WINTER HARDINESS OF EVERGREEN AZALEA (RHODODENDRON CV.) FLOWER BUDS

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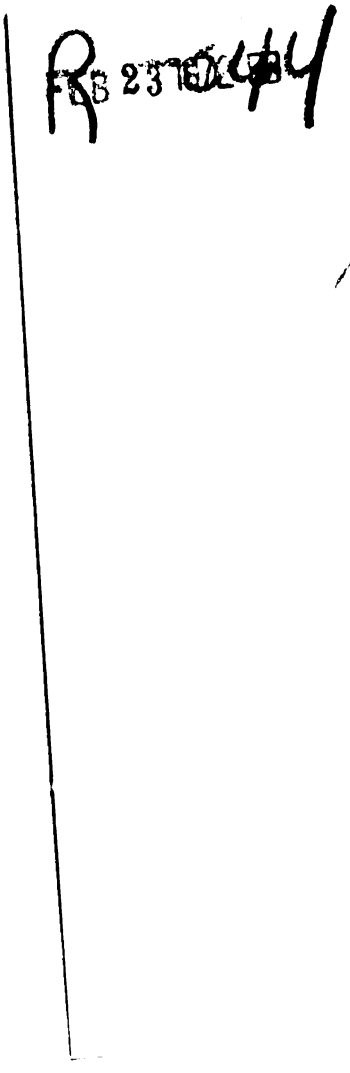

ABSTRACT

WINTER HARDINESS OF EVERGREEN AZALEA
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By

Glen Peirce Lumis

In northern climates of the United States flower buds of evergreen azaleas often do not survive the winter. Since the primary ornamental value of azaleas is their colorful spring bloom, and since there is little specific information about azalea flower bud hardiness, a study was initiated to define the mechanisms most seriously affecting flower bud survival.

During the winters of 3 years (1967-1970) two evergreen azalea cultivars, Rhododendron x 'Maryann' and R. poukhanense (unnamed cultivar), were studied in outdoor and controlled freezing experiments. Experiments were designed to determine: the visual symptoms of injury, when and where injury occurred, cultivar differences, the tissue freezing pattern and the role of ice. The outdoor survey consisted of monthly samples of flower buds and upper stem sections to determine water content and injury. Injury outdoors was assessed by browning of the flower parts. Controlled freezing tests were conducted on plants transferred from outdoors to a 1.5°C growth chamber in early November. Whole plants or, more often, excised twigs were frozen

and thawed at 2°C per hour to a range of temperatures designed to produce a graded series for each cultivar from no injury to complete kill. Survival in the laboratory tests was assessed by a modified neutral red plasmolysis vital staining technique. Ice structure was observed in frozen twigs by sectioning with a sliding microtome mounted in the freezing chamber. To evaluate the stresses in azalea twigs during freezing, a method utilizing molecular probes was developed. This technique, as described in the literature, is based on electrophoretic displacement of indicator molecules in the extracellular continuous liquid system.

The flower buds of R. poukhanense plants grown outdoors were injured during mid winter by low temperatures near or below -20°C. Splits were often evident in the upper stems just below the flower buds. Flower buds or stems of R. 'Maryann' were rarely injured. Water content measurements revealed that upper stems, although not flower buds, of R. poukhanense had a significantly higher water content than those of R. 'Maryann' for the months of December and January.

In the controlled freezing experiments, stem splitting of R. poukhanense was often evident, but could be eliminated by holding excised twigs for 4 days at 1.5°C prior to test freezing. During the 4 day holding period, the stem water content was significantly reduced from 54% to 46%. Initial injury, which occurred at approximately -10°C for R. poukhanense and -15°C for

R. 'Maryann', was evident only in a small section of the apical stem pith. By exposure to lower temperatures, the entire apical stem pith and one or more of the flowers within a bud were injured.

When frozen R. poukhanense stems were sectioned, large splitting ice masses were found in the upper stem vascular tissue. Similar ice formations were not observed in R. 'Maryann'. Throughout the remainder of the stem tissues, ice crystals were of moderate size and evenly dispersed. Freezing progressed as a nonequilibrium process in twigs of both cultivars. This type of freezing pattern indicated that as freezing occurred, there was a rapid decrease of liquid extracellular water and a sudden growth of ice. During freezing, ice formation was independent of decreasing temperature.

Injury to the twigs of both azalea cultivars was the result of the nonequilibrium freezing process, during which ice had an adverse effect by causing cells to contract and slip against the extracellular crystals. Tissues with a high water content are particularly susceptible to injury during an abrupt (nonequilibrium) freeze. In R. poukhanense, rapid ice formation coupled with the high water content of stems caused stem splitting in both outdoor and controlled freezing experiments.

WINTER HARDINESS OF EVERGREEN AZALEA (RHODODENDRON CV.) FLOWER BUDS

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Glen Peirce Lumis

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INTRODUCTION

Growing evergreen azaleas[1] as landscape ornamentals in northern climates of the United States is presently limited, because the plants, particularly the flower buds, are often killed during the winter. This is especially true of recently transplanted plants. Differences in hardiness among cultivars are often apparent whether in a home landscape or a nursery planting. These differences are not always consistant from one year to another or between locations. Evergreen azaleas would be used more widely in cold climates if the plants were more winter hardy. To the nurseryman and also the homeowner, uninjured flower buds for spring bloom are the azalea's greatest asset. Therefore, to the plant breeder, flower bud hardiness is an important character to be incorporated into a new population.

Because of the importance of azalea flower bud hardiness and the lack of specific information, this study was undertaken to define the mechanisms most seriously affecting the survival of evergreen azalea flower buds. In beginning such a study there are some preliminary considerations which can be ascertained from outdoor surveys. However, for more detailed information,

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[1]Azalea is an accepted common name for many species and cultivars of the genus Rhododendron including those cultivars used in this study.

outdoor surveys are inadequate because of uncontrolled environmental factors. A test freezing technique was developed that would produce accurately controlled and consistently repeatable conditions.

This study attempted to answer the following questions about evergreen azalea flower buds:

1. What are the visual symptoms of injury and where do they occur?
2. When does injury occur?
3. Is there variation in the symptoms, location or time of injury; if so, is it important for survival?
4. Are there cultivar differences and what is responsible for the differences?
5. What are the freezing patterns and what is the role of ice?
6. Can injury be prevented?

LITERATURE REVIEW

Foundations of Hardiness

The development of winter hardiness has been very important in determining the distribution of the earth's flora (8, 61, 78). It is clearly established that flowering plants originated in moist tropical areas; thus, their ability to survive cold temperatures is an adapted rather than an ancestral characteristic (8). In order for perennial plants to survive freezing temperatures, certain adaptations have been necessary. Vasil'yev (90) considers three types of adaptations: form, structure and physiology. Growth habit can enable plants to avoid the unfavorable effects of winter. Low growing plants, which are covered by snow for much of the winter, and herbaceous perennials, which restrict their overwintering parts to underground structures, are better able to survive by means of their adapted growth form. The ability of plants to directly withstand unfavorable winter conditions (Vasil'yev's physiologic adaptation) is undoubtedly very important, because without this ability, trees and shrubs would not exist in the north temperate climate. In autumn, northern plants undergo the dramatic event of frost hardening. At this time they quickly acquire a tolerance to freezing even to very low temperatures, while a short time earlier, they would have been killed by temperatures only slightly below freezing. "Only changes of a profound character both

in the physiology of the tree and in the physical and chemical properties of its cells could possibly account for a transition of this magnitude in the capacity of these cells to withstand freezing" (81).

Hardening implies the development of the ability to resist rather than avoid the adverse effects of freezing. In northern climates perennial plants respond to the shortened days and cooler temperatures of fall by becoming hardened or acclimated. By assessing survival after artificial freezing tests, hardening under natural day length and temperature conditions has been shown to be a two step process in woody plants, depending first on short days, then on fall frosts (89). Hardening of woody plants can be accomplished with low temperatures but without short days (26).

Siminovitch and Briggs (80) found that during late summer and early fall the water-soluble protein content of living black locust tree bark increases (in excess of a summer minimum) and that this increase is accompanied by a proportionate increase in hardiness. This relationship was determined by: 1) ringing or girdling the trees at different times from July to September and at increasing heights above the first ring; and 2) the ability of bark cells to withstand injury from extracellular freezing or dehydration. Protein accumulation in the bark appeared to be dependent on the supply of certain factor(s), necessarily derived from the leaves by phloem transport, since the ringed sections were isolated from the roots. These authors were

unable to account for the role of increased proteins, but speculated that they were directly concerned with the mechanism of hardening and may be only a part of an apparent net synthesis of new protoplasmic material.

Heber and Santarius (23) found that freezing of isolated chloroplasts and mitochondria, and also intact cells caused uncoupling of photophosphorylation, observed as a loss of ATP synthesis. This loss of ATP synthesis, which is sufficient to cause cell death, could be prevented by increased sugars in the protoplasm of an intact cell or in direct contact with isolated sensitive proteins. A sugar concentration equivalent to that available in hardened cells, 1 to 2%, was sufficient for protection. The authors suggest that the uncoupling of phosphorylation from electron transport was the result of the removal of water from the membrane system as ice grows. The protective action of sugars was explained on the basis of their ability to substitute for or retain water by hydrogen bonding in structures sensitive to dehydration. Further studies by Heber (22) demonstrated that hardy plant cells were capable of synthesizing specific protein factors which protect membranes against freezing injury. Injury to functional membranes appeared to be a result of membrane structure alteration rather than denaturation of specific proteins. Freezing of unprotected cells caused an irreversible collapse of chloroplast membrane vesicles. Two protein factors were isolated from hardy spinach chloroplasts that were far more effective than sugars in

preventing inactivation of photophosphorylation. Inactivation was used as an index of injury.

In further studies with black locust tree bark, Siminovitch et al. (81) observed that as hardening progressed during the late summer and early fall, there was: 1) an increase in the quantity of cell organelles with a corresponding decrease in the spring; 2) an augmentation of protoplasm and cell structures, including structural changes of the protoplasmic membrane; and 3) an increase in phospholipids and lipoproteins. On the basis of light and electron microscope studies, as confirmation of earlier work, membrane replication was shown to be an important factor in the development of hardiness, although only a part of the total hardening process. Normal hardening, they suggest, is a composite of membrane replication, membrane replication with protoplasmic augmentation, and starch to sugar conversion.

The hardening process is dependent on adaptive mechanisms, as described above, that alleviate injury in tender plants. Hardiness, however, involves more than adaptive changes. Permanent characteristics of a plant such as growth habit or histological features are also important.

Low temperature hardiness of plants has been defined as the ability to survive being cooled (34); the ability to come through the winter successfully (90); the ability to survive ice formation in the tissue (7); or the total capacity to withstand a sequence of environmental interactions sustained in

the interum between cessation of growth in the autumn and its resumption in the spring (91). Levitt (34) describes three distinct types of low-temperature hardiness: chilling, frost and extremely low temperature. Plants which lack chilling hardiness are injured by temperatures slightly above freezing. This is true of many tropical and subtropical plants. Frost hardiness implies the lack of injury at low temperatures attained in the natural environment, while hardiness at extremely low temperatures such as -196^{o}C can be demonstrated only experimentally. This study will deal with frost hardiness, considering winter, cold, frost and low-temperature to be equivalent terms for describing this form of hardiness. In Figure 1, Levitt (36) diagrams the components of winter hardiness.

Winter hardiness has been the subject of extensive investigation, especially with food plants, since earliest times. It is estimated that there are over 5,000 references in the world literature (78) in many fields of biological and physical science.

> Despite man's long association with cold, research on the subject has consisted primarily of scattered studies of resistance and susceptibility of various organisms to injury from freezing. Only in the last twenty or thirty years has real progress been made toward understanding what is involved...(47).

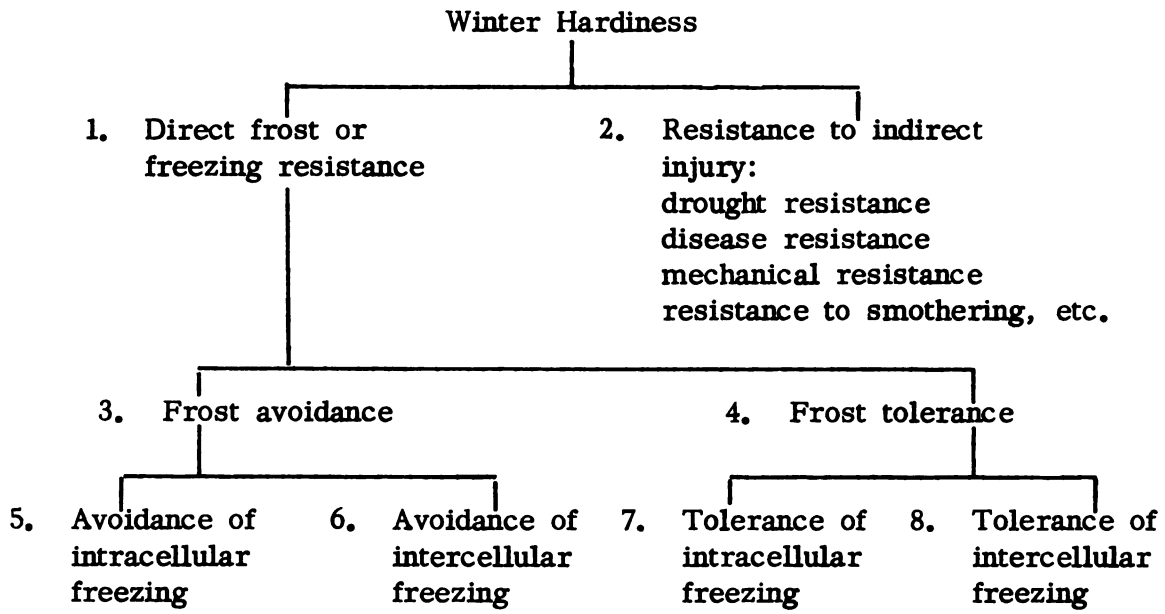


Figure 1. The components of winter hardiness.

Numbers 5 and 8 are the two major components (from Levitt, 1966, p. 496).

Freezing and Ice Formation

Freezing of plant tissues is a physical phenomenon. Lowered temperatures produce the two major effects of rate reduction of physical and chemical reactions, and phase change--specifically, the freezing of water (47). Ice formation is undoubtedly the most important physical change that takes place when plant tissues are cooled to low temperatures, although not the only one. Histochemical and

histomechanical alterations such as dehydration, protein denaturation, electrolyte concentration, mechanical distortion, changes in volume and dissolved gas redistribution are directly attributable to the removal from tissues of liquid water by freezing (86). Mazur (45) emphasizes, "... the rather paradoxical fact that freezing produces straightforward, well-understood physicochemical events; and yet, because of the interaction of the various events, it is a complex process biologically."

Ice Inoculation and Propagation

Plant tissues are usually inoculated by ice nuclei on the surface of leaves or other plant parts such as the bark (19, 25, 50, 70). Under artificial freezing conditions, wheat leaves were found to freeze slowly, in contrast to rapid freezing under natural conditions (84). Initial crystallization was attributed to freezing nuclei in the atmosphere. The presence of condensation or dew on the plant surface insures surface ice formation and subsequent internal inoculation (25). This is useful in artificial freezing tests of plant pieces to prevent appreciable supercooling. After inoculation in hardy plants, freezing is first evident progressing in the vascular system, specifically the non-living xylem (24, 25, 31, 43, 61, 74, 84). Although all water in the outer free space is eventually susceptible to freezing, when non-hardy tissues freeze, initial ice is not restricted to a particular tissue or region, but

appears scattered throughout different tissues (25). The rapidity with which freezing progresses is dependent on the tissues encountered by the ice (74, 84). In leaf tissue of cereals, as studied by Single (84), the crystallization front moved rapidly, virtually unimpended, in the water of the xylem vessels. As the ice moved from the leaves into the stem greater difficulty was encountered, as measured by its forward rate of progress, until the ice front was halted at the node for varying lengths of time. The node interference was presumably due to the complex vascular structure at the node. Interference varied with the temperature, age and condition of the tissue. In spruce needles ice propagation appeared to be impeded by some barrier between the stele and mesophyll (74). This barrier was thought to be the endodermis, since any barrier to water transfer is likely to be a barrier to ice growth.

As winter approaches and plants become hardy, tissue water content usually decreases. This reduces the amount of freezable water and generally increases hardiness (28, 34, 40, 46, 71, 88). McLeester et al. (46) reiterate the finding of previous workers that a lower moisture content influences hardiness by reducing supercooling and decreasing the amount of water readily frozen. The temperature at which ice forms in hardy buds is dependent on their water content; assuming that a higher water content prevents

supercooling, aids inoculation and speeds propagation (29, 61). However, in pine needles the freezing temperature (or supercooling) was unaffected by water content (30). Water volume in needle tracheids had no influence per se on nucleation. The temperature of ice nucleation was an increasing function of needle length, ultimately dependent upon the number and quality of nucleation sites on the cell wall. Cell wall nucleators were not directly demonstrated, but inferred from experiments by other workers with glass capillaries in which freezing was initiated by nucleators on the walls and from experiments with grasshopper legs in which nucleation always started at certain points (30).

Intracellular and Extracellular Freezing

In plant tissues ice can form intracellularly or extracellularly. The type that occurs in any particular situation depends upon such factors as the degree of hardiness, the rate of cooling and warming, and the rate at which crystallization approaches the plasma membrane. Intracellular freezing is nearly always lethal and is characterized by mechanical disorganization of the protoplasm by ice crystals (2, 43, 49, 53, 56, 78, 82). When intracellular freezing occurs, it is believed to be the result of inoculation with crystals from outside the cell (3, 45). Ice formation within the cell is characteristic of non-hardy plant tissues which are either incapable of hardening or not in a hardened condition. Intracellular freezing can also occur

when hardy tissues are cooled quickly, resulting in considerable supercooling (44, 57, 73); however, it is not common in hardy tissues capable of withstanding extracellular freezing, since the plasma membrane is an effective barrier to ice crystal growth (3, 6, 34). Plant cells are able to protect their membrane systems against the harmful effects of freezing by synthesizing specific protein factors which stabilize the membranes and by inducing structural changes in the membrane (22, 80, 81).

The cooling velocity will determine whether cell water will leave during cooling and freeze extracellularly or freeze within the cell (24, 44). Sakai and Yoshida (73) describe three cooling rates they used in studying the survival of thin cortical sections of tree bark. The first and only one pertinent to this study is slow cooling, during which cell viability remains high. The second cooling rate is intermediate, at 15° to $2,000^{\circ}$C per minute, during which cell survival decreases to zero, and the third is rapid cooling, up to $200,000^{\circ}$C per minute, during which cell viability increases to a high percentage. Slow cooling results in large ice crystals that form in the intercellular spaces tending to desiccate and partially collapse the cell. This is a protective effect helping to prevent intracellular freezing provided cell water loss keeps pace with the growing extracellular ice, which is generally the case at slow cooling rates (7, 24, 35, 43, 44, 51). Increased membrane permeability of hardy cells is thus of little consequence when the cooling rate is slow. The extent of

extracellular freezing depends on the water content, permeability and structure of the plant, and environmental factors such as temperature (cooling rate, minimum and duration), soil moisture and humidity (49).

If plant cells are cooled slowly, subsequent slow warming is not harmful (36), survival being independent of the warming rate (73). This is in contrast to cells cooled rapidly. Rapid warming is necessary to insure survival when the cooling rate is high in order to avoid the formation of damaging crystals during warming (44, 72, 73).

When hardy plant tissues freeze they contract, not from external pressure of extracellular ice, but from cell water loss as freezing continues (34, 77). Water diffuses out of the cells as ice forms in the intercellular spaces, because the external vapor pressure drops below that of the protoplasm. As the temperature decreases cell water loss continues until the cells become considerably contracted and sometimes collapsed. The cells contract in proportion to their water loss (77). Siminovitch and Scarth (82) describe freezing induced cell collapse in which opposite sides of the cell come in contact. Similar collapse is illustrated by Glerum and Farrar (17) and attributed to extracellular ice formation. Cell water loss and ice formation do not abruptly cease, but continue progressively at a decreasing rate even at $-30^{o}C$ (34). In some cases ice accumulates in localized masses,

often in transitional zones between different kinds of tissue, and can be injurious (12, 24, 25, 34, 58). Upon thawing, uninjured cells quickly reabsorbe water.

Ice Crystal Size and Structure

The differences in cell viability observed in the many studies of freezing velocity are explained in terms of ice crystal size. Sakai and Otsuka (72) state that the important consideration is not whether ice forms within a cell, but rather the size of the crystals formed. Crystal size and structure are directly related to the cooling and warming rate, minute crystals of particular structure forming at rapid rates and large crystals of more perfect lattice structure forming at very slow rates (39, 43). After freezing has been initiated, the number of nuclei formed, their rate of growth and the size they attain will all depend on the rate of heat removal from the immediate environment (48). Crystal development can be greatly affected by cellular materials that act at the ice-liquid interface. Water soluble cell wall polysaccharides have been isolated from some hardy cereals (56). These polymers, acting as protective materials, interact strongly with and partially block the ice-liquid interface, and interfere with the development of large injurious crystals.

Stress and Freezing Patterns

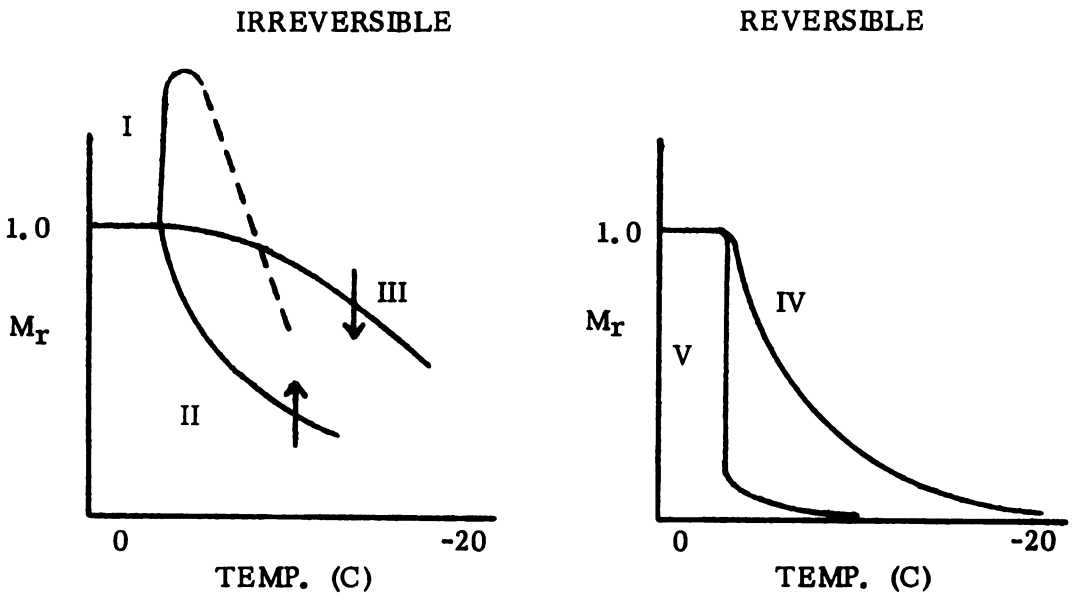
In plant tissues survival is most often limited by the stresses involved

in the formation of ice. Stresses occurring during freezing are determined by the patterns of water redistribution in the tissue. The type of redistribution depends on the type of stress (53, 54), the tissue moisture content, tissue structure and composition, and the conditions under which freezing occurs (56). In order to specifically evaluate stresses in plant tissues during freezing, Olien (53, 54) has developed a technique utilizing molecular probes. This technique is based on electrophoretic displacement of indicator molecules in the extracellular continuous liquid system. Water between protoplasts exists along the cell walls in gradient films. Mobility of the 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. Microscopic observations of dye displacement were used to evaluate mobility in tissue sections. 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. The path of electrophoretic mobility can be restricted to the extracellular continuous liquid system through which ions diffuse freely. Living tissues are effective barriers to internal electrolyte diffusion, unaffected by weak voltages (53). This electrophoretic technique can be used effectively to study freezing stress by determining the

temperature at which ice forms and to identify the different types of freezing processes.

Measurements of conductivity have been used to determine the degree of injury (10, 11), the time when physical changes occur (13, 14), as well as the relationship between temperature and content of extracellular liquid (53, 54). Many factors affect conductivity: resistance of electrical contacts, frequency of alternation, voltage, concentration of electrolytes, etc. Conductivity is used to evaluate changes in liquid content with temperature only when conductivity correlates with mobility, a function of effective viscosity. The research described above began with studies on electrophoretic displacement of copper, silver, amaranth, and a stem rust toxin through wheat leaves (52).

Olien (57) has studied five types of freezing patterns in a variety of plant tissues. These patterns are reproduced in Figure 2. In pattern I, characteristic of tender succulent tissues, ice forms in the intercellular spaces, but rapidly grows into the cells, injuring them and releasing their contents. The pattern is irreversible, in that as the tissue thaws and is again refrozen the pattern can not be repeated, and represents a nonequilibrium process, because the amount of water frozen is not a function of temperature. Extracellular water increases as freezing begins, due to cell laceration from



M_r Relative content of liquid water between protoplasts along the cell walls.

↑ Direction pattern tends to drift at constant temperature.

Freezing Process		Physiological condition	Moisture content	Freezing energy	Killing temperature
I Nonequilibrium, Disjunctive	a.	Tender	Moderate	Low	High
	b.	Hardened	Moderate	High	High
II Semiequilibrium		Semi-hardened	Moderate	Low	Moderate
III Nonequilibrium, Discontinuous		Hardened	Very low (55%)	High	Moderate (-10 to -15C)
IV Equilibrium		Hardened	Low (60 to 70%)	Low	Low (-17C)
V Nonequilibrium Conjunctive		Hardened	High (75 to 80%)	Moderate	Moderate (-1 to -15C)

Figure 2. Patterns of water redistribution during freezing in barley (from Olien, 1969).

intracellular crystal formation. Pattern II occurs in partially hardened barley plants after floral initiation has begun. As freezing progresses the protoplasts break down intermittently. Patterns III, IV, and V are representative of hardened plants. When the moisture is very low (pattern III), freezing is not a continuous process. This results in supercooling and bursts of freezing across histological barriers. Pattern IV is typical of tissue with a low to moderate water content in which freezing occurs gradually as ice continuously develops along cell walls. The content of liquid water decreases in proportion to decreasing temperature. Survival is not affected by ice along the cell wall, because as ice forms the walls contract and the amount of ice is insufficient to cause mechanical damage. Pattern V is characteristic of tissue with a high moisture content, in which freezing of the extracellular water occurs rapidly. The resulting ice structure will determine the extent of mechanical damage. Different stress patterns often occur in neighboring regions of a plant.

Freezing Injury

Plant tissues are injured directly or indirectly by ice formation. Intracellular ice is nearly always lethal, occurring in non-hardy plants or in hardy plants cooled quickly. This type of ice formation lacerates protoplasts, destroying membranes and releasing cellular contents. Under natural conditions

intracellular ice has never been observed in hardened plant tissues (35), although in theory it could happen, as when the south side of a tree is suddenly shaded from the sun on a cold winter day (70, 92). Extracellular ice is generally thought to be a secondary cause of injury rather than producing direct mechanical damage (34). However, extracellular ice can be mechanically disruptive during nonequilibrium freezing (56).

Injury to plant tissue can be of two types: mechanical and/or physiologic. Mechanical injury results when ice crystals form intracellularly or when the quantity of extracellular ice is sufficient to cause splitting or other physical disruption (12, 16, 25, 43, 51, 61). During nonequilibrium freezing, when the amount of water frozen is not a continuous function of temperature, ice grows explosively (56). "Sudden growth of large ice crystals can cause great distortion of cells and tissues as a result of shearing forces generated between crystals and structural components of the tissue" (56). Most explanations of injury to hardy plant tissues are based on physiologic phenomena which are the indirect result of extracellular ice crystal formation. A diagram of the mechanism of frost damage as proposed by Modlibowska (49) is shown in Figure 3.

Frost injury does not occur in supercooled tissues and in tissues with a low moisture content because ice does not form. Both ice formation and injury

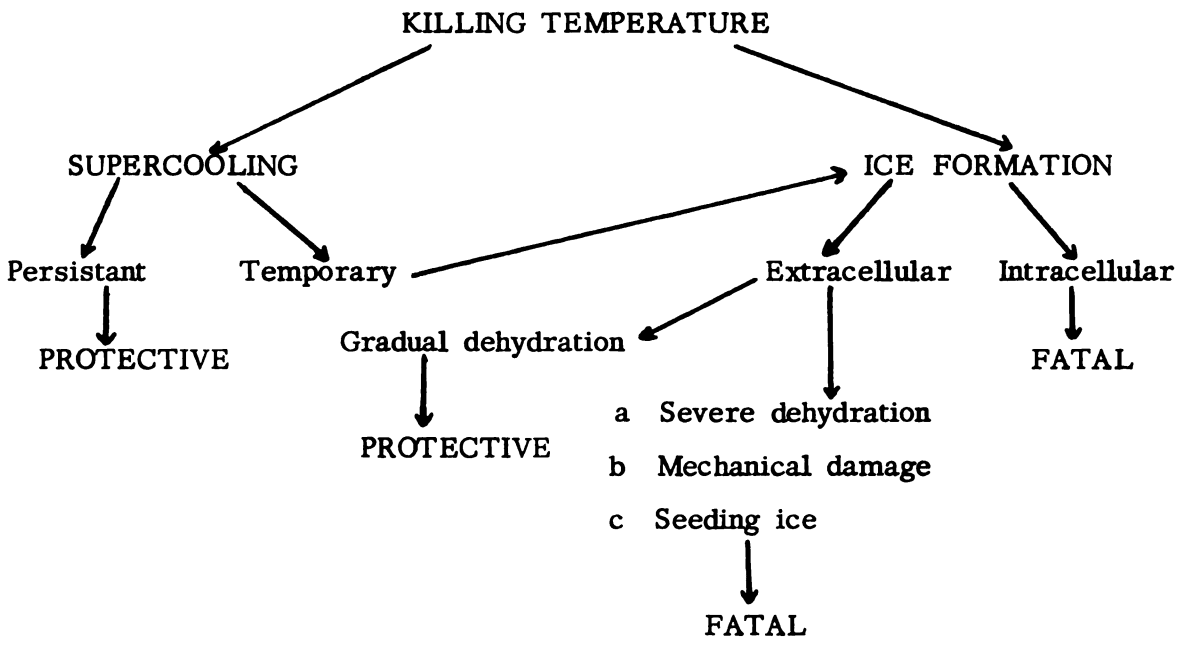


Figure 3. Mechanism of frost damage (from Modlibowska, 1962, p. 180).

increase with decreasing temperature, as determined by the amount of water frozen and the percent of cells alive at progressively lower temperatures (34). Any theory of injury must assume that ice is directly or indirectly responsible for injury. Numerous theories of injury to plant tissues have been put forward. Details of the theories and mechanisms of injury have been reviewed by several workers (34, 36, 45, 48, 49, 61, 77, 82, 90). Two old theories which have been disproven are the "caloric theory" based on a plant's "natural heat" and

the "rupture theory" based on the sound fact that ice expands as it freezes, but not recognizing the fact that cells actually contract upon freezing. These two theories could be called "armchair" theories, since they were proposed without experimental evidence (34). Intracellular freezing is almost without exception lethal, resulting in cell rupture and disorganization. It is extracellular freezing injury that is in need of explaining. Extracellular ice formation has a dehydrating effect, because cell water is lost to the ice forming outside the cell.

Probably the most widely supported theory of injury to plant tissues is cell dehydration, caused by the indirect effect of extracellular ice formation. In fact, many theories basically relate injury to dehydration. There are several consequences of dehydration which could cause injury (45). Removal of liquid water is an obvious consequence of dehydration. This in itself would not seem to be lethal, since all hardy cells undergo water loss during extracellular freezing. Plant cells can survive severe contraction and collapse as a result of water loss, reabsorbing the lost water upon thawing (82). Loss of chloroplast membrane function has been ascribed to alteration of membrane permeability due to dehydrational water loss (23), although some question as to the validity of this hypothesis has been raised (45). Injury from extracellular freezing may result from critical dehydration of the protoplasm, producing changes in the interaction of protoplasmic proteins and

surrounding water (2). This hypothesis is based on the lack of hemolysis of red blood cells until 90% of the cell water crystallizes. A decrease of cell volume accompanies dehydration. This would cause a decreased spacial separation of protoplasmic materials. Levitt (36) discusses an all encompassing hypothesis that attributes injury to structural proteins being forced into close proximity, with the formation of disulfide bonds. These bonds compete with hydration forces as water returns to a cell after thawing, resulting in protein unfolding and denaturation. Little direct evidence is available to support this sulfhydryl-disulfide hypothesis. In fact, it seems inadequate to explain freezing injury in a number of cases with animals, microorganisms and plants (22, 45). Using chloroplast membranes, no oxidation of protein sulfhydryl groups due to freezing could be detected (23). The concentration of cellular solutes is another consequence of dehydration. Injury from concentrated electrolytes and the possible precipitation of proteins is a widely accepted theory for microorganisms and animals (45, 48), if not for plants (35, 36). When red blood cells were frozen in the presence of glycerol, a salt buffer, damage was the result of electrolyte concentration as water was frozen out of solution (as reviewed in 48). For higher plants, the electrolyte concentration and salt precipitation theory is questionable. This theory does not account for a greater resistance to extracellular than to

intracellular freezing, since salt concentration would be the same in both cases, or for the existence of both freezing and thawing injury (36). Hardy cells have not been found to contain more precipitation resistant proteins or to have lower cellular salt content (36). A change of pH is an additional consequence of cell dehydration. There is evidence that progressive freezing of salt solutions causes, among other things, a change in pH which would result in protein precipitation (45, 48). However, pH change may be of questionable importance to hardy plant cells. Distinguishing between desiccation injury, when water prevents stress, and phase change injury, when water is a direct contributor to stress, is possible (57). This distinction and further studies of it may be important in relation to the dehydration theory.

Another theory of injury is based on mechanical damage in which injury results from stresses and strains of cell collapse during extracellular ice formation and, upon rapid thawing, the tearing of protoplasmic surfaces when cell walls quickly regain their original shape. This theory agrees with many known facts such as the relationship between small cell size and survival ability, the reduction in water loss with increasing sugar concentration (resulting in less cell contraction), and the increased resistance of frost-hardy protoplasm to applied pressure (35, 36). But deficiencies of this theory are its inability to explain the greater injury from intercellular freezing, the greater resistance of hardy protoplasm to stresses, and the injury to cells

without rigid cellulose walls (36). Injury from extracellular ice may be attributable to mechanical deformations which affect protoplasts as water is lost, often at temperatures not far below freezing, when protoplast dehydration has not yet reached injurious levels (75). This suggestion is based on the ability of hardened plant cells frozen in protective solutions to survive much lower temperatures, i.e., greater protoplasmic dehydration.

Freezing injury to flower buds of fruit trees has concerned horticulturists for many years. They have found that flower buds are often the most sensitive part of the plant during winter and spring. Because flower bud injury of fruit trees is of considerable economic importance, research was stimulated. Many workers have attempted to correlate such characteristics as water content, varietal differences, size of flower parts, and cell size, composition and maturity to winter injury susceptibility (1, 28, 65, 66, 93). Water content is an important consideration when studying freezing injury; a high water content often leads to injury, not only in flower buds, but in many tissues (28, 46, 54, 60, 61, 93). Splitting and rupturing are often the result (12, 16, 20, 25, 43, 51, 62). Different varieties of many plants, including azaleas, react differently to freezing stress, although the reasons are not well understood (7, 58, 61, 65, 66, 90). For azaleas much of the information on varietal differences and their reaction to freezing stress

consists of unpublished observations by plant breeders, nurserymen and hobbiests. There is little specific information about azalea flower buds. Survival of detached leaves (21) and stem injury symptoms (67) have been studied using Rhododendron species. Graham and Mullin (18) believe that deciduous azalea flowers are able to avoid winter injury by not freezing even at temperatures as low as -40°C. During controlled, rapid freezing, crystallization occurred from -15° to -40°C and was invariably lethal. Injury to floral tissue of azaleas seems to occur during winter months rather than during spring frosts, since azaleas do not bloom in early spring. In contrast, flower buds which begin expanding early in the spring, as those of some fruit trees, are especially susceptible to spring frosts (34, 61, 66).

Survival Evaluation

Evaluating plant survival is an important aspect in any study of hardiness. The development of artificial freezing techniques with chilling chambers (19) and their subsequent sophistication (see Rollins et al., 1962, for literature) have enabled plant survival to be tested quickly and quantitatively, rather than depending on "test winters," which occur on an average of only once every ten years (34). Field survival still remains, however, the ultimate test, being characteristic only of a particular location during a particular

winter.

A number of techniques have been used to determine survival. One of these utilizes browning as a general indicator of injury. This method is often useful as a quick test in the field for determining survival of flower buds of fruit trees when a qualitative evaluation is sufficient. However, in any sort of histological evaluation of survival, the location of browning is no indication of injury to a particular region, since the browning precursors could move from one area to another. Lapins (33) suggests that recovery estimates are useful, because differences in injury to cambium, bark and buds are expressed clearly; the chances of recovery depending on the condition of these vital tissues. Evaluation of protein breakdown products resulting from injury has been used as an index of injury (83). The ability of a plant part to grow and develop following freezing is another method of survival evaluation. Sakai (67) has used this method, although not to the exclusion of other survival tests, believing that the survival of an entire twig cannot be judged on the basis of the viability of one cell type. Another survival evaluation technique consists of determining the number of freezing points of living and dead tissue. Living tissue characteristically has two freezing points while dead tissue has only one (46).

The fact that membrane permeability increases when a protoplast is injured or killed (59) has been the basis for several vitality tests. One

method utilizing this principle was designed by Dexter et al. (10, 11) to measure the electrical conductivity of leachable cellular electrolytes. This has become a common way to evaluate survival. Using intact plants, several workers have studied electrical resistance differences of survival (5, 14, 15, 41, 95). Since cell injury results in a loss of membrane semi-permeability and a release of electrolytes into the intercellular spaces, electrical resistance decreases considerably after injury. Cell plasmolysis and deplasmolysis after freezing are more precise applications of the permeability principle (37, 69, 72, 76, 79, 82). In a hypertonic solution of NaCl:$CaCl_2$ injured cells do not plasmolyze or subsequently deplasmolyze, since the semi-permeability of the plasma membrane no longer exists. Siminovitch and Briggs (79) studied the feasibility of plasmolysis tests in place of actual freezing tests.

Neutral red, a vital stain, the action of which is based on membrane permeability, has been used alone and in conjunction with plasmolysis (37, 38, 72, 73, 79, 82, 83). This dye is useful for studying cell viability, because it easily penetrates cell membranes, is non-toxic and, as a pH indicator, is bright red within the weakly acid cell (4). The ability of living cells to reduce oxidation-reduction indicators is a different type of survival test for which triphenyltetrazolium chloride (TTC) is most often used (42, 63).

A refinement of this test has been proposed in which absorptivity aids visual differentiation between varying degrees of dye reduction (85). Differences in autofluorescence of living and injured cells is an additional survival evaluation method (32).

In order to survive the adverse effects of freezing, plant tissues must be in a hardened condition. Tropical plants are incapable of hardening, but plants of northern climates such as azaleas are able to acclimate. Membrane stabilization and the other physiologic cell processes are important prerequisites of acclimation. Hardiness, however, involves more than adaptive changes during acclimation and does not preclude the possibility of injury. Injury to flower buds and other tissues is dependent on complex physical and chemical reactions to the internal and external environment. The mechanisms of injury and survival are intriguing aspects to pursue.

METHODS AND MATERIALS

Freezing Injury and Survival Study

Outdoor Survey

Plant material and cultural practice: Three evergreen azalea cultivars, Rhododendron x 'Maryann' [indicum x (poukhanense x kaempferi)], R. kaempferi 'Mikado', and R. poukhanense (unnamed cultivar), were purchased as 4 to 5 year old potted or field grown plants from nurseries specializing in azaleas. They were grown in 3 quart plastic pots using a mix or peat moss, Perlite[1] and soil (2:1:1) in a shaded cold frame (50% shade) and arranged in a randomized block design consisting of 4 replications. The plants were fertilized once in early spring with 21-7-7 rhododendron fertilizer (2.6 gm/l) and again in early summer with a 15-45-5 rhododendron fertilizer (3.4 gm/l). A 7 day pen themograph placed in the center of the cold frame recorded air temperature at plant height and soil temperature at 10 cm below the surface throughout the winter.

Sampling method: To determine when flower bud injury occurred, 8 cm twigs were sampled in early to mid-December and after cold periods during the winter over a 3 year period. Within each replication, 5 twigs were cut from each of two randomly selected plants and taken to the laboratory for sectioning. The bud and upper twig were sliced longitudinally with a double-edged razor blade in as many slices as necessary to reveal all the developing

[1]Produced by Zonolite Div., W. R. Grace & Co., Cambridge, Mass.

flowers. Sections were observed to determine the visual symptoms of injury, the tissues affected, at what time during the winter injury appeared, cultivar differences and the consistency of these factors. A hand lens, a dissecting scope and occasionally a light microscope were used for these observations.

At monthly intervals from October to April, the moisture content of flower buds and upper stem sections was measured. Five 5 cm twigs were cut from each of two plants within each replication. Leaves from the twigs were discarded, the buds were snapped from the twigs and placed in stoppered vials, and a 1 cm section from the upper part of each twig immediately adjacent to the bud was cut and placed in a stoppered vial. The vials were quickly taken to the laboratory for weighing, after which they were placed in a drying oven at 65°C. They were dried for 48 hours, then weighed to determine the dry weight.

Laboratory Studies

Plant preparation and freezing procedure: In order to work with naturally hardened plants that had not been injured outdoors, 15 plants each of R. 'Mary-ann' and R. poukhanense were transferred from the cold frame to a growth chamber during early November, where they were held at 1.5°C with a day length of 8 hours and a light intensity of 800 ft-c. Plants were test frozen

in a home style deep freeze modified with a false floor and a fan for air circulation, and equipped with an evaporator pressure regulation (EPR) valve for accurate temperature control. The EPR valve was operated by a small motor controlled by time clocks. A freezing rate of 2^{o}C per hour was used. Before being frozen the material was allowed to equilibrate in the chamber for several hours at -1^{o}C. The material was thawed either in the freezer at 2^{o}C per hour or immediately at room temperature. To prepare the plants for freezing, two techniques were employed. The first was to insulate the pot with styrofoam and glass wool, then place the entire plant into the freezing chamber (Figure 4A). The second and most often used was to cut 10 to 13 cm twigs, placing 3 to 4 twigs in a moist sponge with a plastic bag (Figure 4B), then transfer them to the freezer. With both preparation methods 3 mil copper-constantan thermocouples were either inserted into the upper part of the flower bud or held firmly between the outside of the bud and one of the leaves which cluster tightly around the bud. One thermocouple was attached to each of two buds on the entire plant or 1 to a bud when the moist sponge was used. Additional thermocouples were placed in the soil or sponge, the chamber air stream and an ice water bath. The latter was used as a reference temperature. All thermocouples were attached to a 16 point recording potentiometer.

Figure 4. Plant preparation for freezing injury and survival study.

A. Whole plant with pot insulated.

B. Excised twigs in moist sponge with plastic bag cover.

Figure 5. Diagram of contact arrangements for Experiments I through IV in the freezing pattern study.

A. Contacts: c, 1, 2, 3 and tissue areas: A, B for the R. 'Maryann' twig in Experiment I.

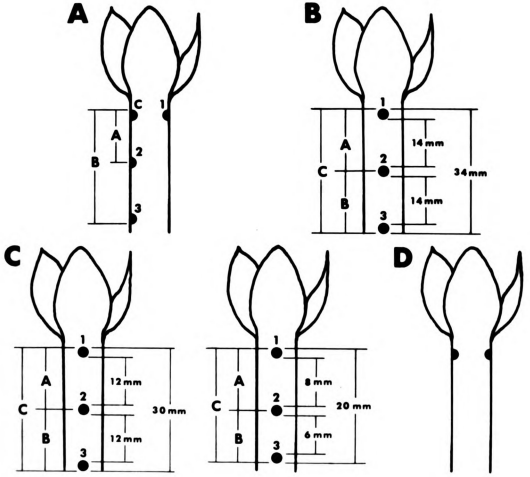
B. Contacts: 1, 2, 3 and tissue areas: A, B, C for the R. 'Maryann' twig in Experiment II.

C. Similar contacts and tissue areas for R. 'Maryann' (left) and R. poukhanense (right) twigs in Experiment III.

D. Contacts for twigs in Experiment IV.


A


B


A
C
1
A
B
2
3
B
1
14 mm
A
2
34mm
C
14 mm
B
3
C
1
12mm
A
2
30 mm
C
12mm
B
3
1
8 mm
A
2
20 mm
C
6 mm
B
3
D

Each freezer run consisted of two entire plants or six sponges, three of each cultivar. Initial experiments to determine the killing temperature for each of the two cultivars were followed by experiments to produce a graded series from no injury to complete kill. The experiments were designed to determine: 1) the visual symtoms of injury; 2) the tissue and area affected; 3) whether injury progressed from one tissue to another; 4) whether the visual symtoms of injury from test freezing were similar to those outdoors; and 5) whether the cultivars reacted differently. After thawing, a twig from each sponge or an entire plant was placed in the greenhouse for evaluation of bud expansion and flowering. The remaining twigs or plants were taken to the laboratory for sectioning and observation. At progressively longer times after thawing, the twigs were sectioned longitudinally with a double-edge razor blade to reveal all the developing flowers and pedicels[1] or longitudinally in such a way as to expose a median section through the upper stem, pedicel and pistil.

Survival determination: Survival was determined by the absence of browning, and by the use of a modified neutral red plasmolysis technique. The neutral red technique alone or in conjunction with plasmolysis has been

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[1] The pedicel of a characteristic azalea inflorescence, umbellate-raceme, is much more conspicuous than the reduced peduncle. Therefore, the term peduncle will not be used.

used previously (37, 38, 72). The most often used neutral red plasmolysis solution was made by adding .5ml of .1% aqueous neutral red to 10 ml of a .35 M sucrose solution buffered to pH 7.2, resulting in 50 ppm neutral red. Two alternative methods replaced the buffered sucrose with: 1) distilled water or 2) .25 or .5 M salt solution ($NaCl:CaCl_2$, 9:1). Longitudinally sliced twigs or thin sections remained in the neutral red solution for 10 to 15 minutes before observation. When sliced twigs were stained, thin sections were cut after staining. A .4% KOH solution was tested with all three methods to destain non-living cells. It was used as a quick dip after which the twigs were rinsed in water. When the buffered sucrose or the salt solutions were used, the twig or a thin section, after staining, was transferred to distilled water to ascertain cell deplasmolysis. However, in reverse, when the distilled water neutral red solution was used, the twigs or sections were transferred to either buffered sucrose or the salt solutions to determine cell plasmolysis.

Moisture content: Samples of moisture content of upper stem pieces from plants held in the growth chamber were taken at two intervals during the storage period as a comparison to plants outdoors. The procedure was identical to that described previously in the outdoor survey. In order to determine the effects of a lowered water content on stem and bud survival,

twigs of both cultivars were cut and inserted in a dry sponge and allowed to remain in the growth chamber for 4 days, after which the twigs were frozen and thawed as described earlier. The twigs were then sectioned to reveal any injury.

Qualitative chemical tests: Tests for starch, pectin, cellulose and lignin were performed in an effort to determine cultivar differences. The procedures used follow the qualitative determination methods described by Jensen (27, Chapter 9) for staining fresh tissue sections.

Ice structure and occurrence: To study ice structure and its occurrence in azalea bud and stem tissues, twigs were frozen to $-9^{o}C$ in sponges as described earlier, then quickly transferred in an insulated container to a working chamber held at $-9^{o}C$. The chamber was equipped with a viewing window and armholes, sliding microtome, microscope, working utensils and interior lighting. Utensils were well insulated with cotton and paper tape to assure their stable temperature during handling. Each bud or stem piece (1 cm long) was frozen to a copper mounting block. A small drop of water was put on the copper block with a probe and the bud or stem immediately laid into the drop and held for several seconds until solid. Additional small drops of water were placed entirely around the specimen, making sure it was held firmly. Before building up the ice, a thermocouple was placed as

close to the area of interest as possible. This was a precautionary measure to insure against otherwise unknown sudden heat rises which could flash thaw part of the specimen. After the material had been frozen securely to the block, unwanted tissue was trimmed away. The microtome was equipped with a stainless steel, injector-type razor blade. Sections thinner than 30 μ were too thin to hold together, and those thicker than 50 μ were too thick for clear microscopic observation. When a good section was cut, a small camel hair brush was used to transfer it to a container of water-saturated mineral oil which was stored in the chamber. Several sections could be stored in this way before they were individually mounted in the oil on a glass slide, covered with a cover slip and arranged on the microscope stage.

Freezing Stress Study

To detect possible differences between the two azalea cultivars, R. 'Maryann' and R. poukhanense, and to determine the freezing pattern in their twigs, several experiments were conducted.[1] By determining the freezing pattern, the speed and ease with which ice forms in the twig can be evaluated. Since there were no previous reports of this type of study with azaleas, it was necessary to determine: 1) the ability of twigs to withstand weak electric

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[1] The techniques described were developed by C. R. Olien (53, 54 and personal communication), and the basis for them was discussed in the Literature Review.

currents; 2) the 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 mobility. Without these any data for freezing patterns would be incomplete.

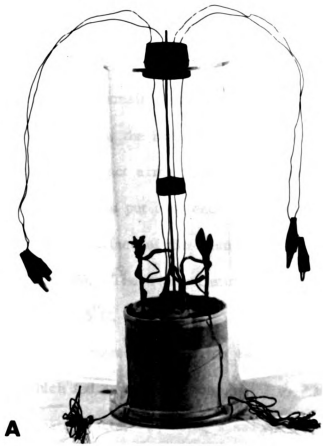
Freezing Pattern Experiments

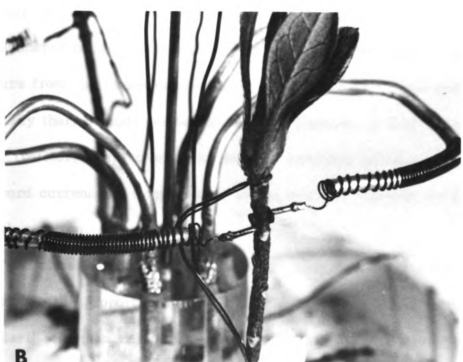
Experiment I: Preliminary freezing pattern: A budded stem 10 cm long of R. 'Maryann' was cut from a plant held in the 1.5°C growth chamber and prepared for the test apparatus. The apparatus shown in Figure 6A consisted of a hollow plexiglass cylinder into which was placed a cardboard container fitted with a moist sponge. Copper wires and solder contact support arms were wedged into a center plexiglass rod. Each 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 (Figure 6B). A small slice of peridermal tissue, 2 mm in diameter, was removed with a razor blade on opposite sides of the stem, 5 mm below the bud. On one side of the stem two additional slices were cut directly below the first and spaced at equal distances, approximately 10 mm apart. This is shown in Figure 5A (page 33). Into these cuts, which became the electrical contacts, a finely divided carbon-water paste was worked with a small brush. Each of the contact points was ringed with silicone to prevent current flow around the

Figure 6. Freezing pattern apparatus.

A. Overall view with cover in place.

B. Close-up of twig contacts.


A


B

the external surface. A small copper-constantan thermocouple was wrapped around the stem just below the bud. Then the stem was inserted into the moist sponge and the contact arms moved into position. A small drop of the carbon-water paste was put onto each platinum contact which was then pushed against the cut surface of the stem and held in place by the coiled wire spring (Figure 6B). These preparations were done quickly in the freezing chamber at 1.5^{o}C.

Wires from the apparatus, one from each contact, connected to a power source which fed a weak DC voltage (.2 to 2 volts) through the stem between the various contacts. By using a switching device the current flow could be routed between the different contacts, using one as a common contact; for example, circuits C-1, C-2 and C-3. Of the 25 switch positions odd numbered points registered current, even numbered points recorded temperature from the thermocouple on the stem. The last three points were a stationary thermocouple inside the freezing chamber, a 1 M resistor and a 100 K resistor, respectively. A pen type recording potentiometer was used to record current and temperature, and it could be adjusted for low, medium or high sensitivity.

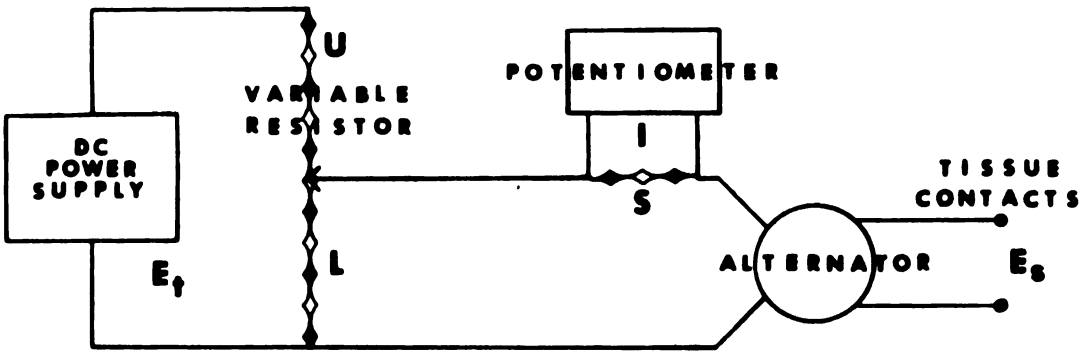
The electrical system is shown schematically in Figure 7A. In order to calculate the proportion of the voltage traveling through the tissue the formula $E_S=(E_t-uI)\frac{L}{L+u}$ was used (see Figure 7A for an explanation of terms).

Figure 7. Schematic diagram of electrical systems for freezing pattern determination in the freezing stress study.

A. Automatic system for Experiments I, IV and V.

B. Manual system for Experiments II, III, VI and VII.

A



E_t = Total voltage

E_s = Voltage in tissue sample

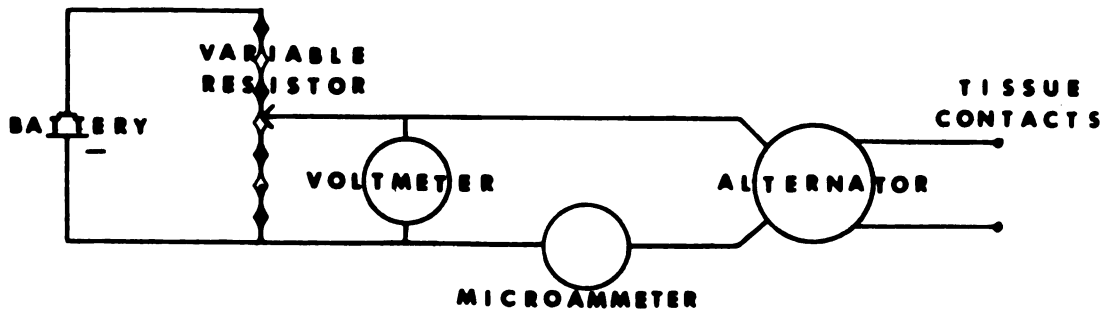
I = Current

L = Lower circuit voltage

U = Upper circuit voltage

S = Shunt resistor for potentiometer sensitivity

B



Voltage could be selected for each circuit by a lettered pin position on a variable resistor. For this experiment, circuit C-1, C-2 and C-3 had pin positions representing .3%, .8% and 1.8% of the total voltage, respectively. With the apparatus in place and the system in full operation, the freezing chamber was held at -1.5°C for several hours. Then the temperature was lowered manually at 5°C per hour to a low of -12°C. Prior to and after freezing, current and voltage were recorded. Resistance was then calculated by using the formula V=IR where V=voltage, I=current in microamps and R = resistance in ohms.

Experiment II: Manual contact check with R. 'Maryann': This experiment was performed to determine whether the platinum contacts affected the recorded freezing pattern. The contacts are pictured in Figure 6B. An apparatus set-up similar to the one described in Experiment I was prepared except that only the three vertical contacts on one side of the stem were used as shown in Figure 5B. There was no contact on the opposite side of the stem. A more simplified electrical system was constructed for this experiment using a 22 1/2 volt battery, voltmeter, microammeter and alternator as shown schematically in Figure 7B. Current flow through circuits 1-2, 2-3, 1-3 was switched manually. The temperature was recorded from an iron-constantan thermocouple with a galvonometer. One set of current and

voltage readings was taken at 1°C and another several hours later after the stem had frozen (-3°C). To reduce the amount of supercooling, a nucleating probe was used. This consisted of a small ball of wet cotton on the end of a wire stored in a freezer until needed. To initiate nucleation, a small drop of water was put on a leaf, then the frozen probe touched to the drop until it froze. In order to evaluate the ability of the azalea stem to withstand electrical current without damage, the voltage at microamp levels from 0 to 9 was recorded at the beginning of this experiment.

Experiment III: Manual contact check with R. 'Maryann' and R. poukhanense: This experiment was an additional check on the extent to which the contacts affected the freezing pattern. It was identical to Experiment II except that another apparatus was added containing a stem of R. poukhanense. Again, the three contacts were arranged vertically as shown in Figure 5C. Voltage and current were recorded and resistance calculated prior to and after freezing as in Experiment II.

Experiment IV: Freezing pattern: With the contacts evaluated, Experiment I was repeated in order to evaluate the freezing patterns of both cultivars. Both R. 'Maryann' and R. poukhanense were used, one twig of each in each of two apparatuses. Two contacts were made on each stem as pictured in

Figure 5D. The electrical system is described in Experiment I and diagrammed in Figure 7A. All circuits had a pin position corresponding to .6% of the total voltage. The freezing chamber was set at -1.5°C for 6 hours after which the temperature was lowered at 2°C per hour to a low of -12°C. After an hour at -12°C the chamber was turned off, allowing the twigs to thaw at 7°C per hour.

Experiment V: Conductivity pattern: This experiment was designed to evaluate injury to the twigs frozen in Experiment IV. Measuring conductivity of leachable cellular electrolytes is a commonly used method of evaluating injury to plant tissue after freezing (10, 11). At the end of Experiment IV, after the twigs had completely thawed, the potentiometer was switched to low sensitivity. This was done so that all four circuit patterns were back in the recording range, since two had gone off the scale. Current per volt values were calculated prior to and after freezing, and after thawing. Several hours after thawing, the twigs were hand sectioned to determine injury and survival.

Current Flow Experiments

In order to study the path of current flow through stem tissue, two experiments were designed using amaranth dye, a negatively charged ion, to evaluate electrophoretic mobility and to serve as a visual indicator of the current path.

Experiment VI: Dye flow in thin stem sections: A thin cross section of the upper stem through the contact point area, as described in Experiment I and pictured in Figure 6B, 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 (Figure 11E). Each paper strip was fastened to a metal contact which led to a voltage source that was regulated by a variable potentiometer to achieve a current level between 0 and 9 microamps. After the desired current level was achieved, a small drop of amaranth dye was spotted on the negatively charged paper strip. By using a switch, the current flow could easily be reversed. As the dye moved through the stem section, its path and relative rate of movement in different tissues were watched through the microscope. The experiment was repeated four times.

Experiment VII: Dye flow in intact stems: This experiment was designed using the same apparatus as in Experiment I and IV of the freezing pattern study (Figure 6A), with a few exceptions. 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 was positioned so it would be held between the stem and the contact. In this way a constant supply of dye was available to be

carried through the plant by the current. The upper leaves were removed from the twig, and the leaf traces plus the entire bud were coated with silicone to prevent transpirational influences on dye flow. As in Experiment VI, the apparatus was connected to a voltage source and variable resistor. 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 for dye flow differences or similarities to Experiment VI. A third apparatus complete with dye reservoir was set up, but without any current, for a check on dye flow as affected by diffusion and transpiration.

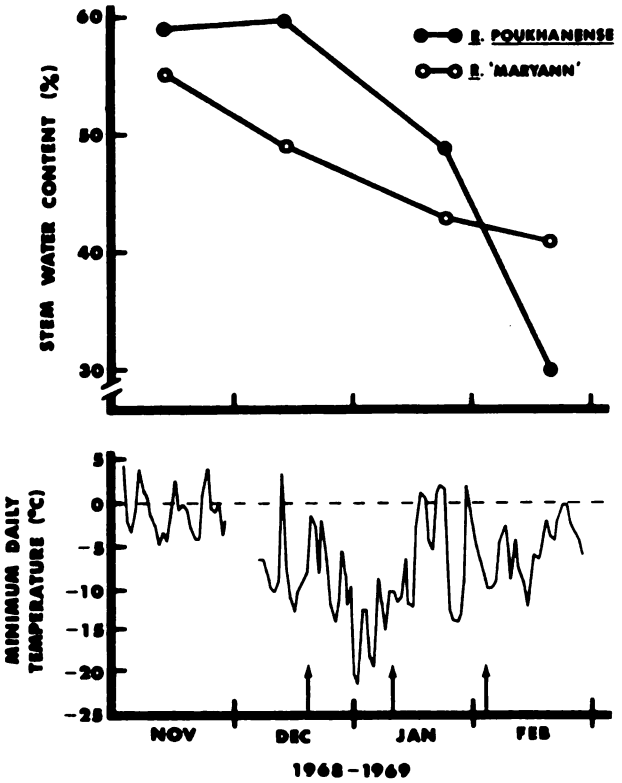
RESULTS

Freezing Injury and Survival Study

Outdoor Survey

During the winters of 1967 through 1970, the flower buds of three cultivars, R. 'Maryann,' R. 'Mikado,' and R. poukhanense, were first injured in late December to early January after exposure to temperatures near and below -20°C; see temperature curves in Figure 8. Many flower buds of R. poukhanense were killed, while only 1/5 of the buds of R. 'Maryann' were injured. Survival of these two cultivars was consistent for each year. However, survival of R. 'Mikado' varied considerably from year to year; high in 1967-1968 and 1969-1970, but low in 1968-1969. An example of bud survival before and after the late December - early January cold period is recorded in Table 1. Before mid December very little bud injury in any cultivar was observed. Continued sampling after early January revealed little further injury. On the basis of the contrast in survival between R. 'Maryann' and R. poukhanense, their desirable growth habits and the inconsistency in survival of R. 'Mikado'; R. 'Maryann' and R. poukhanense were selected for continued outdoor study and for laboratory tests.

Figure 8. Temperature and stem water content data from the outdoor survey for 1968-1969 and 1969-1970. Vertical arrows indicate flower bud injury sampling dates.


STEM WATER CONTENT (%)
60
50
40
30
E. POLYHANENSE
E. 'MARYANN'
MINIMUM DAILY TEMPERATURE (°C)
5
0
-5
-10
-15
-20
-25
NOV
DEC
JAN
FEB
1968-1969

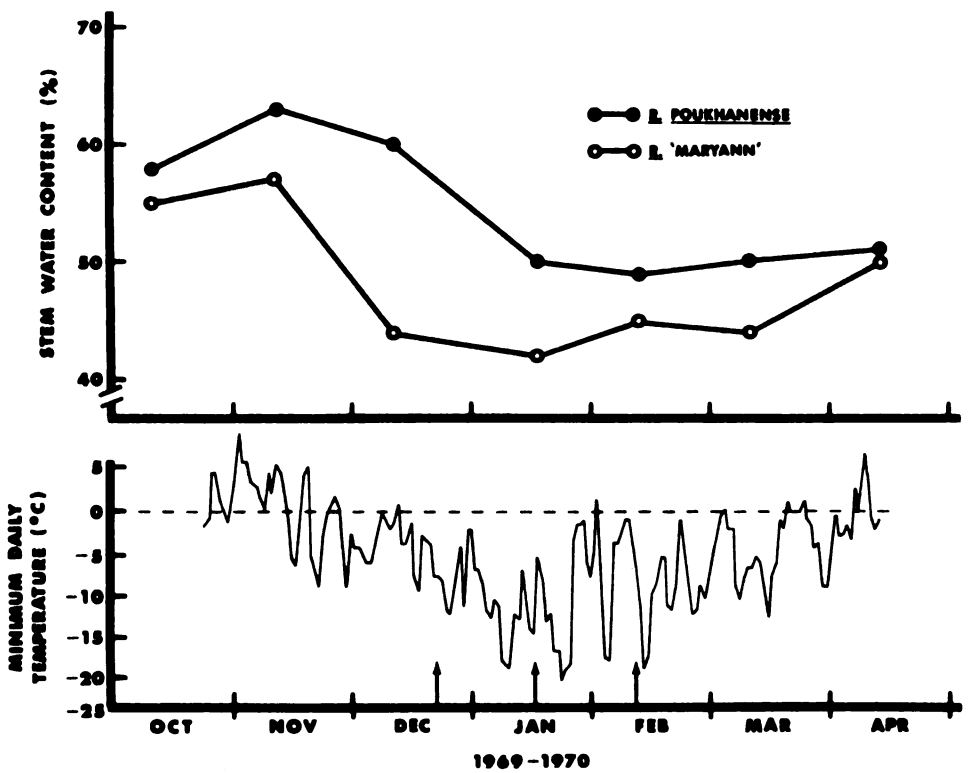

STEM WATER CONTENT (%)
70
60
50
40
E. POLYHANENSE
E. 'MARYANN'
MINIMUM DAILY TEMPERATURE (°C)
5
0
-5
-10
-15
-20
-25
OCT
NOV
DEC
JAN
FEB
MAR
APR
1969-1970

Table 1. Number of flower buds appearing normal or injured at various sampling dates prior to and after low temperature periods for three azalea cultivars.

Date	R. 'Maryann'		R. 'Mikado'		R. poukhanense	
	injured*	uninjured*	injured	uninjured	injured	uninjured
12/18/68	2	58	2	58	3	57
1/10/69	9	51	33	27	48	12
2/3/69	12	48	39	21	54	6
12/21/69	2	46	2	46	3	45
1/16/70	9	39	6	42	39	9
2/11/70	8	40	9	39	44	4

*Total for three replications

From the outdoor surveys the most evident characteristic of low temperature injury to flower buds was browning. This discoloration was usually restricted to the flower and pedicel tissues, although it was occasionally found in the upper part of the stem just below the bud. Browning was first evident at the ovary base and in the vascular tissue. There was no distinguishable variation of the browning pattern between the cultivars. When injury occurred to a particular developing flower within a bud, it was invariably killed; there was no evidence of partially injured flowers. Injury

Figure 9. Photographs of R. poukhanense stems injured outdoors.

A. Longitudinal section with vascular and cortical splits (x 10).

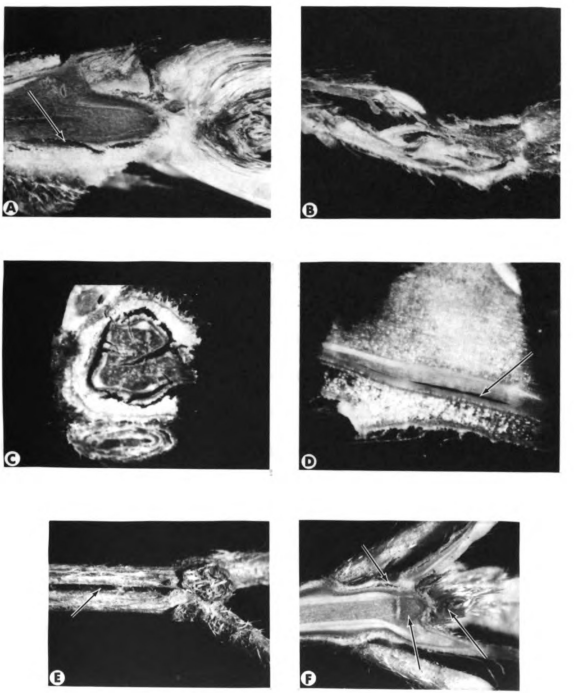
B. Severe upper stem splits (x 4).

C. Cross section with severe splits (x 10).

D. Longitudinal section with split in vascular tissue (x 30).

E. Split in two year old stem (x 2).

F. Longitudinal section in June showing injury in the bud and upper stem, and normal vegetative shoot development (x 4).


A
B
C
D
E
F

to one flower within a bud seemed to have no effect on other flowers in that bud, as buds containing both injured and uninjured flowers could be found. Most buds contain two to three flowers.

Sectioning of injured R. poukhanense twigs revealed splits in the cambial area of the upper stem and pedicel vascular tissue, and in the cortex of the upper stem. As the severity of injury increased, splits occurred throughout the upper stem tissues. Small cortical splits had no effect on the survival or growth of vegetative buds which are borne below the flower bud. Even injury to the flower bud and upper stem had no effect on vegetative bud growth unless stem splitting was severe, as in Figure 9B. In contrast to R. poukhanense, R. 'Maryann' flower buds were injured much less frequently during the winter. When they were injured, no vascular splitting was evident. Photographs depicting injury as described above are presented in Figure 9.

Measurements of water content of upper stem sections during the winter sampling periods revealed that R. poukhanense had a higher water content than R. 'Maryann', (Figure 8). Only during December and January, however, was there a statistically significant difference. In 1970, the difference in March was also significant. Moisture content data are recorded in Table 2. There was no significant difference in flower bud moisture content. Although the

Table 2. Moisture content differences of upper stem sections of azalea cultivars during the sampling periods of 1968-1969 and 1969-1970.

	Fresh-Dry Weight Difference (gm)	
	R. 'Maryann'	R. poukanense
11/14/68	.24	.39
12/13/68**	.16	.30
1/23/69*	.21	.30
2/20/69	.07	.09
10/9/69	.27	.34
11/10/69	.24	.32
12/9/69**	.16	.34
1/16/70**	.12	.25
2/11/70	.11	.14
3/9/70*	.06	.10
4/12/70	.13	.10
6/11/70	.12	.12

*Cultivar difference significant at .05 level.

**Cultivar difference significant at .01 level.

Figures are an average of three replications in 1968-1969 and four replications in 1969-1970.

values resembled those for the upper stems, they were extremely variable between sampling dates and between replications within a sampling date.

Laboratory Studies

Injury and survival determination: Flower buds of intact plants and excised twigs of R. poukhanense were injured, as determined initially by browning, at a consistently higher temperature than the buds of either intact plants or excised twigs of R. 'Maryann.' For R. poukhanense the killing temperatue was approximately -12°C, while for R. 'Maryann' it was approximately -15° to -18°C. Twigs of both cultivars, whether intact or excised, froze at or very near -2°C, provided the cooling rate remained at or less than 2°C per hour and the twigs were frozen in a high humidity atmosphere, (moistened plastic bag). Greenhouse flowering was one method of evaluating survival in the freezing tests. A flowering twig is shown in Figure 11D. The second method utilizing neutral red is described below.

Injury to flower buds in the freezing chamber was characterized by browning. Browning was first evident at the ovary base of injured flowers within three to four hours after thawing. Immediately thereafter, discoloration progressed downward through the vascular system of the pedicel and upper stem, and also upward into the flower parts. Discoloration moved more slowly into the pith. This pattern was consistent between cultivars and similar

Figure 10. Photographs of R. poukhanense flower buds and stems from the laboratory studies.

A. Longitudinal section of injured flower bud with splits and extensive browning (x 12).

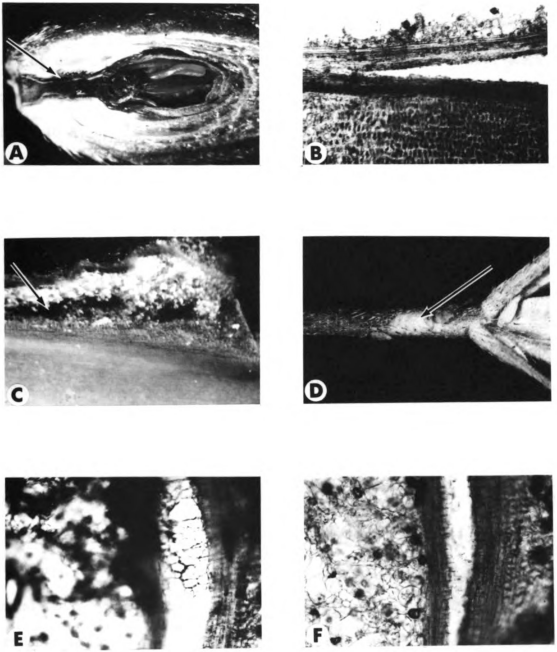
B. Longitudinal stem section with split in vascular tissue (x 200).

C. Longitudinal stem section with cortical splits (x 40).

D. Green colored area on upper stem (x 1.5).

E. Longitudinal stem section with large ice mass in vascular tissue (x 400).

F. Same section after thawing (x 400).


A
B
C
D
E
F

to that observed in the outdoor survey.

Twigs of R. poukhanense from the test freezing chamber, whether intact or excised, commonly had splits in the cambial area of upper stem and pedicel vascular tissue, and occasionally had splits in the cortex, (Figure 10 A, B, C). Small cortical splits had no effect on flower bud or upper stem survival. Both vascular and cortical splitting were similar to, but usually less severe than, that seen outdoors. By contrast, splitting was never seen in R. 'Maryann' twigs. Based on the similar results with intact and excised twigs, the use of whole plants was discontinued.

To produce a graded series of injury, twigs were subjected to a series of temperatures above the killing temperature. There was a range of approximately $3^{o}C$ between the temperature of initial injury and that which caused complete decimation. By using the neutral red plasmolysis technique, injured tissues (frozen well below their killing temperature) could be readily distinguished, because they contained no discernible amount of neutral red except for a slight red tint around the cell walls. Plasmolyzed living cells had very distinct red centers and could be deplasmolyzed, then plasmolyzed again. Neutral red staining results were similar regardless of the solution used (sucrose, salt or water). When water was used, subsequent plasmolysis enabled the living cells to be identified more easily. The KOH destaining solution did not affect neutral red stained cells, while for dead cells the slight red tint was easily removed. On the basis of these results, a living cell was

considered to be one that was stained bright red and plasmolyzed (simply for easy visibility). Both azalea cultivars were characterized by an area in the pith of the upper stem in which there were many more living cells (Figure 11A). This area could be recognized by a green color on the outside of the stem, particularly in R. poukhanense (Figure 10D).

Initial injury in either azalea cultivar, as determined by neutral red staining, was first evident in the pith of the upper stem at the junction where the area of the greater number of living cells adjoins the region of fewer living cells below (Figure 11A). As the injury sequence progressed by exposure to slightly lower temperatures, greater areas in the upper stem pith were injured until the entire pith area was damaged (Figure 11C). Whether twigs were thawed in the freezer at 2^{o}C per hour or at room temperature, there was no difference in survival or injury pattern. Whenever the entire upper stem pith was injured, one or more of the developing flowers was also killed. There was no intermediate step in the sequence between entire pith damage and flower injury. The two occurred simultaniously. Injury to the flower began in the upper pedicel and ovary, then progressed throughout the flower. If, for example, the stamens were not killed by the freezing treatment, they would succumb after other injured flower parts had begun to degenerate. Initial pith injury had no effect on flower bud survival.

Figure 11. Photographs of R. 'Maryann' (A, B, C, E) and R. poukhanense (D) from the laboratory studies.

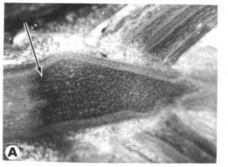
A. Longitudinal section of upper stem stained with neutral red, arrow indicates area of initial injury (x 12).

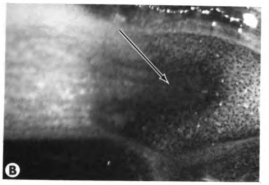
B. Similar section with part of the pith injured (x 18).

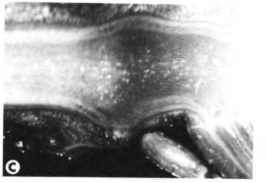
C. Similar section with entire pith injured (x 15).

D. Twig flowering after freezing treatment (x .25).

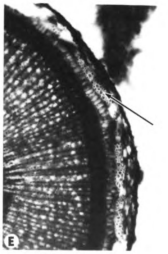
E. Cross section showing paper strip contact, arrow indicates primary path of dye movement (x 100).


A


B


C


D


E

It was necessary to determine survival by staining soon after the freezing test, because within 12 hours after injury, browning and discoloration hindered visual observation. In the initial stages of injury, when only small sections of pith were damaged, browning was no hindrance to determining survival in the remaining stem and bud, since browning of small areas was restricted to that immediate vicinity.

Moisture content: In the growth chamber, R. 'Maryann' and R. poukhanense retained their characteristic difference in water content of the upper stem. Although it was not statistically significant, the difference of 49% for R. 'Maryann' and 54% for R. poukhanense remained throughout the storage period. Both significant and non-significant moisture content differences were recorded in the outdoor survey. By drying the R. poukhanense twigs for 4 days in the growth chamber, the water content was significantly reduced (at .01 level) from 54% to 46%. When the dried twigs were test frozen, there was no evidence of vascular or cortical splitting as was common with fresh R. poukhanense twigs. Dried R. 'Maryann' twigs reacted no differently than fresh twigs. Control twigs, dried but not frozen, were unaffected by the drying, as determined by the neutral red stain and by allowing the twig to flower in the greenhouse. Drying the R. poukhanense twigs also enabled them to survive lower temperatures, nearly those that injured R. 'Maryann' twigs.

Qualitative chemical tests: Throughout all the hand sectioning and observation, no anatomical differences were found between R. 'Maryann' and R. poukhanense. Similarly, none of the qualitative chemical tests produced any positive difference.

Ice structure and occurrence: In azalea cultivars R. 'Maryann,' R. 'Mikado' and R. poukhanense ice was found throughout the twigs. The ovary and other flower parts were very difficult to section while frozen, and therefore, no ice crystals were observed microscopically. Upper stems and pedicels of R. poukhanense contained scattered, large ice massed in the vascular tissue (Figure 10E) and the cortex, causing splitting. Vascular splits formed at the cambium between the xylem and phloem. Both vascular and cortical ice masses occurred at locations where splits had been found in R. poukhanense stems outdoors. No ice masses or splits were evident in R. 'Maryann' or R. 'Mikado' twigs. As the sections thawed, some of the vascular and cortical splits disappeared, that is, when the ice melted, the tissues regained their original positions. Some of the larger vascular and cortical splits remained distinct after thawing. Besides the large splitting ice masses in many of the R. poukhanense stems, small crystals were dispersed through the tissues of all cultivars sectioned.

Freezing Stress Study

Freezing Pattern Experiments

Experiment I: Preliminary freezing pattern: Prior to freezing, the level of current flowing through tissue area A (circuit C-2) and area B (circuit C-3) remained relatively constant. However, as freezing occurred the current dropped sharply and the resistance increased markedly as recorded in Table 3. The twig did not freeze, as indicated by the increased resistance, until the temperature was lowered to -10°C. Tissue areas and circuits were shown in Figure 5.

Total resistance was calculated from the formula V=IR, in which V = voltage in the tissue, I = current flow in microamps (μA) and R = resistance in ohms[1].

Table 3. Electrical values for twig areas of R. 'Maryann' prior to and after freezing in Experiment I.

Temperature	Tissue Area A			Tissue Area B		
°C	I	V	R	I	V	R
1.5	1.39	.18	130	1.18	.40	339
0.5	1.39	.18	130	1.18	.40	339
-1.5	1.25	.18	144	1.08	.40	360
-5.0	0.97	.15	155	0.78	.33	424
-8.0	0.89	.15	169	0.70	.33	472
-10.0	0.18	.15	834	0.18	.33	1833

I = current (μA)
V = voltage
R = resistance (ohms)

[1]All resistance values in this thesis are expressed as ohms x 10^3.

Experiment II: Manual contact check with R. 'Maryann': From the data in Table 4 it is evident that as freezing occurred the total resistance increased considerably, as was noted in Experiment I. Prior to freezing, readings were stable in the three tissue areas, A, B and C; circuits 1-2, 2-3 and 1-3, respectively. By using the nucleating probe, the twig was prevented from supercooling as it did in Experiment I.

Table 4. Electrical values for twig areas of R. 'Maryann' prior to and after freezing in Experiment II.

Tissue area	Prefreeze (1°C)			Frozen (-3°C)		
	A	B	C	A	B	C
Current (μA)	5.0	5.0	5.0	5.0	5.0	5.0
Voltage	4.1	3.5	6.8	15.0	13.1	23.8
Resistance (ohms)	820.0	700.0	1360.0	3000.0	2620.0	4760.0

This experiment also revealed that the platinum contacts accounted for an appreciable part of the total resistance; 24% prior to freezing and 32% after freezing. Contact and tissue resistance were calculated as follows:

Prefreeze

$$2C+R_A+2C+R_B = 1520 \text{ ohms}$$
$$2C+R_C = 1360 \text{ ohms}$$
$$2C = 160 \text{ ohms}$$

$$2C+R_C = 1360 \text{ ohms}$$
$$2C = 160 \text{ ohms}$$
$$R_C = 1200 \text{ ohms}$$

R/mm of tissue = 1200 ÷ 30mm = 40 ohms
40 ohms/mm x 14mm = 560 ohms
2C+560 ohms = 760 ohms
2C = 200 ohms
$2C = \frac{160+200 \text{ ohms}}{2}$ = 180 ohms

$\frac{180 \text{ ohms}}{760 \text{ ohms}}$ = 24% of total resistance

C = contacts
R_A = resistance of tissue area A (area shown in Figure 5B)

Frozen

$2C+R_A+2C+R_B$ = 5620 ohms
$2C+R_C$ = 4760 ohms
2C = 860 ohms

$2C+R_C$ = 4760 ohms
2C = 860 ohms
R_C = 3900 ohms

R/mm of tissue = 3900 ÷ 30 = 130 ohms
130 ohms/mm x 14mm = 1820 ohms
2C+ 1820 ohms = 2810 ohms
2C = 990 ohms
$2C = \frac{860+990 \text{ ohms}}{2}$ = 935 ohms

$\frac{935}{2810}$ = 32% of total resistance

C = contacts
R_A = resistance of tissue area A (area shown in Figure 5B)

The preceeding data do not take into account the contact width which was 2mm. When considered the calculations were as follows:

Prefreeze

Area A = 2C+R/14mm = 830 ohms
Area B = 2C+R/14mm = 690 ohms
1520 ohms

Average of 2C+R/14mm = 760 ohms
R/30 mm = 1360 ohms
R/16mm = 600 ohms
R/mm = 37.5 ohms

Frozen

Area A = 2C+R/14mm = 3000 ohms
Area B = 2C+R/14mm = 2620 ohms
5620 ohms
Average of 2C+R/14mm = 2810 ohms
R/30mm = 4760 ohms
R/16mm = 1950 ohms
R/mm = 122 ohms

C = contact
R = resistance

Whether or not the contact width was taken into account, values for resistance per mm of tissue were similar. In Figure 12 the voltage - current plot indicates that the tissue was unaffected within the range used, since the line remained linear between 0 and 9 microamps. All experiments were carried out within this current range.

Experiment III: Manual contact check with R. 'Maryann' and R. poukhanense: The data for R. 'Maryann', as recorded in Table 5, were similar to those in Experiment II, however, the percent of resistance attributed to the contacts was less; 19% unfrozen, 28% frozen. Calculations similar to those shown in Experiment II revealed:

Prefreeze

Average resistance for two contacts = 98 ohms
Resistance /mm of tissue = 34 ohms
Resistance attributed to contacts = 19%

Frozen

Average resistance for two contacts = 612 ohms
Resistance /mm of tissue = 125 ohms
Resistance attributed to contacts = 28%

Table 5. Electrical values for twig areas of R. 'Maryann' prior to and after freezing in Experiment III.

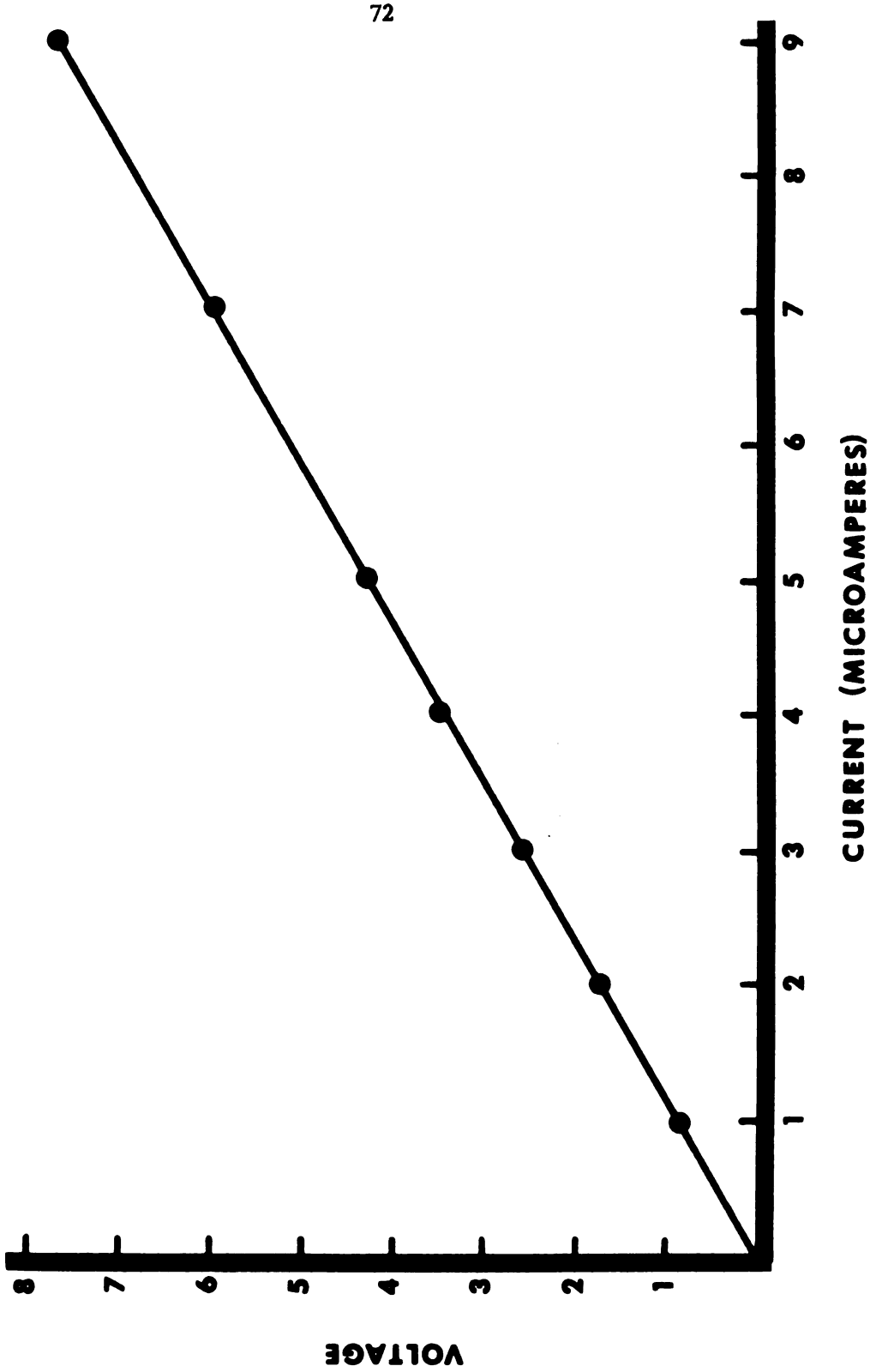
	Prefreeze (1°C)			Frozen (-3°C)		
Tissue area	A	B	C	A	B	C
Current (μA)	5.0	5.0	5.0	5.0	5.0	5.0
Voltage	2.8	2.4	4.8	11.0	10.8	19.0
Resistance (ohms)	560.0	480.0	960.0	2200.0	2160.0	3800.0

The data for R. poukhanense, as recorded in Table 6, were similar to those for R. 'Maryann' in that resistance markedly increased as the stem froze. However, the contact resistance accounted for a much greater part of the total resistance. Calculations similar to those in Experiment II revealed:

Prefreeze

Average resistance for two contacts = 120 ohms
Resistance /mm of tissue = 25 ohms
Resistance attributed to contacts = 37%

Figure 12. Current passing through unfrozen R. 'Maryann' stem as affected by voltage applied.


VOLTAGE
1
2
3
4
5
6
7
8
CURRENT (MICROAMPERES)
1
2
3
4
5
6
7
8
9

Frozen

Average resistance for two contacts = 1120 ohms
Resistance /mm of tissue = 110 ohms
Resistance attributed to contacts = 56%

Table 6. Electrical values for twig areas of R. poukhanense prior to and after freezing in Experiment III.

	Prefreeze (1°C)			Frozen (-3°C)		
Tissue area	A	B	C	A	B	C
Current (μA)	5.0	5.0	5.0	5.0	5.0	5.0
Voltage	2.0	1.2	2.6	11.5	8.4	14.2
Resistance (ohms)	400.0	240.0	520.0	2300.0	1680.0	2850.0

This experiment further demonstrated that contact resistance does contribute to the freezing pattern. But with the contacts accounted for, there was still a great increase in resistance of the tissue as it froze.

Experiment IV: Freezing pattern: Data, temperature and current, from this experiment are recorded in Table 7. Again there was a sharp rise in resistance as freezing occurred. Values for contact resistance were taken from Experiment III, since no accurate evaluation of contacts could be made in this experiment. Contact resistance was considered to be no better than the values for R. 'Maryann' in Experiment III prior to and after freezing (98 and 612 ohms, respectively). Prior to freezing the tissue contributed only

Table 7. Data (temperature and current) and calculations of M_r in Experiment IV.

Twig	Temperature (°C)	Current (μA)	Absolute resistance (x 10^3)	Absolute tissue resistance* (x 10^3)	Corrected current** (μA)	Relative current (μA)	Relative Viscosity	M_r***
R. 'Maryann' #1	-2.0	1.44	139	41	.025	1.00	1.00	1.00
	-1.8	.23	870	258	.004	.12	1.02	.12
	-2.5	.18	1111	499	.002	.08	1.01	.08
R. poukhanense #1	-2.0	1.71	117	19	.053	1.00	1.00	1.00
	-1.2	.26	770	158	.006	.12	1.03	.12
	-2.0	.18	1111	499	.002	.03	1.00	.03
R. 'Maryann' #2	-2.0	1.48	135	37	.027	1.00	1.00	1.00
	-3.2	1.48	135	37	.027	1.00	1.03	1.03
	-3.5	.20	1000	388	.003	.10	1.07	.11
	-4.0	.18	1111	499	.002	.07	1.06	.07
R. poukhanense #2	-2.0	1.86	107	9	.111	1.00	1.00	1.00
	-2.0	.26	768	156	.007	.06	1.00	.06
	-2.5	.18	1111	499	.002	.02	1.01	.02

* Total resistance minus average resistance for two contacts before and after freezing (98 and 612 x 10^{-3}ohms, respectively).

** 1÷absolute tissue resistance.

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

30% to the total resistance, while after freezing the tissue contribution was 50%. The final column of data in Table 7 is presented as the relative content of liquid extracellular water (M_r). As freezing occurred the M_r dropped very sharply approaching zero, indicating that the amount of liquid water decreased very rapidly (Figure 13). Both of the curves for R. poukhanense fell to a slightly lower level than the curves for R. 'Maryann'. Before freezing was completed, the temperature rose an average of 1.2°C for the two R. poukhanense twigs and .5°C for both R. 'Maryann' twigs.

In Figure 14 the characteristic freezing pattern of azalea twigs is compared to that of cherry and apple twigs (9). When the rate of temperature change was slow (2°C/hr), the path of current flow in azalea twigs was quickly blocked, as a result of the intercellular liquid being converted to ice within several minutes after freezing had begun. However, freezing is a much slower process in the cherry and apple twigs. After nearly an hour ice continued to accumulate as indicated by the much more gradual decrease in current flow.

Experiment V: Conductivity pattern: As can be seen in Table 8, the microamp per volt values for R. poukhanense were much higher after thawing than before freezing. Values for R. 'Maryann', however, were similar before freezing and after thawing. Hand sectioning several hours after completing the experiment disclosed that the bud and upper stem of R. poukhanense twig #2 were dead (as determined by extreme browning); having been injured by

Figure 13. Freezing patterns of R. 'Maryann' (M) and R. poukhanense (P) twigs.

M_r = relative content of liquid extracellular water.

A. Twigs from apparatus 1.

B. Twigs from apparatus 2.

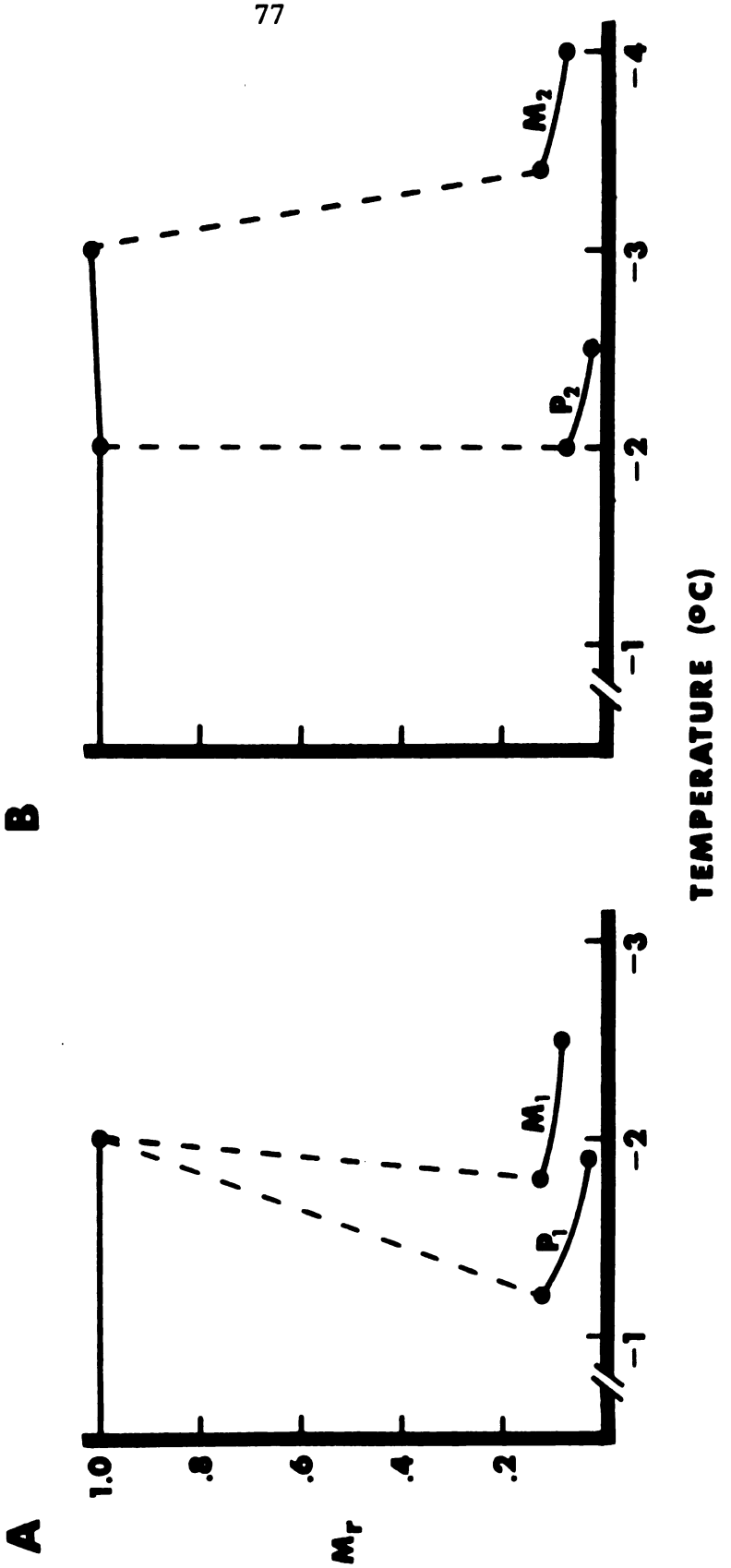
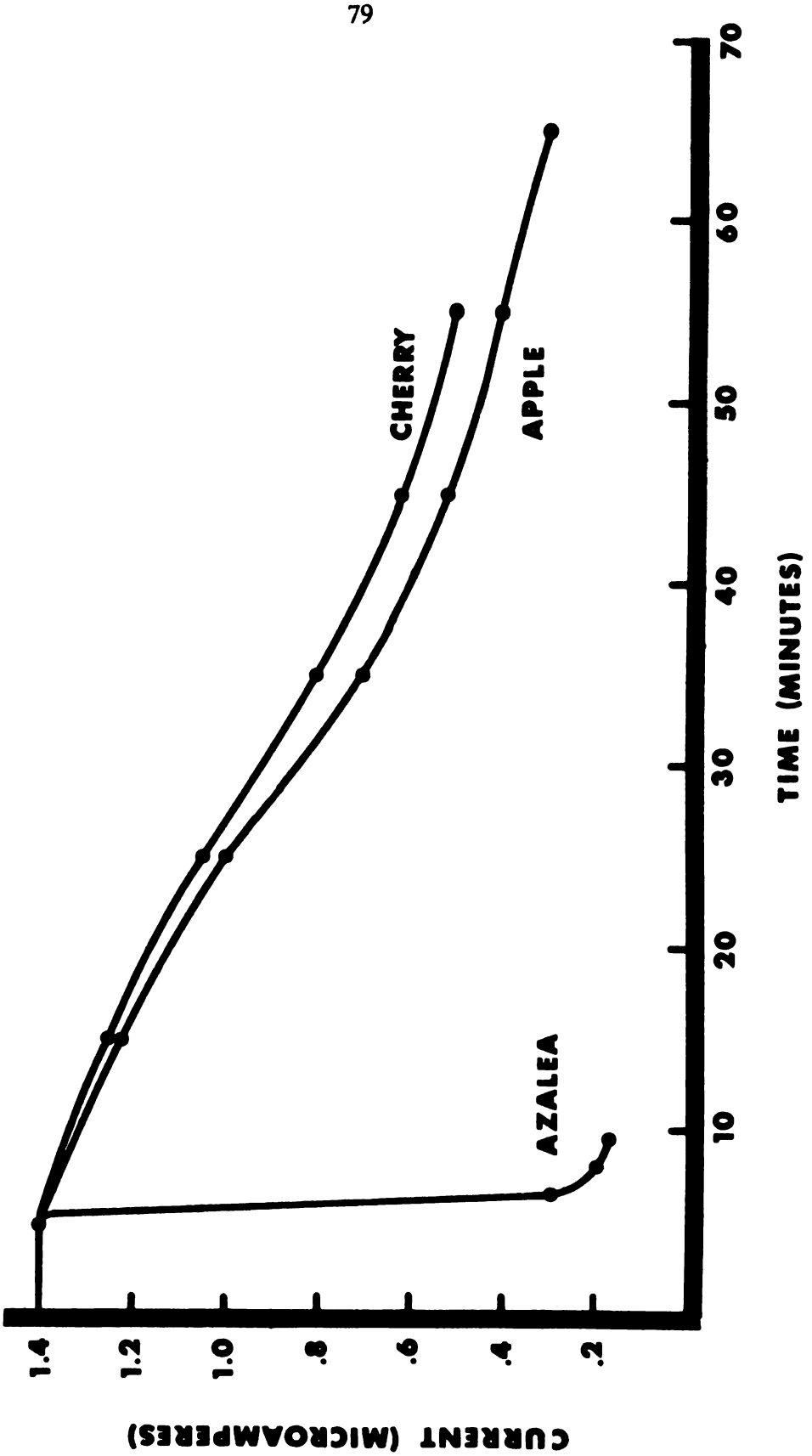

A
B
1.0
.8
.6
.4
.2
M_r
M_1
P_1
M_2
P_2
-1
-2
-3
-4
TEMPERATURE (°C)

Figure 14. Freezing pattern comparison among azalea, apple and cherry twigs [from Dennis, et al. (9)].


CURRENT (MICROAMPERES)
1.4
1.2
1.0
.8
.6
.4
.2
TIME (MINUTES)
10
20
30
40
50
60
70
CHERRY
APPLE
AZALEA

the freezing treatment. This is reflected in the much higher thawed value for R. poukhanense twig #2 in Table 8. When R. poukhanense twig #1 was sectioned only slight injury could be found in the upper stem pith. The bud had not been injured. Neither of the R. 'Maryann' twigs were injured by the freezing treatment as indicated by sectioning and by the very small differences between prefreeze and thawed values in Table 8.

Table 8. Electrical values for R. 'Maryann' and R. poukhanense twigs prior to and after freezing, and after thawing in Experiment V.

	Prefreeze		Frozen		Thawed	
Twig	Temp (°C)	Current per volt	Temp (°C)	Current per volt	Temp (°C)	Current per volt
R. 'Maryann' #1	-2.0	7.2	-2.5	(.9)*	3.5	9.7
R. poukhanense #1	-2.0	8.6	-1.8	(.9)	3.5	15.2
R. 'Maryann' #2	-2.0	7.4	-4.0	(.9)	3.5	7.8
R. poukhanense #2	-2.0	9.3	-2.0	(.9)	3.5	28.5

*Values lower than recorder scale

Current Flow Experiments

Experiment VI: Dye flow in thin stem sections: After moving along the negatively charged strip and reaching the section, the amaranth dye very quickly began moving extracellularly around the stem in the outer cortex just

beneath the periderm (Figure 11). The dye may have flowed in the periderm also, but could not be seen, because of the dark color of the periderm. Very soon after moving into the cortex, the dye could be seen on the positive contact, having traversed the section via the cortex. Within 2 to 5 minutes amaranth began to accumulate near the negative contact and migrate inward into the phloem and xylem. As the dye progressed inward across the vascular tissue, it tended to accumulate at the boundary between the xylem and the pith. No dye could be seen in the pith. After 1 hour the entire stem section, outside the pith, was stained lightly with the amaranth, with greatest concentrations adjacent to the negative contact. When small notches were cut into the periderm, simulating the contact areas described in the freezing pattern experiments, the flow of dye was similar to that in sections with intact periderm. Thus, the periderm was no barrier to the dye movement or current flow.

Mobility of the amaranth was proportional to the current applied, whether measured on the paper strip or through the tissue. If the current was reduced by one-half, the dye flow was similarly reduced. The rate of flow was much more rapid through cortex than through other tissues. When the current direction was reversed, the dye was nearly removed from the cortex, but very little was dislodged from the phloem.

Experiment VII: Dye flow in intact stems: In this experiment the dye flow pattern was very similar to that observed in the thin stem sections of

Experiment VI. After 5 hours a thin section cut through the contact area disclosed that considerable dye had accumulated throughout the periderm and cortex. The phloem was darkly stained for only one-half of the stem circumference, while the xylem was heavily stained only in a band in a line with the negative contact. A light coloration could be seen throughout the section in areas not heavily stained. There was slight vertical dye movement, after 5 hours, restricted to the width of the contact slice in the periderm cortex, phloem and xylem. After 20 hours the dye reservoir was empty. Very dark concentrations of dye were massed in the periderm, cortex, phloem and xylem adjacent to the positive contact. The stem without current had a small amount of dye extending 2mm above and below the contact. None of the dye had moved laterally beyond the cortex.

DISCUSSION

Acclimation

For plants to survive the adverse effects of freezing, their cells must be in a physiologically hardened condition. Not all plants are able to harden or acclimate as evidenced by most tropical plants which are incapable of surviving freezing or temperatures near freezing. Azaleas and other perennial plants of northern climates are able to acclimate in response to late summer and early fall environmental conditions. Thus, the azaleas studied were not injured by fall frost. However, such cultural practices as late fertilization could induce fall frost susceptibility. Susceptibility to spring frosts is often a critical problem, particularly in some fruit crops, because the plants flower while the danger of frost is still imminent. Spring frosts are of no consequence to evergreen azaleas, since they flower after the danger of frost. The evergreen azaleas studied are adapted to fall and spring environmental conditions in mid Michigan.

Freezing Stresses

When studying tissue freezing patterns, it is important to know that the pattern is characteristic of the living, unaltered tissue and not the result

of some effect induced by the measurement procedure. The electrophoretic mobility technique has been shown to be reliable in measuring the relative amount of extracellular water remaining liquid as freezing progresses (53, 54). Provided low voltage (less than 5 volts per cm) of low frequency is used, the path of mobility is restricted to the extracellular continuous liquid system through which ions diffuse freely. Electrical conductivity can be used to evaluate changes in liquid content in temperature only when conductivity correlates with mobility, a function of effective viscosity.

Conditions of the experiment must be controlled so as not to affect the freezing pattern. The cooling rate should be slow (1-2^{o}C/hour) and the twigs should be inoculated in order to avoid supercooling. Since test conditions were rigidly controlled, they were eliminated as a factor affecting the freezing pattern in these studies.

The ability of azalea tissue to withstand low voltage, low frequency direct current was shown by the fact that tissue resistance was independent of the voltage applied. At higher voltages the resistance would have decreased with increasing voltage; an indication of electrolyte leakage from the cells. Azalea tissue was, thus, unaffected by the current applied (less than 9 microamps).

In the freezing pattern experiments the contact resistance was far from negligible. Nevertheless, the contact resistance could be subtracted from the

total, leaving a greater amount of resistance accounted for by the tissue. Even though the resistance of contacts was appreciable, the freezing pattern remained unchanged.

Since the freezing pattern technique is based on electrical conductivity as a measure of mobility (a function of effective viscosity), it is important to show a direct relationship between conductivity and mobility. Amaranth dye was used as a visible indicator of mobility. Mobility was found to be directly proportional to current flow in azalea tissue.

Determining the path of current flow is an additional important consideration when studying freezing patterns. The path of current flow in azalea twigs, as determined by the amaranth mobility, was primarily through the periderm and cortex tissues and secondarily through the vascular tissue. This means that the observed freezing patterns occurred for the most part in the periderm and cortex. However, Dennis et al. (9) found the freezing pattern of azalea to be similar for the wood (xylem and pith) and for the bark (periderm, cortex and phloem). The determined freezing pattern is thus representative of the entire stem.

From the preceeding discussion, there can be little doubt that the freezing patterns observed were characteristic of the azalea tissue and not a result of the system or conditions of measurement. Since the pattern was a conjunctive nonequilibrium one, it was obvious that the twigs were not tender, but physiologically hardy. If the twigs had been tender, the freezing pattern would have

been distinctly different as a result of protoplast rupture. The tissue did not supercool. The pattern would have been a drifting one if areas of the stem had been isolated by barriers to ice growth and had supercooled while adjacent tissues froze.

Both azalea cultivars had a nonequilibrium freezing pattern. This type of pattern indicated that as freezing occurred, there was a rapid readjustment of extracellular water and a sudden growth of ice. During nonequilibrium freezing, ice is directly involved in stress. A nonequilibrium freezing pattern was unexpected in the azalea twigs, because previous work with cherry and apple twigs (9) and barley leaves (53, 54) showed a smooth equilibrium pattern. Nonequilibrium patterns have been found to be characteristic of certain hydrated tissues. In barley, the leaves and upper crown tissues had equilibrium patterns, while roots and lower crown tissues had nonequilibrium patterns (54). During nonequilibrium freezing water is an important consideration. A difference in water content between the two cultivars was evident. The exotherm for the R. poukhanense stems was nearly double that for R. 'Maryann'. Another indication of a water content difference was the breaking point in the freezing curves. The transition from the nonequilibrium to the lower equilibrium portion of the curves occurred at a lower M_r (relative content of liquid extracellular water) value for R. Poukhanense.

As was evident from the abrupt drop in conductivity during freezing of azalea twigs ice quickly blocked the path of current flow. Ice formation was independent of decreasing temperature. This suggests that there was little interaction between much of the extracellular water and the cell wall. By not being associated with the cell wall much of the water was readily available for freezing immediately after initial inoculation (ca. $-2^{o}C$). The lower part of the curve was an equilibrium pattern where ice formation was a function of decreasing temperature; however, at this point, very little water remained in the liquid state. In contrast to azalea, apple and cherry twigs followed a smooth equilibrium pattern during which ice formation was a continuing function of decreasing temperature (9). This suggests a much greater interaction between the free water and the cell wall in apple and cherry stems.

An interesting observation was made by Dennis et al. (9) that the freezing patterns of azalea and also cherry was unchanged whether the tissue was dead (autoclaved or boiled) or alive. Thus, the pattern of ice formation bears little relation to the physiological state of the tissue. However, when the tissue structure of azalea was disrupted by grinding or slivering the pattern changed from nonequilibrium to equilibrium. This strongly suggests that structural pecularities of the azalea stem were responsible for the nonequilibrium freezing pattern.

A conjunctive nonequilibrium freezing pattern indicated that injury from

the growth of ice between cells was possible because of critical stress. Freezing 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. Tissues with a high water content are particularly susceptible to injury during an abrupt (nonequilibrium) freeze. Since ice formation was independent of decreasing temperature and there was little interaction between free water and the cell wall, water froze before it had time to move and before any tissue adjustment could occur. Rapid ice formation coupled with a high water content could cause splitting. Even at a lower water content ice crystals could have an adverse effect by causing cells to contract and slip against the extracellular crystals.

In the twigs of both azalea cultivars moderate sized ice crystals were dispersed throughout the tissues. Evenly distributed crystals are characteristic of tissues which freeze quickly. Kinetics inhibitors which interfere with ice crystal structural development have been isolated from certain varieties of winter cereals (55). Similar inhibitors are not suspected in either azalea cultivar because of the size of dispersed ice crystals.

Water content and ice crystal development appear to be very important factors affecting survival of the evergreen azaleas studied. The possibility of protoplasmic properties directly affecting survival should not be overlooked.

However, cultivar differences in protoplasmic resistance to freezing stress are not significant because the difference is a function of the amount of ice dispersed between cells. Also, the cultivar difference is minimized when the water content is adjusted to a comparable level in both cultivars.

Upper stem water content was an obvious difference between the two azalea cultivars. Rhododendron poukhanense had a significantly higher water content than R. 'Maryann' during December and January. The water content of R. 'Maryann' dropped sharply between November and December, while for R. poukhanense it remained constant or dropped only slightly. For the December sampling periods of both years, the average water content was 46% in R. 'Maryann' stems and 60% in R. poukanense stems. Any reason for this different behavior would be speculative since environmental conditions were similar for both cultivars. Varietal differences in water uptake from the soil and atmosphere, transpiration, translocation, and respiration might account for water content differences (87). In a study of Pinus root development during fall and winter, Wilcox (94) described the formation of a suberized layer enclosing the apical region and developing along the endodermis. If a similar situation occurred differentially in the two azalea cultivars, restricted water uptake might have accounted for the observed difference. Rhododendron was among several genera found to contain the suberized layer (96).

Since the ice formation was accomplished so rapidly in azalea twigs, water had no time to move away from critical tissues to areas of accumulation. If such a movement had occurred, cells (for example, pith) at a distance from the nucleation center would not have been injured by the proximity of ice. By contrast, water in cherry stems had time to move to centers of nucleation as freezing progressed, producing large crystals and occasionally "glaciers" in the cortex. When cherry stems were cooled rapidly and water had less time to migrate, crystals appeared more like those in azalea (9).

When the water content of stems was very high (ca. 60% as in R. poukhanense), severe splitting often resulted. Severe stem splitting injured both vegetative and flower buds, because of vascular disruption and the drying effect of open splits. Stem splitting can also be caused by differential contraction of woody tissues. Contraction, which is the result of temperature fluctuation, and cell dehydration and water movement as ice forms, can induce considerable strain between frozen and thawed areas. In large woody stems the outer tissues freeze before the central ones, thereby causing greater tangential than radial shrinking (61). Stem splitting can also result from expansion as large ice masses form and continue to increase in size as the temperature decreases. This type of splitting was seen in R. poukhanense and is of greater importance in azalea twigs than differential contraction.

When the water content was moderately high, splitting of R. poukhanense was still evident, but the splits were small and confined to the vascular and cortical tissues. Small ice masses were seen in these tissues. Minor splits, particularly in the vascular tissue of the pedicel, could adversely affect the flower parts. These minor stem splits had little if any effect on vegetative bud survival or subsequent growth.

When the water content was moderate, no splitting was evident. Splitting was, thus, a function of the water content and the resulting ice formation as tissues froze. Twigs of R. 'Maryann' and those of R. poukhanense that had been dried were in the moderate moisture range. The lack of visible splits did not mean that ice could not have a direct effect on injury. Moderately small ice crystals were found to be dispersed throughout azalea stem tissue. As frozen tissue sections thawed, there was some distortion or change in shape of the section. Cells or groups of cells could be seen to reorient slightly in relation to adjoining cells or tissues. Similar distortion must have occurred during freezing. The effect of this distortion would be a grinding action as cells slipped between extracellular ice crystals. As the temperature slowly decreased, the ice that was dispersed throughout all tissues became more rigid and increased in size as cell dehydration continued. Also, stress increased as tissues contracted due to cell desiccation. The resulting shearing forces as ice accumulated and cells moved to accommodate the

increasing ice could injure the living cells. Since splitting was not observed in floral parts, damage was most likely the result of sensitivity to forces of the formation and growth of ice. This kind of injury was a result of non-equilibrium freezing and the formation of moderate sized crystals throughout the tissue.

Ice may not have a direct injurious effect if the water content is sufficiently low. Under low water conditions the ice crystals might be small, and contraction of cells as a result of frost dehydration could be relatively harmless. The tissue structure would affect the crystal dispersion more than the crystals would affect the tissue. Azalea twig water content was well above this low range.

Differences in survival between the two azalea cultivars resulted from the difference in water content. Rhododendron 'Maryann', with the lower water content (moderate), was injured at a lower temperature than R. poukhanense. Survival of R. poukhanense could be increased by lowering its water content from high to moderate. Thus, water content is related to killing temperature. By lowering the water content, large splitting ice masses were avoided and the stress of rapid crystal development throughout the tissue was quite likely lessened. Water plays a very important, if not crucial, role in the survival of the evergreen azalea twigs studied.

Freezing Injury Evaluation

Browning, an obvious sign of injury in azalea flower buds, is a good criterion for field surveys, but an unreliable indicator of specific injury. Although browning is symptomatic of low temperature injury, it provides neither evidence of the location of initial injury, nor differentiation between direct injury (caused by low temperatures) and indirect injury (caused by dead cells adversely affecting their living neighbors). Some tissues become discolored without being injured, while others may be injured but develop no discoloration. The color or color precursors sometimes move from one area to another, thereby, giving a false indication of the tissue actually injured. After browning has developed, it hinders visual observation. Consequently, a means of determining cell injury soon after treatment and prior to browning was necessary. Neutral red, when used alone or in conjunction with cell plasmolysis, proved to be an effective and valid indicator of living cells. Both the ability of cells to retain the neutral red and their ability to plasmolyze and deplasmolyze were indicative of uninjured cells. Injury was also validly evaluated by comparing conductivity values prior to freezing and after thawing. Sectioning and neutral red staining confirmed injury or the lack of it.

By using the neutral red plasmolysis technique the upper stem pith was found to be injured prior to and at a higher temperature than the flower.

Initial injury was restricted to a small number of cells. Pith tissue of azalea twigs was most susceptible to the stress of extracellular ice formation. The pith parenchyma cells are not long lived and could be expected to die during the first winter with little effect on other tissue. Since browning or injury did not progress from one area to another, injured pith cells had little effect on their living neighbors or on vegetative and flower bud survival. By being restricted to pith tissue initial injury occurred at some distance from vital flower parts.

Recovery from freezing injury that would enable the flower to develop for show if not for function did not occur. Without a functional pedicel and pistil, both of which were usually injured, the flower did not expand and grow in the spring. There was no evidence of recovery that enabled the flower to bloom.

In summary, survival of the evergreen azaleas studied was influenced by the combined effects of water content and the rather violent nonequilibrium freezing process that was induced by structural peculiarities of the twig. A high tissue water content had the effect of intensifying the stress during freezing and the resulting injury. The mechanism most critically affecting survival, then, is the nonequilibrium freezing process, which is not caused by environmental or physiological conditions, but rather by the basic structural arrangement of the tissue.

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