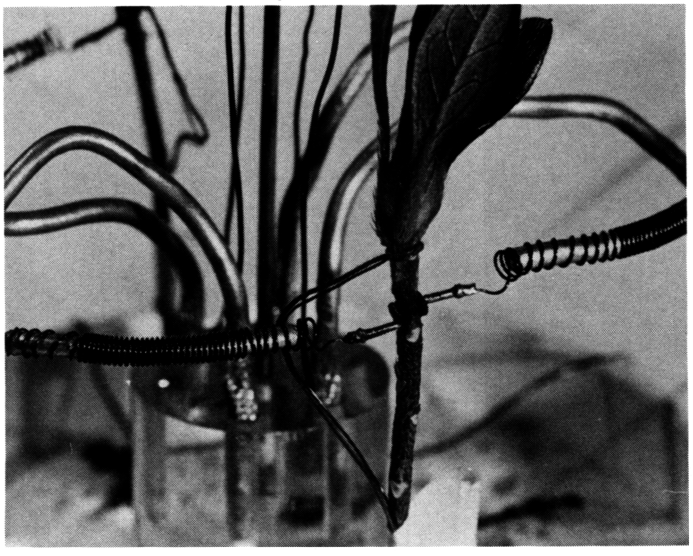

Fig. 1. Contact arrangement to determine freezing pattern.

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[4]Contact resistance was calculated by subtracting the total resistance of area C from that of areas A + B.

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against the twig cuts. Each contact point was ringed with silicone to prevent current flow around the external twig surface. A small thermocouple was attached to the twig just below the flower bud. These preparations were completed quickly in the freezing chamber at 1.5°C. An equilibration period of at least 2 hours at -1.5°C preceded each experiment.

For each experiment, contact wires were attached to a low-voltage, low-frequency (less than 5 cps) direct current power source. Two electrical measurement systems were used. The first system (Fig. 2A) utilized a selective sensitivity recording potentiometer for continuous temperature and current plotting. The second and more simplified system (Fig. 2B) replaced the recording potentiometer with a voltmeter and microammeter. Temperature in the latter system was recorded from a galvanometer.

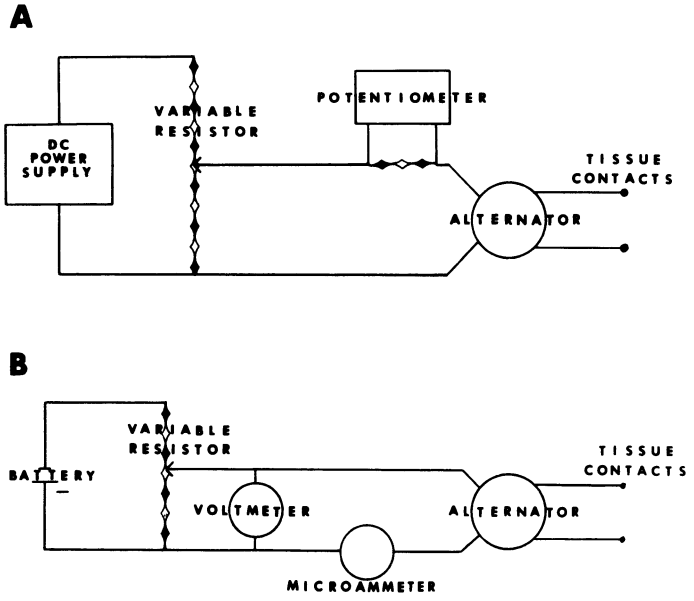

Fig. 2. Schematic diagrams of electrical systems used to determine freezing pattern, A. automatic and B. manual.

Contact resistance checks. Three apparatuses were prepared, 2 with a single 'Maryann' twig each, the third with a 'Poukhanense' twig. Three vertical contacts were placed on 1 side of each stem 14 mm apart (Fig. 3). Contact 1 was uppermost and placed 4 mm below the flower bud. Current flow between contacts 1-2, 2-3, and 1-3 (tissue areas A, B, and C respectively) was controlled manually. The electrical measurement system shown in Fig. 2B was used. Current and voltage readings were taken during the equilibration period, then the temperature was slowly lowered (2°C per hour) and additional readings were taken after the stem had frozen (-3°C). To reduce the amount of supercooling, which could alter the freezing pattern, a small drop of water was placed on a leaf adjacent to the flower bud and nucleated with a frozen probe. At the beginning of this experiment the voltage was recorded as a function of current from 0 to 9 microamps. Resistance was calculated from the formula V = IR.

Freezing patterns. One twig, each of 'Maryann' and 'Poukhanense' was placed in each of 2 apparatus using 2 contacts (Fig. 1) and the automatic measurement system (Fig. 2A). Temperature and current were recorded continuously during equilibration and cooling to -12°C.

Current path experiments. A negatively charged ion from amaranth dye was used to evaluate electrophoretic mobility and to serve as a visual indicator of the current path through stem tissue.

Dye flow in thin stem sections. A thin cross section of the apical stem through the contact point area was mounted in water-saturated mineral oil under a cover slip on a glass slide held on a microscope stage. A tapered strip of moistened cellulose paper touching the tissue extended from each side of the section beyond the cover slip. Each paper strip was fastened to a metal contact which connected to a voltage source that was regulated by a variable resistor to achieve a current level between 0 and 9 microamps. By using a switch, the current flow could be reversed. A small drop of amaranth dye was spotted on the negatively charged paper strip at various current levels. As the dye moved through the stem section, its path and relative rate of movement in different tissues were watched through a microscope. The experiment was repeated 4 times using stems of 'Maryann' and 'Poukhanense'.

Dye flow in intact stems. Dye flow was measured in the apparatus described earlier with a few modifications. One of the platinum contacts was coated as usual with the carbon paste. Before the second contact was coated with a diatomaceous earth paste, a small cotton wick leading to a reservoir of amaranth dye was positioned so it would be held between the stem and the contact. The upper leaves were removed from the twig, the leaf traces plus the entire bud were coated with silicone to reduce transpiration influences on dye flow. A current level of 9 microamps was maintained for 5 hours in one apparatus and for 20 hours in another, after which thin stem cross sections above, through and below the contact region were scrutinized under a microscope. A third apparatus complete with dye reservoir was prepared, but without a power source, to check dye flow as affected by diffusion and transpiration.

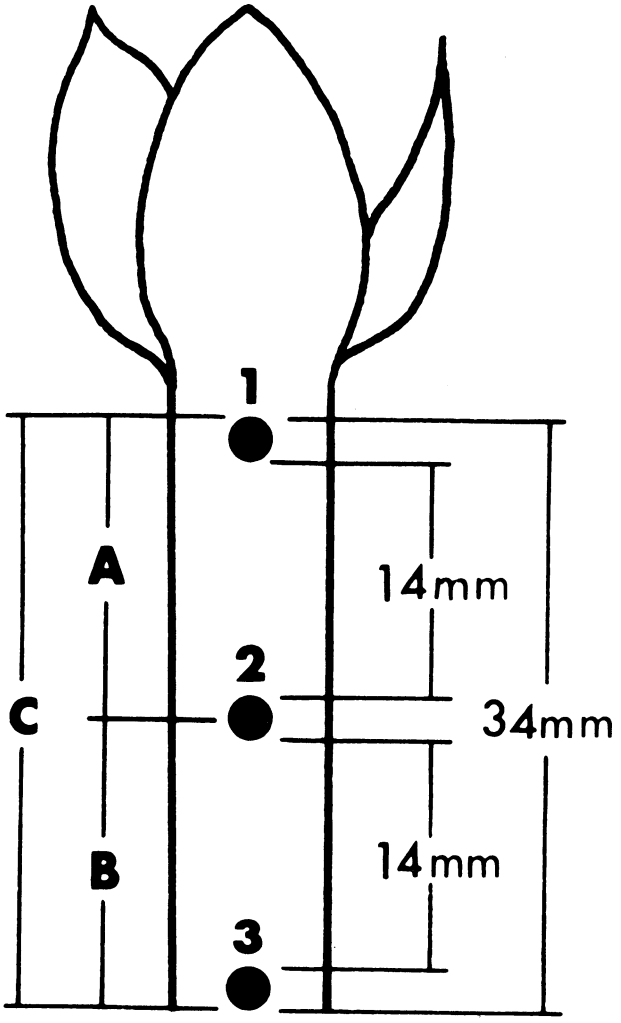

Fig. 3. Diagram of contact arrangement to test resistance.

Table 1. Electrophoretic mobility for tissue areas of 3 azalea twigs prior to (1.5°C) and after freezing (-3°C) at a constant current of 5μA. Data not corrected for contact resistance.

Tissue area[z]	Voltage			Resistance (X10^{-3} ohms)		
	A	B	C	A	B	C
'Maryann' #1						
Prefreeze	4.1	3.5	6.8	820.0	700.0	1360.0
Frozen	15.0	13.1	23.8	3000.0	2620.0	4760.0
'Maryann' #2						
Prefreeze	2.8	2.4	4.8	560.0	480.0	960.0
Frozen	11.0	10.8	19.0	2200.0	2160.0	3800.0
'Poukhanense' #1						
Prefreeze	2.0	1.2	2.6	400.0	240.0	520.0
Frozen	11.5	8.4	14.2	2300.0	1680.0	2850.0

[z]Tissue areas A, B, and C correspond to 3 vertical twig contact points spaced 14 mm apart.

Results

Contact resistance checks. Prior to freezing, the rate of current flow through twig tissue areas A (contacts 1-2), B (contacts 2-3) and C (contacts 1-3) fluctuated only slightly. As freezing occurred the current dropped sharply and the resistance increased markedly. These changes were characteristic of both 'Maryann' and 'Poukhanense' twigs (Table 1). After freezing there was no variation in current flow. The platinum contacts accounted for an appreciable part of the total resistance.[4] Prior to freezing, contact resistance was 28% and 24% for 'Maryann' twigs 1 and 2, and 37% for 'Poukhanense' twig 1. After freezing, contact resistances were 37%, 33%, and 56% respectively. Even though contact resistance was appreciable it could be accounted for and had no effect on the recorded freezing pattern. Contact width (2mm) had an insignificant effect on contact resistance calculations.

Within the current range we used, azalea twigs were unaffected since the current per volt relationship remained linear. Tissue resistance was thus independent of voltage applied.

Freezing patterns. A sharp drop in current and rise in resistance occurred with freezing (Table 2). Because of the contact configuration no accurate evaluation of contact resistance could be made. Therefore, contact resistance values were taken from the previous experiment, considering them to be no better than those for 'Maryann' twig 2, prior to and after freezing (126 and 722 x 10^{-3} ohms respectively). The relative content of liquid extracellular water (M_r) decreased rapidly in all twigs when freezing occurred (Fig. 4). Rapid ice formation without appreciable physical tissue disorganization has been termed nonequilibrium freezing (12).

Dye flow. The amaranth dye very quickly moved extracellularly around the stem in the outer cortex just beneath the periderm (Fig. 5). Dye flow could not be seen in the periderm because of its dark color. Within 45 seconds after moving into the cortex, the dye could be seen on the positive contact, having traversed the section via the cortex. Two minutes later the dye could be seen migrating inward into the vascular tissue and slowly moving toward the positive contact. When small notches were cut into the periderm, simulating the contact areas on intact stems, the dye flow was similar to that

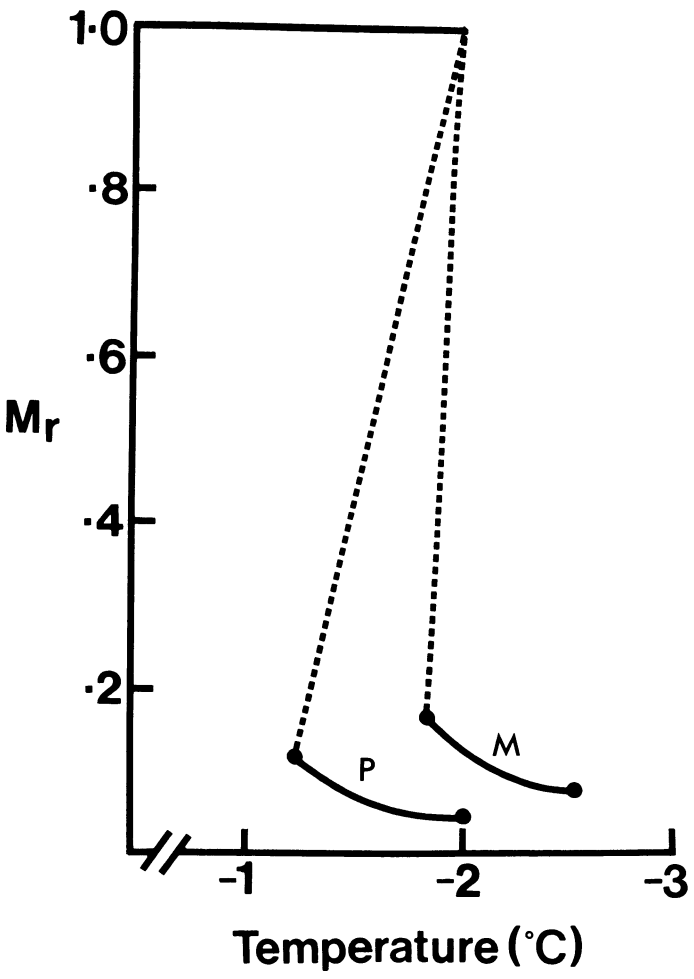

Fig. 4. Freezing patterns of 'Maryann' (M) and 'Poukhanense' (P) twigs. Pattern development represents less than 5 minutes. M_r, relative content of liquid extracellular water.

Table 2. Sample data (temperature & current) and calculation of M_r prior to and after freezing for 1 set of freezing pattern apparatus. The temperatures represent a time sequence not exceeding 5 minutes, with no external temperature change after freezing started

	Temperature (°C)	Current (μA)	Total resistance (X 10^{-3} ohms)	Tissue resistance[z] (X 10^{-3} ohms)	Corrected current[y] (μA)	Relative current (μA)	Relative viscosity of water	M_r[x]
'Maryann'	-2.0	1.44	167	41	.025	1.00	1.00	1.00
	-1.8	.23	980	258	.004	.16	1.02	.16
	-2.5	.18	1221	499	.002	.08	1.01	.08
'Poukhanense'	-2.0	1.71	145	19	.053	1.00	1.00	1.00
	-1.2	.26	880	158	.006	.11	1.03	.11
	-2.0	.18	1221	499	.002	.04	1.00	.04

[z]Total resistance minus average resistance for 2 contracts before and after freezing (126 and 722 x 10^{-3} ohms, respectively.

[y]1 ÷ tissue resistance.

[x]Relative content of liquid extracellular water (relative current x relative viscosity).

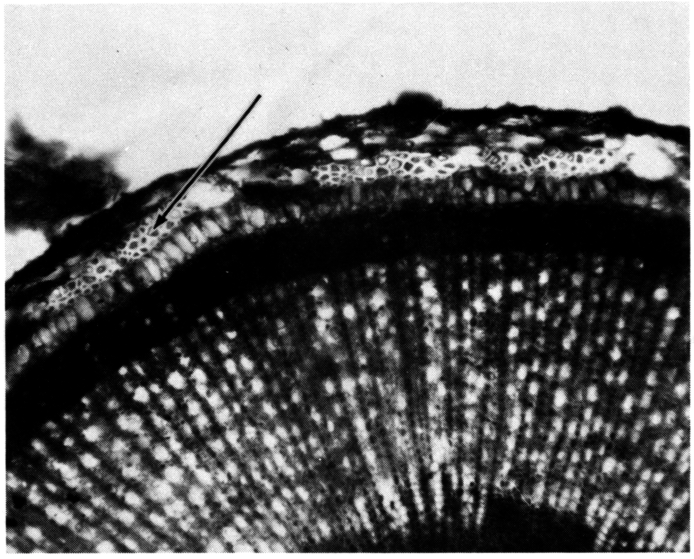

Fig. 5. 'Maryann' apical stem cross section from current path experiment showing paper strip contact. Arrow indicates primary path of dye movement (x 100).

in sections with intact periderm. As current flow through the section was reduced by one-half the dye flow was similarly reduced.

Amaranth movement within intact stems was similar to that observed in thin sections. Much dye had accumulated throughout the periderm and cortex after 5 hours. The vascular tissue was stained for less than one-half of the stem circumference. The periderm, cortex, and vascular tissue were very darkly stained after 20 hours. Vertical dye movement was less than 2 mm in both the 5- and 20-hour stems. In the stem without current no dye had moved laterally beyond the cortex or vertically in excess of 2 mm.

Discussion

The electrophoretic technique is useful to evaluate changes in extracellular water during freezing when conductivity correlates with mobility. Water redistribution with decreasing temperatures gives rise to freezing stresses. Stress is a function of the amount of water involved, the abruptness of freezing, the location of the process within the plant, and the freezing energy. Under nonequilibrium conditions freezing induces considerable physical strain and may cause tissue disruption.

Although the periderm and cortex were primarily responsible for the observed freezing patterns, earlier work (1) has shown that freezing patterns of these tissues are representative of the entire apical stem. Thus the freezing patterns of the azalea twigs were characteristic of living, unaltered tissues and not induced by the measurement apparatus.

As determined previously (1) the nonequilibrium freezing pattern in azalea twigs is the result of a limited interaction between cell wall and the water in the outer free space. Undefined structural peculiarities of the twig, disrupted by grinding the tissue, were found to be critical.

Although the 2 clones differed somewhat in hardiness ('Poukhanense', -12°C and 'Maryann', -18°C) there was little difference between their freezing patterns. The greater exotherm of 'Poukhanense' was due to the higher water content of that clone (54% or more). Also, after freezing the amount of extracellular liquid water in R. 'Maryann' twigs was twice that in *R. poukhanense,* although values for both were only a small fraction of the initial water content prior to freezing. These clonal differences could not account for the observed hardiness differences.

Water content is an important consideration during nonequilibrium freezing since the associated freezing energy is often high. Nonequilibrium patterns have been found to be characteristic of hydrated tissues (12, 13). The moisture contents of twigs we used were considerably higher (49% and 54% for 'Maryann' and 'Poukhanense' respectively) than those reported for other acclimated winter hardy woody tissue (10). As a result of ice crystal growth and restricted water diffusion due to rapid crystallization, excess energy is available for tissue disruption. Tissues with a high water content, such as the stems used here, are particularly susceptible to disruptive injury during a nonequilibrium freeze. Physical disruption does occur in twigs of 'Poukhanense' (8).

Injury from a nonequilibrium freeze could occur as glaciers formed in hydrated tissues soon after freezing began or from dehydrational stress at lower temperatures. We did not investigate the effects of the freezing pattern on survival but our data indicate that the nonequilibrium pattern may induce physical stress during the initial stages of freezing. This, along with other types of injury, would limit survival at lethal temperatures. Thus the nonequilibrium pattern seems to be an important contribution to injury in azalea twigs. These findings will help to define survival in terms of freezing patterns.

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